Schlafen-1 Causes a Cell Cycle Arrest by Inhibiting Induction of Cyclin D1*

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Schlafen-1 (Slfn-1), the prototypic member of the Schlafen family of proteins, was described as an inducer of growth arrest in T-lymphocytes and causes a cell cycle arrest in NIH3T3 fibroblasts prior to the G1/S transition. How Slfn-1 exerts its effects on the cell cycle is not currently known. We report that synchronized murine fibroblasts expressing Slfn-1 do not exit G1 when stimulated with fetal calf serum, platelet-derived growth factor BB (PDGF-BB) or epidermal growth factor (EGF). The induction of cyclin D1 by these stimuli was blocked in the presence of Slfn-1 as well as all downstream cell cycle processes. Overexpression of cyclin D1 in growth-arrested, Slfn-1-expressing cells induced an increase in cell cycle consistent with this protein being the biological target of Slfn-1. Activation of the mitogen-activated protein kinase pathway by EGF or phorbol 12-myristate 13-acetate was unaffected by Slfn-1 expression. PDGF signaling was, however, almost completely blocked. This was due to a lack of PDGF receptor expression in Slfn-1-expressing cells consistent with Slfn-1 blocking the cell cycle in G1 where PDGF receptor expression is normally down-regulated. Finally, overexpression of Slfn-1 inhibited the activation of the cyclin D1 promoter. Slfn-1 therefore causes a cell cycle arrest during G1 by inhibiting induction of cyclin D1 by mitogens.

Regulation of mammalian cell proliferation by extracellular mitogens is controlled by receptor-initiated signaling pathways that ultimately converge on the cell cycle machinery, thereby mediating a G1/S transition (1). These signaling pathways are tightly controlled at many levels and by a variety of negative regulatory molecules.

Schlafen-1 (Slfn-1)1 is a recently described protein, which has been shown to impair thymocyte development and cause a G1 arrest when expressed in fibroblasts (2). It belongs to an expanding family of proteins, including at least eight distinct members in the mouse. Slfn1 can be ordered into three distinct groups based on the length of the C terminus: short forms (Slfn-1 and Slfn-2), intermediate forms (Slfn-3 and -4), and long forms (Slfn-5, -8, -9, and -10). Although all Slfn1 encode a common N-terminal AAA domain reported to be involved in GTP/ATP binding (3–5), long form Slfn1 possess several motifs found in members of the superfamily I of DNA/RNA helicases. These helicases have been reported to be involved in many aspects of DNA/RNA metabolism, such as DNA repair (6) or transcriptional/translational regulation (7, 8).

Slfn1 have previously been shown to be differentially regulated during T-cell development, after infection with the intracellular bacterial pathogens Brucella (9) or Listeria (10) and following lipopolysaccharide (LPS) or interferon-γ-mediated cell cycle arrest in bone marrow-derived macrophages (10). Only Slfn-1 was shown to cause a growth arrest in murine fibroblasts, with Slfn-5, -8, -9, and -10 expression having no effect in these cells (10).

Entry of quiescent cells into the cell cycle is triggered experimentally by growth factors, many of which act via receptor tyrosine kinases (11). In these systems, binding of a growth factor to its cognate receptor induces trans-phosphorylation on adjacent intracellular receptor chains (12), facilitating Src homology-2 (SH-2) domain protein interactions and activation of downstream pathways, such as the Ras/mitogen-activated protein (MAP) kinase pathway (13, 14) and PI3 kinase/Akt pathways (15–17). Both of these signals co-operate critically in the up-regulation and stabilization of D-type cyclins (18–26), which couple to G1 cyclin-dependent kinases (CDKs)—4/6. These active cyclin/CDK complexes phosphorylate retinoblastoma protein (27) leading to the activation of E2F family transcription factors. Once activated, these transcription factors induce the expression of genes that mediate transition of the cell into S phase with the initiation of DNA replication (11, 28).

In this study we have analyzed the effect of Slfn-1 on signaling by a variety of mitogens. We demonstrate that Slfn-1 inhibits the induction of cyclin D1 by fetal calf serum (FCS), epidermal growth factor (EGF), phorbol 12-myristate 13-acetate (PMA), and platelet-derived growth factor (PDGF). It does not block the activation of the Ras/MAP kinase or Akt pathways by EGF or PMA. Signaling by PDGF-BB is, however, inhibited, due to a mid-G1-associated down-regulation in PDGF receptor expression. Our study identifies mitogen-driven cyclin D1 induction as a target of Slfn-1 in its ability to cause a mid-G1 arrest in the cell cycle.

EXPERIMENTAL PROCEDURES

Materials—PDGF-BB and anti-FLAG M1 monoclonal antibody were obtained from Sigma. Anti-PDGF receptor A/B and anti-phospho-tyrosine antibody (4G10) were obtained from Upstate Biotechnology. All other antibodies were obtained from Cell Signaling Technologies. All other reagents were obtained from Sigma.

Cell Culture and Transient Transfection—Stock cultures of NIH3T3 murine fibroblasts stably transfected with FLAG-tagged Slfn-1 in a tetracycline-inducible system (18-9 cells previously described) (2) were
Schlafen-1 Inhibits Cyclin D1 Induction

RESULTS

Slnf-1 Expression Inhibits Serum-induced Mitogenesis in NIH3T3 Murine Fibroblasts and Epithelial Cells—Previous work on Slnf-1 examined its effect on cell growth using asynchronous cell populations in the presence of FCS (2, 10). We first confirmed this result using NIH3T3 murine fibroblasts stably transfected with Slnf-1 in a tetracycline-inducible system. Asynchronous cells were counted and assayed for DNA content from 0–72 h with and without Slnf-1 expression. Cells not expressing Slnf-1 increased in numbers over 72 h (Fig. 1A, white bars), whereas cells expressing Slnf-1 ceased proliferation by 24 h post-induction (black bars). No cell death was evident in Slnf-1-expressing cells over this time course. Slnf-1 expression was detected in lysates from induced cells (Fig. 1B).

Slnf-1 growth-arrested cells were examined by flow cytometry to validate the nature of the cell cycle arrest. Cells were serum-starved for 24 h then induced for Slnf-1 expression and stimulated with FCS for 24 h. The DNA content of these cells was then analyzed after fixation and staining with propidium iodide. Slfn-1-expressing cells did not enter into S-phase of the cell cycle even after waiting 72 h (Fig. 1C, right-hand side). Slfn-1 expression was driven by T7 RNA polymerase (Promega) for 31 cycles at 62.2°C annealing temperature. Primers used were: β-actin forward, 5′-TGGAAATCCTGT-GGCATCACCTGA; β-actin reverse, 5′-TAAAGGAGACGTCAAGAC; AGGTG-pro forward, 5′-CGACCTAGACGGAAGCTCC; PGF-βr forward, 5′-TGCCATACCTTCCACGGCA; PGF-βr reverse, 5′-GGATTCCCAAAAGGACGACA; cyclin D1 forward, 5′-CACAAGCCGCTTTTCTTCCA; cyclin D1 reverse, GACCACCTCTTCCTCCAC; Slnf-1 forward, CCAGATGCTCTGTTGAAGA; and Slnf-1 reverse, GCTAAAGACAT-AGAGACCTTG.

Cloning—NIH3T3 cells were seeded at 1 × 10^4 in a 10-cm dish and were serum-starved overnight to synchronize cells in G0. Cells were stimulated the following day with 20 ng/ml EGF for 6 h, and cells were harvested. Total RNA was extracted using TRI reagent (Sigma) as per manufacturer’s instructions. Total RNA was reverse-transcribed, and cyclin D1 was amplified using primers designed against the database sequence of the coding region (p:2792/4088): forward primer, CCCTGCGATAGGAACACGTCTCTG; reverse primer, ATAAAGATGGGCCGCTCAGATGTCCACATC; cyclin D1 cDNA was cloned into the XhoI and NotI sites of pcDNA3.1. The constructs was sequenced and confirmed to contain murine cyclin D1.

GENE EXPRESSION—CHO cells were chosen as a commonly used cell line, as CHO cells would have a greater relevance to cancer biology. To do this we chose the Chinese hamster ovary (CHO) cell line as a commonly utilized, transfecable epithelial cell model. CHO cells were transiently co-transfected with Slnf-1- and EGFP-expressing plasmids, and cell populations were examined for EGFP positivity as an indication of proliferation. Transfected CHO cells

Flow Cytometry—Cells were harvested by trypsinization, centrifuged at 2,000 rpm for 5 min, and washed with 5 ml of sterile PBS. Cells were then centrifuged at 2,000 rpm for 4 min and fixed in 2 ml of 70% ethanol at 4°C for 1 h. After fixation, cells were washed twice with 5 ml of PBS and incubated in 1 ml of PBS containing 10 μl of RNase A 10 mg/ml (DNase free) for 1 h at 37°C. Cells were then centrifuged at 2,000 rpm for 4 min, and pellets were resuspended in 1 ml of PBS containing 40 μg/ml propidium iodide. Stained cells were then analyzed using a FACS Calibur Flow Cytometer (BD Biosciences, Mountain View, CA) and CellQuest propidium iodide. Stained cells were then analyzed using a FACSCalibur flow cytometer with the X-Amin filter.

LUCIFERASE ACTIVITY—Luciferase activity was determined as luminescent units using the Promega system. Luciferase activity is expressed as luciferase units per 10-cm dish consisting of 4 g of pEGFP-N1 and 4 g of pcDNA3-empty vector, pcDNA3.1-Slnf-1, or pcDNA3.1-cycD1. Cells were cultured for 48 h before harvesting for flow cytometry analysis.

FIRE POLARITY—To further confirm that Slnf-1-expressing cells did not pass G1, we examined the phosphorylation state of cdc2 (Tyr-15), which occurs after entry into S-phase (30, 31). Cells not expressing Slnf-1 exhibited cdc2 Tyr-15 phosphorylation ~15 h after stimulation of serum-starved cells with FCS (Fig. 1D, first panel, left-hand side, lane 4). Slnf-1-expressing cells did not exhibit any Tyr-15 phosphorylation at this time point (first panel, right-hand side, lane 8). These data confirmed that Slnf-1-expressing cells do not enter into S-phase of the cell cycle.

ALTERATIONS IN THE EXPRESSION PATTERN—Special care was taken to avoid artificial differences in cell proliferation. Cells not expressing Slnf-1 exhibited an increase in G0/G1 and a decrease in S and G2 phase populations (Fig. 1A, right-hand side, black bars). Slfn-1-expressing cells do not enter into S-phase of the cell cycle.
FIG. 1. Slfn-1 causes a G₀/G₁ arrest in murine fibroblasts cultured in FCS. A, 18-9 cells were seeded in duplicate at 2.5 × 10⁵ cells per 10-cm dish. Cells were left untreated (0 time point), induced for Slfn-1 expression by withdrawing tetracycline or left suppressed with tetracycline for 24–72 h as indicated. Cell counts from duplicate samples are shown. B, lysates from cells treated as for A were assayed for Slfn-1-FLAG expression by Western blotting, probing with an anti-FLAG antibody. C, cells seeded as before except were synchronized by serum starvation for 24 h. Cells were then stimulated with 10% FCS without or without the induction of Slfn-1 expression for 24 h. Samples were then harvested, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. D, cells were seeded as before, induced for Slfn-1, or left suppressed for 24 h and then serum-starved for a further 24 h. Cells were then stimulated for 0–15 h with 10% FCS, harvested by lysis in protein sample buffer. Lysates were then separated by SDS-PAGE and immunoblotted with the indicated antibodies. These data are representative of three separate experiments.
were assayed for Slfn-1 expression by Western blotting (Fig. 2A, upper panel, lane 2). Cells transfected with EGFP alone exhibited 13.9 ± 0.59% EGFP-positive cells when assayed by flow cytometry (Fig. 2B, left-hand panel). Conversely, samples transfected with EGFP and Slfn-1 exhibited a significant decrease (7.725 ± 0.6%) in EGFP-positive cells (right-hand panel) indicative of decreased cell growth. The data are represented graphically below the flow cytometry profiles. These data indicate that Slfn-1 causes a growth arrest in CHO epithelial cells similar to fibroblasts.

Slfn-1 Expression Inhibits Growth Factor-mediated Mitogenesis in NIH3T3 Murine Fibroblasts—We next examined the effect of Slfn-1 expression on cell growth using the defined growth factors PDGF-BB and EGF. Cells were serum-starved for 24 h to obtain synchronization in G0 and stimulated with 20 ng/ml PDGF-BB or 20 ng/ml EGF. Induction of Slfn-1 abolished an increase in cell numbers over 72 h in response to PDGF-BB or EGF stimulation (Fig. 3, A and C). As shown in Fig. 3 (B and D), on stimulation with PDGF-BB or EGF, cells not expressing Slfn-1 exhibited a decrease in the G2/G1 population with a concomitant increase in the S- and G2-phase populations as cells entered into and passed through the cell cycle. Cells expressing Slfn-1 exhibited a sharp increase in the G2/G1 population and a sharp decrease in the S- and G2-phase populations. These data demonstrate for the first time an inhibitory effect of Slfn-1 on mitogenic signaling activated by specific growth factors.

Slfn-1 Inhibits Mitogen-mediated Cyclin D1 Induction—A key, rate-limiting event in mitogenic signaling leading to S-phase entry is the induction of cyclin D1. We first examined levels of cyclin D1 in non-synchronized cells expressing Slfn-1 over 0–48 h. Control cells not expressing Slfn-1 possessed approximately similar levels of cyclin D1 24 h after seeding cells in 10% FCS-containing medium (Fig. 4A, panel 2, compare lanes 1 and 2). Slfn-1-expressing cells, however, exhibited a clear decrease in cyclin D1 levels (compare lanes 2 and 3). After 48 h, control cells showed an increase in expression of cyclin D1 with an additional band of higher molecular mass corresponding to phosphorylated cyclin D1 (lane 4), whereas Slfn-1-expressing cells exhibited no increase in either form (lane 5). Conversely, the level of cyclin D3 was not significantly affected by the presence of Slfn-1 (panel 3). We have observed that cyclin D3, unlike cyclin D1, is not up-regulated by mitogenic stimulation consistent with previously published observations that it is expressed constitutively throughout the fibroblast cell cycle (33). Similarly, levels of the D-type cyclin binding kinases CDK4 and -6 were normal in presence and absence of Slfn-1 (panels 4 and 5).

We next examined the levels of two late G2 cyclins: cyclin E2 and cyclin A. These cyclins are classically expressed after the induction of D-type cyclins following the activation of E2F by cyclin D/CDK4/6 complexes. Indeed, the expression of these genes has been shown to be dependent on E2F binding sites in their respective promoters (34–36). Decreased levels of cyclin E2 were observed 24 h after Slfn-1 induction (panel 4, compare lanes 2 and 3) with a more pronounced decrease at 48 h (compare lanes 4 and 5). Cyclin A was not detected in the presence of Slfn-1 at 24 h (panel 5, compare lanes 2 and 3) and 48 h (compare lanes 4 and 5).

Cyclin B1 is the major mitotic cyclin the levels of which rise in G2 and show peak expression in M phase (37–39). We observed a decrease in the expression of this protein in cells expressing Slfn-1, both at 24 h (panel 6, compare lanes 2 and 3) and 48 h (compare lanes 4 and 5) post-induction. Levels of CDC25B, a key G2/M regulatory phosphatase, were also examined. Levels of non-phosphorylated CDC25B were observed both in the presence and absence of Slfn-1 at 24 h (panel 9, compare lanes 2 and 3). However, a decrease in total and phospho-CDC25B were evident at 48 h post-Slfn-1 expression (compare panels 4 and 5). Because CDC25B levels and activity increase during G2/M (40) we suggest that extended arrest during G2 by Slfn-1 may be responsible for the turnover of the protein at this later time point. No phosphorylation of the DNA damage checkpoint kinase Chk-1 (Ser-296) was observed in the presence of Slfn-1 (data not shown). These data, taken as a whole, indicate that Slfn-1 expression causes a dysregulation of cyclin expression associated with a G2 arrest.

We examined the ability of mitogenic stimuli to induce cyclin D1 expression in synchronized cells. In cells not expressing Slfn-1, each stimulus induced cyclin D1 expression over 6–18 h (Fig. 4, B–E, second panel, left-hand side in each case). Removal of tetracycline induced Slfn-1 expression, which was maintained over the time course (Fig. 4, B–E, first panel, right-hand side in each case). Slfn-1 inhibited this induction by all mitogens tested (Fig. 4, B–E, second panel, right-hand side in each case). Furthermore, the downstream consequences of this inhibition were demonstrated by a decrease in the phosphorylation of retinoblastoma protein on Ser-780 (Fig. 4, B–E, third panel, compare right- and left-hand side in each case), which is known to be dependent on cyclin D1-CDK4/6 activity (41).

Slfn-1 has previously been shown to be induced by LPS treatment of primary macrophages (10). LPS treatment of macrophages has also been shown to cause a growth arrest in these cells, a process associated with a dramatic decrease in cyclin D1 expression (42). We were interested in determining whether these two events might be correlated. RAW 264.7 macrophages were stimulated with LPS for 6 h, and total RNA was extracted from these cells. Semi-quantitative RT-PCR analysis was performed probing for Slfn-1, cyclin D1, and β-actin. Cells stimulated with LPS exhibited an increase in Slfn-1 expression at 6 h post-stimulation (Fig. 4F, upper panel, compare lanes 1 with 2) and a decrease in cyclin D1 expression (middle panel, compare lanes 1 with 2). These data confirm that LPS-induced Slfn-1 expression in macrophages is associated with an inhibition in cyclin D1 expression.

We next wanted to test whether the Slfn-1-mediated inhibition of cyclin D1 transcription was the cause of the growth arrest observed in the NIH3T3 fibroblast Slfn-1 growth arrest model. We therefore determined whether overexpression of cyclin D1 could induce growth in Slfn-1-arrested cells. For this purpose, the murine cyclin D1 coding sequence was cloned from NIH3T3 cell cDNA into the mammalian expression vector pCDNA3.1. NIH3T3 cells were transfected with the pCDNA3.1-cyclin D1 plasmid and a strong 36-kDa band was observed when blots were probed using an anti-cyclin D1 antibody (see Fig. 6G).

We next determined the effect of transient cyclin D1 expression on Slfn-1-mediated growth arrest in NIH3T3 cells. The flow cytometry software was first calibrated with EGFP-negative, non-transfected cells. Slfn-1-expressing, arrested populations co-transfected with EGFP and pCDNA3.1 empty vector contained 28.1 ± 0.53% EGFP positive cells (Fig. 6H, left panel). Interestingly, cells co-transfected with EGFP and pCDNA3.1-cyclin D1 exhibited a significant increase to 40.595 ± 1.53% EGFP-positive cells (right panel). These data indicate that cyclin D1 expression causes proliferation in cells that are growth-arrested by Slfn-1 indicating that overexpressing cyclin D1 can overcome the growth-arresting effect of Slfn-1. This provides further evidence that the growth-inhibiting effect of Slfn-1 is due to an inhibition of cyclin D1 expression.
**FIG. 2.** Slfn-1 causes a growth arrest in epithelial cells. **A**. CHO cells were seeded at $1 \times 10^6$ cells per 10-cm dish, were transfected with 4 μg of pEGFP-N1 and 4 μg of pcDNA3.1-Slfn-1, were harvested, and analyzed for Slfn-1 expression by Western blotting. **B**. CHO cells were seeded at $1 \times 10^6$ cells per 10-cm dish and allowed to recover for 24 h. Cells were then transfected with 4 μg of pEGFP-N1 with 4 μg of pcDNA3.1 (left-hand panel) or 4 μg of pcDNA3.1-Slfn-1 (right-hand panel). CHO cells were then harvested 48 h later and analyzed for Slfn-1 expression by Western blotting. Transfected cells were then analyzed by flow cytometry. Non-transfected cells were analyzed first to determine the cut-off FL-1 fluorescence for EGFP-negative population. Duplicate transfected cell populations were then analyzed using CellQuest software to determine the population of EGFP-positive cells. Data are also presented in table format (below). These data are representative of three separate experiments.
Slfn-1 Has No Effect on EGF- or PMA-mediated Ras/MAP and Akt Pathway Activation but Inhibits PDGF-BB-mediated Activation of These Signals—After determining that Slfn-1 inhibits cyclin D1 induction by mitogens, we next investigated the integrity of signals that lead to the activation of the cyclin D1 gene in these pathways. ERK-1/2 and Akt activation have been extensively characterized as the key mediators of receptor tyrosine kinase-driven cyclin D1 induction (21, 23, 25) and stabilization (18), respectively.

The activation of these early mitogenic signals by EGF and PMA were first examined. As shown in Fig. 5A, Slfn-1 had no significant effect on the activation of MEK-1/2, ERK-1/2, or Akt (as assayed by immunoblotting using phospho-specific antibodies) by EGF (panels 2, 4, and 6, respectively, compare lanes 6–8 with lanes 2–4). Slfn-1 also had no effect on MEK-1/2 and ERK-1/2 activated by PMA (Fig. 5B, panel 2 and 4, respectively, compare lanes 6–8 with lanes 2–4). These data suggested that Slfn-1 inhibited EGF and PMA-mediated cyclin D1 induction without affecting the early events of mitogenic signaling.

The ability of ERK-1/2 to phosphorylate downstream targets was next examined. Using an elk-1 transactivation assay, we found that Slfn-1 had no effect on the activation of this transcription factor when driven with RasHa or MEK1 (Fig. 5C, compare bars 1 and 2 with bars 3 and 4). The lack of effect of Slfn-1 on Ras/MAP kinase pathway activation by EGF activation and also elk-1 activation indicates that Slfn-1 is targeting a process downstream of ERK-1/2 and elk-1 activation, but prior to cyclin D1 induction.

**Fig. 3.** Slfn-1 inhibits PDGF-BB-and EGF-mediated proliferation of NIH3T3 cells. 18-9 cells were seeded in duplicate at 2.5 × 10⁵ cells per 10-cm dish and serum-starved overnight. Cells were then stimulated for the indicated times with 20 ng/ml PDGF-BB (A) or EGF 20 ng/ml (C) without (white bars) or with (black bars) induction of Slfn-1. After this period, cells were harvested and counted, plotting cell counts from duplicate samples. Harvested cells from the 0- and 24-h time points of PDGF (B)- and EGF (D)-stimulated samples were also fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. DNA content statistics are also presented in table form. These data are representative of three separate experiments.

**Table 1.** Effect of Slfn-1 on PDGF-BB and EGF-mediated proliferation of NIH3T3 cells.

| Time (hrs) | Slfn-1 (-) | Slfn-1 (+) |
|-----------|------------|------------|
| PDGF-BB   |            |            |
| 0         | 65.97 ± 3.2| 58.10 ± 3.3|
| 24        | 12.13 ± 0.99| 14.34 ± 0.45|
| 48        | 15.00 ± 1.5 | 17.58 ± 1.45|
| 72        |            |            |

**Table 2.** Effect of Slfn-1 on EGF-mediated proliferation of NIH3T3 cells.

| Time (hrs) | Slfn-1 (-) | Slfn-1 (+) |
|-----------|------------|------------|
| EGF       |            |            |
| 0         | 58.04 ± 3.82| 65.53 ± 2.4|
| 24        | 15.74 ± 1.22| 12.71 ± 0.3|
| 48        | 11.77 ± 1.9 | 16.81 ± 0.55|
| 72        |            |            |
FIG. 4. Slfn-1 inhibits cyclin D1 induction by mitogens. A, 18-9 cells were seeded at a density of 2.5 × 10^5 cells per 10-cm dish. Slfn-1 was induced or left suppressed, and cells were cultured for 48 h without serum starvation. Samples were harvested at 0, 24, and 48 h by lysis in sample buffer. After SDS-PAGE, proteins were immunoblotted with the indicated antibodies. B–E, cells were seeded as in A. Slfn-1 was induced for 24 h, and cells were serum-starved for a further 24 h. Cells were stimulated with indicated mitogens for 6–18 h and were harvested by lysis. Proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies. Results are representative of three separate experiments. F, Raw 264.7 macrophage cells were seeded at 1 × 10^6 cell per well in 6-well dishes. Cells were stimulated with 100 ng/ml LPS and harvested at 0 and 6 h. Total RNA was isolated from cells and RT-PCR analysis was carried out to determine the expression levels of Slfn-1, cyclin D1, and β-actin. G and H, 18-9 cells were seeded at 2.5 × 10^5 cells per 10-cm dish. Cells were then transfected with 4 μg of pEGFP-N1 with 4 μg of pcDNA3.1 (left panel) or 4 μg of pcDNA3.1-cyclin D1 (right panel). Cells were then cultured with Slfn-1 expression for 24 h. Cells were harvested and analyzed for cyclin D1 expression by Western blotting, probing with an anti-cyclin D1 antibody (G). Cells were also analyzed by flow cytometry. Non-transfected cells were analyzed first to determine the cut-off FL-1 fluorescence for EGFP-negative population. Duplicate transfected cell populations were then analyzed using CellQuest software to determine the population of EGFP-positive cells (H). These data are representative of three separate experiments.
Interestingly, as shown in Fig. 6A, induction of Slfn-1 abolished activation of MEK-1/2, ERK-1/2, and Akt by PDGF-BB (panels 2, 4, and 6, respectively, compare lanes 6–8 with 2–4). This indicated that both PDGF-BB-activated Ras/MAP kinase and Akt pathways were inhibited by Slfn-1. We were interested to determine why PDGF-BB-activated Ras/MAP kinase and Akt pathways were inhibited, whereas those activated by EGF or PMA were not. We first examined the effect of Slfn-1 on PDGF receptor phosphorylation. As shown in Fig. 6B, PDGF-BB caused a dramatic increase in the phosphorylation of the PDGF receptor (second panel, left-hand side). Slfn-1-containing cells exhibited a profound decrease in this response (second panel, right-hand side). The dramatic decrease in receptor phosphorylation in the presence of Slfn-1 was, however, concomitant with a decrease in total receptor levels (third panel, compare lanes 5–8 with 1–4). No effect was observed on EGF receptor levels in the presence of Slfn-1 (Fig. 6B, compare lanes 1 and 2).

To determine how Slfn-1 was exerting its effect on levels of the PDGF receptor, we examined its effect on the transcriptional activity of the PDGFβ promoter. Induced and non-induced cells were transfected with a construct containing 1.4 kb of DNA sequence upstream from the transcription initiation site of the murine PDGFβ promoter linked to a firefly luciferase reporter and assayed for activity 24 h later. Results are representative of three separate experiments.

FIG. 5. Slfn-1 has no effect on EGF or PMA-mediated mitogenic signaling to the Ras/MAP kinase and Akt pathways. 18-9 cells were seeded at 2.5 × 10⁵ cells per 10-cm dish and were induced for Slfn-1 expression for 24 h. Cells were serum-starved for a further 24 h and then stimulated from 0–60 min with 20 ng/ml EGF (A) or PMA 100 nM (B). Proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies. Results are representative of three separate experiments.

PDGF receptor chains were conformed by semi-quantitative RT-PCR analysis. As shown in Fig. 6D, demonstrates that Slfn-1 causes a significant decrease on the activity of this promoter. Decreases in both the PDGFRα and PDGFRβ receptor chains were confirmed by semi-quantitative RT-PCR analysis. As shown in Fig. 6E, Slfn-1 expression for 48 h caused a decrease in both receptor chains (compare lanes 1 and 2 in both cases). This indicates that the decrease in PDGFR levels and therefore the activation of PDGFR-mediated mitogenic signaling was likely to be due to a decrease in the expression of both of these genes in the presence of Slfn-1.

It has previously been reported that PDGF receptor levels are high in G₀, decrease on entry of cells into G₁ (43–45), and do not return to normal levels until after cell division. EGF receptor expression has been reported to remain constant throughout the cell cycle (46, 47). We were therefore interested to determine if the effect of Slfn-1 on PDGF could be explained solely in the context of a Slfn-1-mediated mid-G₁ arrest. Fig. 6F shows that if cells are cultured with serum and Slfn-1 is induced (standard protocol as used in previous experiments), a decrease in PDGF expression is evident because the cells have entered into G₁, when PDGF levels are low, and have been arrested by Slfn-1 (left-hand panel, compare lane 2 with lane 1). Induction of Slfn-1 in cells held in G₀ (by serum starvation for 24 h prior to Slfn-1 induction), where PDGF levels are high, had no significant effect on PDGF expression (right-hand panel, compare lanes 2 to lane 1). As expected, the level of EGFR expression was unaffected in either protocol (second panel in either case). Interestingly, PDGF-BB stimulation of cells held in G₀ resulted in the phosphorylation of ERK-1/2 with and without Slfn-1 expression (Fig. 6G, panel 2, compare lanes 1–4 with 5–8) consistent with the lack of effect on EGF-induced ERK-1/2 activation. These data suggest that the dramatic decrease in PDGF levels was due to the arrest of cells by Slfn-1 at a point in G₁ following the down-regulation of this receptor. These results further support the conclusion that Slfn-1 causes a mid-G₁ arrest by inhibiting cyclin D1 induction by mitogens.
To determine if the decreased induction of cyclin D1 was mediated at the level of gene expression, we first examined the activity of the cyclin D1 promoter. 18-9 cells induced for Slfn-1 expression (Fig. 7A, left-hand side) and CHO cells transfected with Slfn-1 (Fig. 7A, right-hand side) were transfected with a fragment of the cyclin D1 promoter corresponding to /H11002/1745 bp upstream of the transcriptional start site coupled to a luciferase reporter. Slfn-1 expression in both cases caused an inhibition of the activity of this fragment (Fig. 7A).

We also tested a series of cyclin D1 promoter fragments from /H11002/1,745 to /H11002/271 in NIH3T3s. These fragments were transcriptionally active in cells not expressing Slfn-1 (Fig. 7A, white bars). However, Slfn-1 reduced the activity of all the promoter fragments tested.

**FIG. 6.** Slfn-1 inhibits PDGF-BB-mediated mitogenic signaling to the Ras/MAP kinase and Akt pathway due to PDGFR down-regulation associated with mid-G1 arrest. A, 18-9 cells were seeded at 2.5 x 10^5 cells per 10-cm dish and were induced for Slfn-1 expression for 24 h. Cells were serum-starved for a further 24 h and then stimulated from 0–60 min with 20 ng/ml PDGF-BB. Proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies. Results are representative of three separate experiments. B, cells were as for A then stimulated with 20 ng/ml PDGF-BB over 30 min. Cell lysates were prepared, and proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies. For PDGFR analysis, the receptors were immunoprecipitated from 500 μg of whole cell lysates overnight with 5 μg of anti-PDGF receptor antibody prior to SDS-PAGE and immunoblotting. Results are representative of three separate experiments. C, cells were serum-starved and induced as for A. Lysates were prepared and assayed for the EGF receptor by Western blotting, probing with an anti-EGF receptor antibody. D, 18-9 cells were seeded at 1.25 x 10^6 cells/well in 24-well plates and Slfn-1 expression was induced 24 h later. After a further 24 h, cells were transfected with 800 ng of PDGFβ -1.4 kb promoter luciferase construct. Luciferase activity was assayed after 48 h. E, total RNA was isolated from control cells and cells expressing Slfn-1 for 48 h. RT-PCR analysis was carried out to determine the expression levels of PDGFβαβ and β-Actin. F, cells were seeded at 2.5 x 10^6 cells per 10-cm dish and were induced for Slfn-1 expression for 24 h, then serum-starved for 24 h and harvested by lysis (left-hand panel, lanes 1 and 2). Cells were also serum-starved for 24 h, induced for Slfn-1 for a further 24 h, and harvested by lysis (right-hand panel, lanes 3 and 4). Samples were assayed for expression of Slfn-1, PDGFR, and EGFR as indicated. These data are representative of three separate experiments. G, 18-9 cells were seeded at 2.5 x 10^5 cells per 10-cm dish, were serum-starved for 24 h, and induced for Slfn-1 for a further 24 h. Cells were then stimulated with 20 ng/ml PDGF-BB for indicated times and were harvested by lysis. Proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies. Results are representative of two separate experiments.
to a low basal level (black bars). Also, no increase from this basal activity of the 1745-bp fragment was observed when the promoter was driven by RasHa, MAP/ERK kinase-1 (MEK-1), or EGF (data not shown). These data indicated that Slfn-1 was having a general inhibitory effect on the functioning of the cyclin D1 promoter that was not associated with a specific site between 1745 bp to 271 bp.

The activity of two transcription factors, nuclear factor κB (NFκB) and “activating protein-1” (AP-1), known to be involved in cyclin D1 gene induction, were tested in analogous luciferase systems. Both assays demonstrated the normal basal activity of these transcription factors in the presence of Slfn-1. The activity of a construct containing 2.2 kb of the cyclin E promoter was also inhibited in the presence of Slfn-1 (Fig. 7C and D), consistent with the data on the expression of this protein. These data indicated that Slfn-1 causes an inhibition of the cyclin D1 promoter without affecting the activity of two transcription factors involved in the regulation of this promoter. We are currently investigating the precise mechanism whereby Slfn-1 inhibits the activity of the cyclin D1 promoter focusing on the possibility of a global effect.

**DISCUSSION**

Slfn-1 causes a G1 arrest in both thymocytes and fibroblasts, and its expression is also correlated with resting states in T-cells (2). However, the mechanism of action of Slfn-1 or any other Slfn family member has not been reported to date. We have demonstrated that Slfn-1 inhibits not only FCS-induced cell growth, but also cell growth induced by specific mitogens, such as PDGF-BB and EGF. We have also demonstrated that expression of Slfn-1 inhibits the growth of CHO epithelial cells.

A key growth factor sensor and a rate-limiting signal required for progression from G1 into S-phase is the induction of D-type cyclins (48–54). We have found that this response is inhibited by Slfn-1 along with downstream events such as late G1 cyclin induction and post G1-related events. Slfn-1 had no effect on other cell cycle proteins such as CDK4/6. Moreover Slfn-1 did not inhibit the expression of cyclin D3.

There are a number of pieces of evidence that indicate that Slfn-1 causes a decrease in cyclin D1 levels by inhibiting the transcription of the cyclin D1 gene. First, we have observed that Slfn-1 inhibits the activation of a series of cyclin D1
promoter fragments, reducing their activity to a low basal level. This inhibition of the cyclin D1 promoter was observed in CHO cells where Slfn-1 caused a similar growth arrest. These data suggest that Slfn-1 may not be targeting a specific transcription factor but rather a critical process in the transcription of the cyclin D1 gene. Alternatively, its inhibitory action may be exerted by acting as, or modulating the activity of, a negative regulatory factor which targets the minimal promoter fragment, because all the fragments tested were equally inhibited. We are currently attempting to further characterize the nature of this inhibition.

Unsurprisingly, the expression of Slfn-1 inhibited the cyclin E promoter consistent with an inhibition of cyclin D1 expression. Finally, we have observed that transient overexpression of cyclin D1 in growth arrested Slfn-1-expressing cells causes an increase in cell growth, which appears to validate the hypothesis of the growth arrest being specifically due to an inhibited induction of this cyclin.

Signaling to the cyclin D1 promoter by growth factors is dependent on the activation of the Ras/MAP kinase pathway (20, 21). The activation of the MAP kinase pathway by EGF and PMA was not affected by Slfn-1. PDGF signaling to the Ras/MAP kinase and Akt pathways, however, were inhibited by Slfn-1. This was shown to be due to low PDGFR expression during G0, as observed by others (45–45), because, when cells were held in G0, Slfn-1 did not inhibit PDGFR expression. The EGF receptor was not affected, explaining the lack of effect of Slfn-1 on EGF-mediated Ras/MAP kinase and Akt pathway activation. The effect of Slfn-1 on the PDGFR expression can therefore be explained by Slfn-1-mediated mid-G1 arrest due to an inhibition of cyclin D1 induction. Our results presented here indicate that the target of Slfn-1 is downstream of ERK-1/2 activation and does not involve inhibition of elk-1, AP-1, or NF-κB activation, transcription factors previously reported to be important activators of the cyclin D1 promoter (21, 55, 56).

Other studies support our conclusion that Slfn-1 is targeting D-type cyclin induction downstream of early mitogenic signals. Circulating peripheral T-cells exhibit continual activation of their early mitogenic signaling systems due to continued engagement of self-major histocompatibility complex molecules in the periphery, a process necessary for their survival (57). Indeed, due to this engagement, these cells exhibit constitutive phosphorylation of ζ chain of the T-cell receptor and ZAP-70 (58). These activated early mitogenic signals do not, however, result in a productive growth signal. Peripheral T-cells contain high levels of Slfn-1 mRNA, and activation of these cells with anti-CD3/CD28 causes a decrease in Slfn-1 expression (2) at the same time that mitogenic signals become productive and proliferation is observed. Our data strongly suggest that this may also be due to an inhibition of mitogen-driven induction of D-type cyclins by Slfn-1 in these cells. Geserlick and colleagues (10) have recently reported that interferon-γ and LPS-mediated growth repression is correlated with increases in Slfn-1 expression via, most likely, secondary stimulation by type I interferons and induction of IRF-1, respectively. Interferon-γ and LPS-mediated growth arrest in macrophages has previously been linked to decreased cyclin D1 expression by unknown means (42). We have also observed that LPS growth arrested RAW 264.7 macrophages exhibit an increase in Slfn-1 expression 4–8 h post-stimulation, an event that is correlated with a decrease in cyclin D1 expression and growth arrest. These data suggest that Slfn-1 expression may also be the means by which LPS mediates a growth arrest by inhibiting cyclin D1 expression.

The association of Slfn1s with interferons raises the possibility that these proteins may be part of the anti-viral interferon response. Because S-phase induction is a prominent strategy used by viruses to enhance viral propagation, inhibition of D-type cyclin induction would prevent this process and limit viral infection. Interestingly, poxviruses encode a sequence with strong similarity to host short-form Slfn1s. The purpose of this protein is as yet unclear. Indeed, we have found that expression of this viral Slfn in NIH3T3 cells does not cause an overt growth arrest phenotype despite significantly higher levels of expression than Slfn-1 in a similar system. This raises the intriguing possibility that its effects may be antagonistic to host Slfn1s induced during a viral infection. We are currently investigating this possibility.

In conclusion, Slfn-1 and potentially other Slfn family members cause a growth arrest by repressing mitogen-driven D-type cyclin expression. This may be a key mechanism to limit cell growth in different contexts, including during T-cell development or in LPS/interferon-γ-mediated growth arrest in macrophages and infection with intracellular bacteria or viruses.

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Schlafen-1 causes a cell cycle arrest by inhibiting induction of cyclin D1.

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Several blots were inadvertently duplicated in Figs. 1D, 2A, 4E, and 6A and G. These errors have now been corrected and do not affect the results or conclusions of this work.