In vitro addition of stem cell factor (SCF) to c-kit-expressing A1–A4 spermatogonia from prepuberal mice stimulates their progression into the mitotic cell cycle and significantly reduces apoptosis in these cells. SCF addition results in a transient activation of extracellular signal-regulated kinases (Erk)1/2 as well as of phosphatidylinositol 3-kinase (PI3K)-dependent Akt kinase. These events are followed by a rapid redistribution of cyclin D3, which becomes predominantly nuclear, whereas its total cellular amount does not change. Nuclear accumulation of cyclin D3 is coupled to transient activation of the associated kinase activity, assayed using the retinoblastoma protein (Rb) as a substrate. These events were followed by a transient accumulation of cyclin E, stimulation of the associated histone H1-kinase activity, a delayed accumulation of cyclin A2, and Rb hyper-phosphorylation. All the events associated with SCF-induced cell cycle progression are inhibited by the addition of either a PI3K inhibitor or a mitogen-activated protein-kinase kinase (MEK) inhibitor, indicating that both MEK and PI3K are essential for c-kit-mediated proliferative response. On the contrary, the anti-apoptotic effect of SCF is not influenced by the separate addition of either MEK or PI3K inhibitors. Thus, SCF effects on mitogenesis and survival in c-kit expressing spermatogonia rely on different signal transduction pathways.

The tyrosine kinase receptor encoded by the c-kit gene and its ligand stem cell factor (SCF) play a fundamental role in gametogenesis (1). Most mutations of either c-kit or SCF genes (W and Steel mutations, respectively) result in the loss of primordial germ cells in the embryonal gonad, whereas some Steel mutations affect gametogenesis after birth (2–3). c-kit expression is high in primordial germ cells and is down-regulated in germ cells of the fetal gonad at around 13.5 days postcoitum (4). It is resumed in perinatal oocytes at the end of meiotic prophase and in proliferating spermatogonia at around 6 days postpartum (5–7). In the adult testis, c-kit expression is absent in undifferentiated spermatogonia (8), high in differentiating spermatogonia from type A1 to B (5–7, 8), and turned off in meiotic and postmeiotic cells (6–7). A truncated form of the c-kit kinase, possibly playing a role during sperm-induced egg activation at fertilization, is expressed during spermiogenesis (9–12).

c-kit expression in differentiating spermatogonia has led to the hypothesis that the SCF/c-kit interaction is required for the proliferation and/or survival of these cells. Several lines of evidence support this hypothesis. In vivo injection of antibodies directed against the extracellular region of c-kit selectively blocks proliferation and induces apoptosis of c-kit expressing type A spermatogonia but not of c-kit negative undifferentiated spermatogonia (7, 13). Furthermore, a mutation in the c-kit docking site for the p85 subunit of phosphatidylinositol 3-kinase (PI3K), introduced by a knock-in strategy, causes a dramatic reduction of the spermatogonial population in the prepuberal testis (14–15). A loss of spermatogonia during postnatal development is also observed in a peculiar Steel mutation, S177H (3). Finally, in vitro addition of SCF, which is expressed by Sertoli cells (16–17) under FSH control (17–18), selectively stimulates DNA synthesis in type A but not in type B spermatogonia (17, 19).

The series of molecular events leading to G1 progression, G1/S transition, and mitosis have been established in several somatic cell types synchronized in G0 through serum starvation (20–23). Synthesis of D-type cyclins and the assembly and nuclear translocation of cyclin D/cyclin-dependent kinase 4/6 (cdk4/6) complexes is required for commitment to G1 entry, whereas the consequent cyclin E accumulation and activation of the associated cyclin-dependent kinase 2 (cdk2) allows progression through G1 (20–23). Cyclin D/cdk4/6 complexes trigger initial phosphorylation of the retinoblastoma protein (Rb) and titrate cdk2 inhibitors (cip1/kip1 family), thus de-repressing cyclin E/cdk2 activity. Hyperphosphorylation of Rb by cyclin E/cdk2 is followed by release of the Rb-associated transcription factor E2F, which activates cyclin E transcription in a positive feedback loop, allowing the burst of cyclin E accumulation and activity in a narrow window coincident with the G1/S transition. E2F transcriptional activity is required to elicit timely induction of genes required for S phase progression, such as cyclin A2. Progression through the S phase coincident with the appearance of cyclin A2/cdk2 activity is followed by rapid down-regulation of cyclin E levels (20–23).

We report evidence that SCF acts as a mitogenic factor in cultured c-kit-expressing spermatogonia and that both mitogen-activated protein kinase kinase (MEK)- and PI3K-dependent pathways are required for the proliferative response. The
mitogenic effect is not accompanied by an increase in total cellular amount of cyclin D3 (24), but it is associated with a rapid change in its subcellular localization. We also show that SCF is an anti-apoptotic factor for spermatogonia, but the MEK- or the PI3K-dependent pathways are not sufficient on their own to promote the survival response.

**EXPERIMENTAL PROCEDURES**

**Isolation and Culture of Mouse Spermatogonia**—Spermatogonia were obtained from either 5/6- or 8-day-old Swiss CD-1 mice, as reported previously (17). Spermatogonial stem cells and proliferating but undifferentiated spermatogonia are the prevalent germ cell types at 5–6 days of age, whereas differentiating (type A–A,) intermediate, and type B) spermatogonia predominate at 8 days of age (25–26). Briefly, germ cell suspensions were obtained by sequential collagenase-hyaluronidase-trypsin digestions of freshly withdrawn testes from 20 animals. To release cells completely, after the trypsin treatment, the pellet was resuspended in 1 ml of culture medium and then brought to 20 ml with culture medium adding 2 mg/ml DNase and 10% fetal calf serum. Cell suspension was plated in Petri dishes (5 ml/dish) for 3 h in a humidified incubator at 32 °C.

**RESULTS**

DNA synthesis was studied by [3H]thymidine incorporation followed by autoradiography as previously described (17). In these experiments, incubation with [3H]thymidine was performed during the last 4 h of the 24 h culture period.

**Western Blot Analysis and Antibodies**—Cells were harvested and homogenized in 40 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 10 mM β-glycerophosphate, 0.1 mM sodium vanadate, and 1/100th (v/v) of a pre-formed protease inhibitors mixture (P8340, Sigma). Total cellular proteins were transferred to polyvinylidene difluoride membranes after SDS-PAGE. Membranes were blocked with PBS buffer containing 5% fat-free milk and 0.1% Tween 20 for 1 h at room temperature and then hybridized with primary antibodies. After hybridization with secondary antibodies conjugated to horseradish peroxidase, the immunocomplexes were detected with Supersignal West Pico detection reagent (Pierce). Primary antibodies used are as follows: anti-phospho-Akt and anti-Akt rabbit polyclonal antibody (Ser-473) antibody kit, catalog number 9270, New England Biolabs Inc.; anti-phospho Erk1/2 mouse monoclonal antibody (sc-7383, Santa Cruz Biotechnology, Inc.); anti-c-Myc rabbit polyclonal antibody (sc-788, Santa Cruz Biotechnology, Inc.); anti-phospho Erk1/2 mouse monoclonal antibody (sc-7383, Santa Cruz Biotechnology, Inc.); anti-cyclin D3 mouse monoclonal antibody (sc-6283, Santa Cruz Biotechnology, Inc.); anti-cyclin E rabbit polyclonal antibody (sc-6283, Santa Cruz Biotechnology, Inc.); anti-cyclin A2 rabbit polyclonal antibody (sc-751, Santa Cruz Biotechnology, Inc.); anti-Rb (aa 332–344) mouse monoclonal antibody (14001A, PharMingen); anti-Rb (carboxyl terminus) rabbit polyclonal antibody (sc-50, Santa Cruz Biotechnology Inc.); anti-phospho-Akt and anti-Akt rabbit polyclonal antibody (catalog number sc-6246, Santa Cruz Biotechnology, Inc.); anti-c-Myc rabbit polyclonal antibody (sc-788, Santa Cruz Biotechnology, Inc.); anti-cdk4 goat polyclonal antibody (sc-260-G, Santa Cruz Biotechnology, Inc.).

**Immunoprecipitation and Cdk5 Kinase Assays**—2 x 10⁶ viable cells were harvested and homogenized in 40 μl of a modified lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10%
TABLE I  
**MEK- and PI3K-dependent stimulation of DNA synthesis and cell cycle progression induced by SCF in spermatogonia from 8-day-old mice cultured for 24 h**

|                | Control | SCF | U0126 | LY-294002 | SCF + U0126 | SCF + LY-294002 | AG490 | SCF + AG490 |
|----------------|---------|-----|-------|-----------|-------------|-----------------|-------|------------|
| % ³H-labeled cells  
(n = 8)         | 19.58 ± 3.87 | 34.90 ± 2.01 | 19.52 ± 3.13 | 18.92 ± 1.63 | 15.02 ± 5.44 | 13.92 ± 4.15 | 23.57 ± 2.98 | 43.92 ± 0.82 |
| % cells in metaphase  
(n = 5)          | 1.08 ± 0.31 | 2.94 ± 0.50 | 0.62 ± 0.24 | 0.62 ± 0.21 | 1.12 ± 0.32 | 1.31 ± 0.30 | ND   | ND         |

`a` Percentage ± S.D. of ³H-thymidine labeled nuclei observed out of 500 cells in autoradiographic slides. See Ref. 17 for further experimental details.

`b` Statistical analysis (analysis of variance test) was performed using a program PSI-plot 3.0 from Polysoftware International, p values versus control, p < 0.0001.

`c` Statistical analysis (analysis of variance test) was performed using a program PSI-plot 3.0 from Polysoftware International, p values versus control, p < 0.5.

`d` Percentage ± S.D. of cells with nuclei showing condensed metaphase chromosomes out of 500 cells stained with Giemsa (see Fig. 1).

`e` Statistical analysis (analysis of variance test) was performed using a program PSI-plot 3.0 from Polysoftware International, p values versus control, p < 0.01.

`f` ND, not determined.

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**RESULTS**

**SCF Stimulates Cell Cycle Progression of c-kit Expressing Spermatagonia through Both MEK and PI3K Signals**—Cultures of germ cells obtained from 8-day-old mice are particu-
larly enriched in differentiating spermatogonia (25, 26), which express high levels of c-kit (5–8, 14). Fig. 1 shows that, after 24 h of culture, several nuclei with characteristic features of apoptosis, such as reduced size and intense chromatin staining, can be observed in untreated cells. In SCF-treated cultures, the frequency of such cells is clearly reduced (see the last paragraph of this section), and a clear increase in the number of mitotic figures (nuclei showing condensed metaphase chromosomes) can be appreciated. These data confirm that SCF is required to maintain the proliferative state of differentiating spermatogonia cultured in vitro (17, 24).

We studied DNA synthesis and cell cycle progression in these cultures by using [3H]thymidine incorporation and metaphase counting. SCF induces a 2-fold increase in the number of [3H]thymidine incorporating cells and a 3-fold increase of metaphase counts with respect to the control after 24 h of culture (Table I). We also analyzed the effects of SCF addition in germ cell populations from 5- to 6-day-old mice, when undifferentiated spermatogonia are the predominant cell types (25, 26), and c-kit expression is not detectable (5, 7, 8, 27, 28). No stimulation of cell cycle progression was observed in these cells (% of 3H-labeled cells in control cultures, 9.20 ± 0.05; in SCF-treated cultures, 9.40 ± 1.85; % cells in mitosis in control cultures, 1.25 ± 0.35; in SCF-treated cultures, 0.70 ± 0.30).

c-kit signaling pathways activated in cell cycle progression have been shown to involve PI3K, MEK, and Janus-activated kinase 2 (JAK2) in different cell types (14, 15, 29, 30). In mouse spermatogonia, PI3K activation has been shown to be involved in SCF-dependent proliferation (14, 15, 24); however, the possible involvement of MEK- and JAK2-dependent pathways has not been studied. To investigate whether these c-kit-activated signaling pathways mediate the mitogenic activation of spermatogonia observed in vitro, the proliferation assays were performed in the presence of inhibitors selective for each of the three different pathways: the MEK inhibitor U0126, the JAK2 inhibitor tyrphostin AG490, and the PI3K inhibitor LY294002 (Table I). The inhibition of the MEK pathway abolished the SCF-induced increase in both [3H]thymidine incorporation and metaphase counts, demonstrating that the integrity of this pathway is required for SCF induction of mitogenesis. Inhibition of PI3K pathway also abolished SCF mitogenic effect, indicating that both MEK and PI3K pathways are required. On the contrary, inhibition of JAK2 signaling had no effect on SCF-stimulated [3H]thymidine incorporation.

**SCF Activates Both Extracellular Signal-regulated Kinases (Erk)1/2 and Akt Kinases in c-kit-expressing Spermatogonia**

Since the MEK and PI3K inhibitors were effective in the inhibition of SCF-induced proliferation of spermatogonia, we studied the Erk1/2 and PI3K activation pathways induced by SCF.
Expression of cyclins specifically expressed during the G1/S phase by Western blot. SCF addition did not modify the levels of cyclin D3, a D-type cyclin that is predominantly expressed in proliferating spermatogonia (31, 32), at any time point studied (Fig. 3A), nor c-Myc levels (Fig. 3B), which are often up-regulated during mitogenic stimulation in other cell types (20–23). However, the levels of cyclin E were up-regulated after 1 h from SCF addition and decreased after 3 h (Fig. 3C). Cyclin A2, which is expressed in proliferating spermatogonia (33), was up-regulated between 10 and 16 h after SCF addition, and it returned to the control levels after 24 h (Fig. 3D).

To exclude the possibility that in vivo exposure of spermatogonia to endogenous SCF had already caused sustained expression of cyclin D3 prior to their isolation for the in vitro culture, we stimulated spermatogonia with SCF following an overnight incubation in the absence of the growth factor. Even under these conditions, cyclin D3 did not increase upon SCF stimulation at any time point checked (Fig. 3E). The levels of cyclin A2, however, reached a maximum about 12 h after SCF addition (Fig. 3F).

**SCF Treatment Causes a Transient Increase in the Activity of Cyclin D3- and Cyclin E-associated Kinase Activities**—The increase of cyclin E and subsequently of cyclin A2 levels in the absence of detectable cyclin D3 quantitative modifications prompted us to investigate whether the activity of cyclin D3- cdk4 complex was affected by SCF addition. It is known that D-type cyclins induced by growth factors activate cyclin-dependent kinases (cdk4 and cdk6) to initiate Rb phosphorylation, which is then completed by cyclin E/ cdk2 and cyclin A/ cdk2 (20–23). Western blot analysis of spermatogonial extracts with a mouse cross-reactive monoclonal anti-Rb antibody directed against aa 332–344 of human Rb showed that hyper-
phosphorylation of Rb (the slower migrating bands) started to be detectable as early as 2 h after SCF addition and reached a plateau after 16 h (Fig. 4A). Similar results were obtained using a polyclonal anti-Rb antibody directed against a peptide corresponding to 15 aa at the carboxyl terminus of human Rb (data not shown). This indicates that the different mobility of Rb in SCF-treated and control samples is actually due to changes in the phosphorylation state of Rb and not to a change in its molecular size due to proteolytic cleavage at the carboxyl terminus (a phenomenon which is often associated to apoptotic death in some cell types). Rb was timely hyperphosphorylated after SCF treatment also in spermatogonial cultures that had been subjected to overnight growth factor deprivation (Fig. 4B).

In order to test whether the increase of Rb phosphorylation was due to cyclin D3/cdk4 activation by SCF, cell extracts from spermatogonia were immunoprecipitated with anti-cyclin D3 antibodies, and then a kinase assay using GST-Rb as a substrate was performed. Fig. 5A shows a significant increase of cyclin D3-associated Rb-kinase activity after 1 h of stimulation with SCF, which became less evident after the 2nd h of culture. As a control, we performed similar kinase assays on cyclin D3 immunoprecipitates using histone H1 as a substrate, and no stimulation of H1 phosphorylation was observed after SCF addition (data not shown).

U0126 or LY294002 abolished SCF-induced stimulation of cyclin D3-associated Rb kinase activity (Fig. 5B), but they did not modify total cellular levels of cyclin D3 and cdk4 (Fig. 5C). Thus, both the MEK and the PI3K pathways converge at the level of regulation of cyclin D3-dependent kinase activity, rather than at the level of cyclin D3 or cdk4 synthesis or stabilization.

As expected from the time course of cyclin E accumulation in SCF-treated cells (Fig. 3C), cyclin E/cdk2 kinase activity, monitored using histone H1 as a specific substrate, was strongly induced after 1 h in the presence of SCF and decreased to the control levels after 4 h of stimulation (Fig. 5D). SCF-induced cyclin E accumulation was also abolished after pretreatment with either U0126 or LY294002 (Fig. 5E).

SCF Induces Nuclear Localization of Cyclin D3 through MEK- and PI3K-dependent Pathways—The subcellular localization of cyclin D3 was studied by immunofluorescence experiments. As shown in Fig. 6, the immunofluorescence staining of spermatogonia using an anti-cyclin D3 antibody revealed that SCF induced a marked increase in nuclear localization of the
SCF-induced nuclear accumulation of cyclin D3 in spermatogonia is coincident with a transient induction of its associated histone H1 kinase activity, followed by induction of cyclin A2 (a marker of the S phase) at later times, and progressive hyperphosphorylation of Rb.

The observation that SCF mitogenic stimulus provokes such a rapid activation of the G1/S transition in differentiating spermatogonia is in agreement with pioneering autoradiographic studies by Monesi (37) on DNA synthesis in these cells, showing that duration of the “resting phase preceding DNA synthesis” (i.e., the G1 phase) is very short, ranging between 2 and 3 h.

Inhibition of either MEK or PI3K signaling completely abolished SCF-induced increases in nuclear localization of cyclin D3, cyclin D3-associated Rb-kinase activation, cyclin E induction, and cell cycle progression in c-kit-expressing spermatogonia. Thus, the contemporaneous activation of both these pathways by SCF is essential to trigger G1/S transition in these cells.

MEK and PI3K cooperation in promoting cell proliferation has been explained by the observation that MEK-dependent Erk stimulation often promotes the synthesis whereas PI3K-dependent Akt activation leads to the stabilization of D-type cyclins (20–23). Here we show a novel effect of the cooperation between these two pathways, culminating in modulation of the subcellular localization, rather than of total cellular levels, of a D-type cyclin.
It has been shown that Erk activation can trigger a transient induction of p21Cip1/Waf1 (38), which in turn can play a positive role in the assembly, in the nuclear translocation, and in the activation of cyclin Dcdk4/6 complexes (39). However, we found that p21Cip1/Waf1 is barely detectable in spermatogonia at early times of culture, and SCF treatment does not cause any increase in its cellular levels (data not shown). Alternatively, Erk-dependent pathways might regulate phosphorylation of cyclin D3 residues homologous to Thr-156 of cyclin D1, whose mutation is known to inhibit nuclear import of the cyclin D1/cdk4 complexes (40).

The nuclear localization of D-type cyclins is also regulated by the PI3K pathway through the inhibition of glycogen synthase kinase 3β exerted by Akt. Indeed, inhibition of glycogen synthase kinase 3β-dependent phosphorylation of cyclin D1 at the Thr-286 residue is coupled to the maintenance of nuclear localization of this cyclin during the G1/S transition (41–42). We suggest that a similar mechanism may regulate cyclin D3 localization in response to SCF.

It has been reported that mouse spermatogonia isolated from 5-day-old mice and propagated on a feeder layer for an undefined period express higher levels of cyclin D3 when stimulated with SCF, and this would correlate with stimulation of DNA synthesis (24). In the present study we demonstrate that primary cultures of spermatogonia freshly isolated from 8-day-old mice are fully responsive to SCF, but no increase in cyclin D3 levels can be observed, even when SCF treatment is performed after overnight growth factor deprivation. Moreover, we found that freshly isolated spermatogonia from 5- to 6-day-old mice are not stimulated by the growth factor. The possibility exists that, even though the cell population used by Feng et al. (24) should not express c-kit at the beginning of culture (5, 7), it could eventually acquire SCF responsiveness during the coculture period.

Our data are in agreement with two recent reports (14, 15) in which mutant mice were generated in which the c-kit codon tyrosine 719 (the docking site for the p85 subunit of PI3K) was altered. It has been reported that mouse spermatogonia isolated from 8-day-old mice after 24 h of culture. However, the anti-apoptotic effect observed in vitro was not inhibited by the separate addition of either the MEK- or of the PI3K inhibitor, whereas both inhibitors on their own can impair the mitogenic response. Thus, the Erk1/2 activation and the PI3K-mediated Akt activation that we observed in cultured spermatogonia are not essential for SCF inhibition of apoptosis. Distinct SCF-activated signal transduction pathways must be involved in the pro-survival response, since even the simultaneous addition of both MEK- and PI3K inhibitors does not completely suppress SCF anti-apoptotic effect. In agreement with our in vitro observations, abolishment of c-kit-mediated PI3K signaling in c-kit Y719F knock-in mice was not associated to increased apoptosis in spermatogonia at 8 days of age (14).

In conclusion, our data indicate that soluble SCF stimulates proliferation of c-kit-expressing and differentiating type A1 spermatogonia in vitro through both MEK- and PI3K-dependent pathways, by triggering nuclear relocation of cyclin D3 and a rapid G1/S transition. Moreover, they show that the SCF-mediated proliferative and survival effects on spermatogonia depend on the activation of different combinations of intracellular signal transduction pathways.

TABLE II

| SCF-triggered inhibition of spontaneous apoptosis in spermatogonia from 8-week-old mice cultured for 24 h is partially impaired by the simultaneous block of MEK and PI3K inhibitors |
|---------------------------------|
| Control |
| SCF + U0126 |
| SCF + LY294002 |
| SCF + U0126 + LY294002 |
| SCF + AG490 |
| % apoptotic cellderived * (n = 5) || 38.42 ± 7.06 | 16.52 ± 3.71a | 16.15 ± 2.92b | 15.81 ± 4.30b | 27.33 ± 1.77c | 12.35 ± 4.02b |

* Percentage ± S.D. of cells showing TUNEL-positive staining observed out 500 cells in slides subjected to in situ detection of cell death (see Fig. 7).

** Statistical analysis (analysis of variance test) was performed using a program PSI-plot 3.0 from Polyswift International. p values versus control: p < 0.005.

* Statistical analysis (analysis of variance test) was performed using a program PSI-plot 3.0 from Polyswift International. p values versus control: p < 0.05.

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Signaling through Extracellular Signal-regulated Kinase Is Required for Spermatogonial Proliferative Response to Stem Cell Factor

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