Stem cell factor activates telomerase in mouse mitotic spermatogonia and in primordial germ cells

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Summary
The discovery of sterility in the descendants of telomerase-null mutant mice, owing to the lack of spermatogonia proliferation, has drawn attention to the role of telomerase activity in mouse spermatogenesis. Since spermatogonia proliferation is under Kit ligand control, we explored its possible role in the regulation of telomerase activity. We show that Kit ligand induces telomerase activity in mitotic spermatogonia and increases the mRNA levels of both the catalytic subunit form and the telomerase RNA template. The increase of telomerase activity by Kit ligand is blocked by the presence of the PI3K inhibitor LY294002. Kit-positive proliferating male primordial germ cells (PGCs) show low levels of telomerase activity, but they increase telomerase activity upon Kit ligand stimulation. Diplotene-arrested growing oocytes that re-express Kit do not increase telomerase activity upon Kit ligand stimulation. Our data suggest that the induction of telomerase by Kit ligand may contribute to the self-renewing potential of male germ cells and of PGCs.

Key words: Kit, Kit ligand, Telomerase, Germ cells, Meiosis, Proliferation, PI3K

Introduction
Telomerase is a ribonucleoprotein that adds hexameric repeats to the mammalian telomeres to compensate for the loss of basepairs that occurs after subsequent rounds of DNA replication. This enzyme consists of an RNA-dependent DNA polymerase (TERT), which synthesizes the hexameric repeats on the 3’ end of the telomeres using a RNA molecule (TR) as a template (Blackburn et al., 1991; Greider et al., 1996). Progressive shortening of the telomeric ends, which occurs in the absence of telomerase, has been suggested to act as a mitotic checkpoint that contributes to cell senescence and mortality (Harley, 1991). Telomerase activity has been shown to be present in human and mouse tumors, immortalized cell lines, stem cells of self-renewing tissues and germ cells (Kim et al., 1994; Wright et al., 1996). Human somatic cells do not express telomerase activity, whereas most mouse somatic cells express detectable amounts of telomerase (Kipling, 1997). However, homologous recombination of the TR gene does not alter the normal phenotype of the knock-out mice, and primary cells obtained from these animals can be oncogenically transformed after tumorigenic viral infection (Blasco et al., 1998), which may suggest that telomerase activity may not be limiting for tumorigenesis in the murine system. Interestingly, after several generations of breeding, the telomerase-null mice develop progressively worse effects on the reproductive and hematopoietic organs (Lee et al., 1998). In particular, homozygous male mice show a reduced testis weight compared with control animals, absence of germ cells within the tubules, impairment of long term renewal of hematopoietic stem cells and increased apoptosis of activated splenocytes, suggesting that telomerase is involved in the control of normal cell growth and survival.

In the mouse testis, telomerase activity has been reported mainly in proliferating spermatogonia (type A spermatogonia), and it is downregulated in the differentiating spermatocytes and spermatids and is no longer present in spermatozoa (Ravindranath et al., 1997; Eisenhauer et al., 1997). As a result of telomerase activity, sperm cells have long telomeres, of about 10-20 kb in humans and 50 kb in mice, that apparently do not shorten with the aging of the organism (Allsopp et al., 1992). Type A spermatogonia are pluripotent stem cells in the testis, which undergo many rounds of duplications giving rise to type B spermatogonia and to other type A spermatogonia. The mechanisms of germ cell mitogenesis are poorly understood, but we have recently shown that stem cell factor (Kit ligand, Kitl) can induce 3H-thymidine incorporation in type A, Kitexpressing proliferating spermatogonia (Rossi et al., 1993). In light of the recent reports that spermatogonia lacking telomerase undergo arrest of mitosis and apoptosis, we investigated whether Kitl is able to regulate spermatogonial stem cell telomerase activity. Since Kitl is a survival/proliferation factor for proliferating primordial germ cells (PGCs) but not for Kit-expressing growing oocytes, we investigated its role in regulating telomerase activity in these cell types.

Materials and methods
Germ cells isolation
Male PGCs were obtained from 12.5, 14.5 and 15.5 day post-coitum (dpc) CD-1 embryos according to De Felici and McLaren (De Felici and McLaren, 1982). Briefly, gonads were collected in PBS and...
incubated for 15 minutes in PBS-EDTA. Germ cells were released in PBS+BSA (1 mg/ml) by puncturing the gonads with fine needles under a stereomicroscope. Cells were then collected by a mouth-operated micropipette and cultured in suspension for 24 hours in 10% FCS with D-MEM added, in the presence or absence of 100 ng/ml Kitl. An equal number of surviving cells, which were trypan-blue negative, at each experimental point were immediately frozen. Contaminating somatic cells were less than 10% of the total, as judged by both alkaline phosphatase staining and nuclear morphology of Giemsa-stained cells. Growing oocytes were obtained from 10 day post-natum (dpm) mice by puncturing ovaries with fine needles under a stereomicroscope. At the beginning of the culture (T0) and after 24 hours of culture in the presence or absence of Kitl, groups of 15 viable oocytes were collected in 5 μl of PBS+BSA and immediately frozen. The occurrence of germinal vesicle breakdown (GVBD) was observed under a stereomicroscope.

Spermatogonia were obtained from 7-8 days old Swiss CD-1 mice, as previously reported by Rossi et al. (Rossi et al., 1993). Briefly, germ cell suspensions were obtained by sequential collagenase-hyaluronidase-trypsin digestions of freshly withdrawn testes. A 3 hour period of culture in 10% FCS with E-MEM added was performed to facilitate adhesion of contaminating somatic cells to the plastic dishes. At the end of this pre-plating treatment, which was considered to be T0, enriched germ cell suspensions were rinsed from FCS, and spermatogonia were then cultured in E-MEM supplemented with 2 mM Na-pyruvate and 1 mM Na-lactate in the presence or absence of Kitl (100 ng/ml, Genzyme). To obtain mitotic indexes, nuclear morphologies from at least 10^3 cells per treatment were assessed by microscope observations of the culture dishes previously fixed in 3:1 methanol-acetic acid and stained with Giemsa, according to Meistrich et al. (Meistrich et al., 1973). The purity of the spermatogonia was about 80% after the pre-plating treatment. During the 24 hours of culture, the contaminating somatic cells attached to the dish, whereas spermatogonia did not adhere and were recovered in the suspension at a purity higher than 90%. At different times of culture an equal number of surviving cells, judged as trypan-blue negative, have been fixed or frozen to analyse nuclear morphology, telomerase RNA template expression by RT-PCR, in situ hybridization and telomerase activity.

Where indicated, cells were also incubated for 1 hour before Kitl addition with 10 μM LY294002 (#270-038-M005, Alexis).

**Probe preparation for in situ hybridization**

The plasmid-containing human telomerase RNA template sequences (560 bp) was kindly provided by Geron Corp., Menlo Park, CA, USA. To generate sense and antisense riboprobes, the plasmid was linearized by NheI or EcoRV, respectively. 32S-UTP-labeled single-stranded RNAs were synthesized according to the manufacturer’s (Stratagene) conditions. Transcripts were then alkali hydrolyzed to generate probes of an average length of 200 nucleotides and then ethanol precipitated. The probes were then dissolved to a final concentration of at least 5×10^5 cpm/μl in 10 mM DTT.

**In situ hybridization**

Cells were seeded onto poly-L-lysine-coated slides, fixed in 4% paraformaldehyde and then air-dried. The hybridization procedure was performed according to Jannini et al. (Jannini et al., 1994). Briefly, cells were incubated overnight at 50°C in hybridization solution containing 5×10^6 cpm/μl of the telomerase probe. The day after, slides were incubated in successive formamidine/SSC washes and then dehydrated and exposed to Kodak NTB-2 emulsion for 2 weeks. The microautoradiographs were then developed and counter-stained with Toluidine blue.

**TRAP assay**

This method is based on PCR amplification of telomerase extension products and was performed as previously described (Piatyszek et al., 1995). Briefly, cells were lysed in 400 μl of ice-cold extraction buffer (0.5% 3-(cholamidopropyl)-dimethyl ammonio-1-propanesulfonate, 10 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 5 mM mercaptoethanol, 0.1 mM 4-(2-aminoethyl)-benzene-sulfonfluoride hydrochlorolrine and 10% glycerol). To increase the extraction strength of the lysis buffer, experiments performed with fetal germ cells were carried out using a modified extraction buffer according to Norton et al. (Norton et al., 1998). Preliminary assays were carried out in order to assess the relationship between the concentration of cell extracts and the amount of TRAP product obtained, which was not at saturation using 200 cells. 4 μl of the cell extracts, corresponding to 200 viable cells, was then used for the spermatogonia and fetal male germ cells TRAP assays in 50 μl of the reaction mixture consisting of 20 mM Tris-HCl (pH 8.3), 68 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 0.05% Tween 20, 0.1 g of TS (AATCCGTCCAGCAGAGTT) primer, 0.5 M T4 gene 32 protein, 50 μM of each deoxyribonucleotide triphosphate, 2 units of Taq DNA polymerase and 0.2 μl of 32P dCTP (3000 Ci/ml, DuPont NEN Research Products, Boston, MA). Each reaction was carried out in a single PCR tube containing 100 ng of CX-Ext oligonucleotide (5'-GTGCCCTTACCCTTACCCCTTACTACC-3') sealed at the bottom of the tube by a wax barrier. Samples were incubated at room temperature for 20 minutes to allow telomerase to extend TS primer, followed by 31 cycles of PCR amplification of the telomeric products. 45 μl of the reactions was loaded on a 10% non-denaturing polyacrylamide gel and identified by autoradiography. 6 μg/ml of RNase A (Boehringer Mannheim) was added to the reactions to confirm the specificity of the telomerase products, which disappeared in the presence of RNase A. The TRAP assay on oocytes was performed according to Eisenhower et al. (Eisenhower et al., 1997). Cell extracts corresponding to 15 oocytes for each treatment were used in each assay.

An assay of alkaline phosphatase activity as an internal control for the quality of the cell extracts performed using a commercially available kit (SIGMA) as previously described (Fiyatsek et al., 1995).

**RT-PCR experiments**

1 μg of total RNA, obtained from an equal number of viable spermatogonia after 24 hours of culture, in the presence or absence of 100 ng/ml of Kitl, was reverse transcribed by Mo-MuLV reverse transcriptase using random hexamers in 20 μl of reaction mixture (ImpronIT reverse transcription system, Promega). To discriminate bands originating from contaminating DNA, another set of reactions was performed omitting the reverse transcriptase. Preliminary RT-PCR experiments were performed for each set of primers in order to evaluate the conditions under which PCR amplification was in the logarithmic phase. Step cycle amplifications were performed diluting 1 μl of the cDNAs in 50 μl of PCR mixture (Taq polymerase, Promega) using TERT and TR murine primers (mTERT and mTR respectively) designed on the basis of the published sequences: mTERT A (5'-CTCTGTGCGCCGCAAGCCAGTACC3'); mTR B (5'-ACGTGAAGG CACACTTGTA-CT-3') (Greenberg et al., 1998); mTR A (5'-ACTTCCAGCGGGCAGAAAGCT-3'); and mTR B (5'-CTGACAGGGCCGCTCTTTC (Blasco et al., 1998), Hprt (Rossi et al., 1993) and Kit-specific primers (5'-TATGGCATAGGAACCCGGCCTGAG3; 3'-CATCTCTGATGTCCTGCGTCG) for the expected size of the amplified band is 293 bp) were used as internal controls of gene expression. The conditions for amplification of mTERT were 35 cycles at 94°C for 1 minute, 58°C for 30 seconds and 72°C for 45 seconds, and the expected size of the amplified band is 776 bp. The conditions for amplification of mTR were 35 cycles at 94°C for 1 minute, 52°C for 30 seconds and 72°C for 30 seconds; expected size of the amplified band is 183 bp). Conditions for Hprt and Kit
amplifications were 30 cycles at 94°C for 1 minute, 58°C for 30 seconds and 72°C for 45 seconds.

**Results**

**Kitl modulates telomerase in type A spermatogonia**

Telomerase activity has recently been demonstrated in mitotic spermatogonia, and its downregulation has been shown in the more differentiated germ cells (spermatocytes and spermatids) (Ravindranath et al., 1997). To investigate whether Kitl was able to regulate telomerase activity in spermatogonia, cell extracts obtained from freshly isolated spermatogonia or following culture in the presence or absence of Kitl were analysed using the TRAP assay (Fig. 1A). A high level of telomerase activity was detected in freshly isolated spermatogonia, which showed a mitotic index of 6.1% (±2.5), whereas after 24 hours of culture a significant decrease in the activity was observed in the surviving cells, which showed a mitotic index of 1.8% (±1). The presence of Kitl in the medium was able to maintain the telomerase activity almost at the same level of the freshly isolated spermatogonia, upregulating the enzyme activity and the mitotic index (5.4±0.5) with respect to the control. The increase of telomerase activity following Kitl treatment was not caused by an increase in spermatogonia survival, as an equal number of trypan-blue-negative cells were analysed in each experiment. Since the spermatogonial population that we used in these experiments includes the whole range of spermatogonial differentiation stages (undifferentiated, typeA, intermediate and typeB), it cannot be excluded that the relative ratios of the subpopulations might change upon culture. Addition of RNase A to the reactions completely abolished the 6 bp ladder typical of telomerase activity. When cell extracts were tested for the presence of alkaline phosphatase activity, an enzyme that has stability similar to telomerase (Pyitiaszek et al., 1995), comparable levels of activity were found in all the samples tested (data not shown). To investigate if the induction of telomerase activity by Kitl was caused by a net increase and not just by maintenance of the activity observed in the freshly isolated cells, we starved spermatogonia for 24 hours and then added Kitl. It was possible, in fact, that at the beginning of the culture we were using cells that had already been exposed in vivo to the stimulation of endogenous Kitl. In Fig. 1B, a TRAP assay shows that although starved cells show decreased levels of activity (lane 24h- Kitl) or with the addition of Kitl (48h+ Kitl). Enzyme activity was assessed by the TRAP assay in the presence (+) or absence (−) of RNase A.

**Fig. 1.** Telomerase activity is induced by Kitl in mitotic spermatogonia. (A) Spermatogonial cell extracts were obtained from cells that were freshly collected (T0) or after 24 hour in culture without (control; 24 hr) or with the addition of Kitl. Enzyme activity was assessed by the TRAP assay in the presence (+) or absence (−) of RNase A. (B) Spermatogonial cell extracts were cultured for 24 hours (24h) in the absence of Kitl and then for further 24 hours in culture without (48h- Kitl) or with the addition of Kitl (48h+ Kitl). Enzyme activity was assessed by the TRAP assay in the presence (+) or absence (−) of RNase A.

To verify whether Kitl was also influencing telomerase at the expression level, RNA from spermatogonia cultured for 24 hours with or without Kitl was analysed by RT-PCR using mTR- and mTERT-specific primers. A 183 bp band corresponding to the mTR amplification product was amplified only from Kitl-treated cell; omission of RT in the reverse transcription step resulted in no signals being obtained (Fig. 2A). PCR amplifications using mTERT-specific primers showed a strong increase in the corresponding 776 bp amplified band in the Kitl-treated sample compared with the control (Fig. 2B,C). RNA integrity was assessed by Hprr amplification (Fig. 2B). Kit primers were used to show that equal amounts of RNA extracted from spermatogonia were analysed (Fig. 2C).
mTR expression was also studied by in situ hybridization on spermatogonia immediately following pre-plating (T0) and after 24 hours of culture in the presence or absence of 100 ng/ml of Kitl. The riboprobe was synthesized from a human TR cDNA, which shares 65% sequence homology with the murine gene. In Fig. 3A,E, it is shown that mTR template is strongly expressed in spermatogonia but that such expression is downregulated after in vitro culture (B,F). Kitl addition completely restores mTR template expression (C,G). Panels D and H shows a negative control with a sense probe. The increase of silver grain density in the Kitl-treated cells further confirms that the cells were Kit-expressing spermatogonia, even though, owing to the in situ hybridization procedure, cellular morphology could not be well preserved.

**Telomerase activity is modulated by the PI3-K pathway in mitotic spermatogonia**

Activation of the Kit receptor in mouse spermatogonia has been shown to be mediated by the PI-3K-Akt signal transduction pathway (Blume-Jensen et al., 2000; Kissel et al., 2000). Therefore we studied the effect of the PI-3K inhibitor LY294002 on telomerase activity. Spermatogonia were preincubated for 1 hour in the presence of 10 μM LY294002 and then cultured for 24 hours in the presence or absence of 100 ng/ml Kitl. The presence of the inhibitor slightly influenced the basal levels of telomerase activity; however it blocked the induction mediated by Kitl (Fig. 4) as well as spermatogonia proliferation (10 μM LY294002=1.5%±0.9 versus 10 μM LY294002+Kitl=1.9±0.7) in agreement with our results (Dolci et al., 2001) and those by Feng et al. (Feng et al., 2000).

**Kitl induces telomerase activity in male primordial germ cells**

Prospermatogonia develop within the fetal testis from PGCs. After gonadal colonization, PGCs continue to proliferate, and by 13.5 dpc in the male they enter mitotic arrest, which lasts until few days after birth, and differentiate into prospermatogonia (McLaren, 1983). PGCs express high levels of Kitl receptor (Kit), whereas prospermatogonia do not express Kit until they have differentiated in spermatogonia (Manova et al., 1990). Since telomerase activity is present in mitotically active prepuberal spermatogonia and it is induced by Kitl, we investigated whether mitotically active male PGCs and mitotically arrested prospermatogonia showed telomerase activity. TRAP assays were performed on cell extracts corresponding to 200 trypsin-blue-negative germ cells from 12.5 dpc male gonads (mitotic germ cells),either freshly isolated (T0) or after 24 hours of culture in the absence or presence of 100 ng/ml Kitl, and on germ cell extracts from 14.5 and 15.5 dpc male gonads (arrested prospermatogonia). In Fig.
Telomerase activity is not modulated in Kit-positive oocytes

Kit is also re-expressed in female fetal germ cells when oocytes reach the diplotene stage, throughout oocyte growth and acquisition of meiotic competence (Manova et al., 1993). The function(s) of Kit/Kitl during this period have not been fully understood, although some results indicate that it can play a role in oocyte growth (Manova et al., 1993; Ismail et al., 1996). To test whether telomerase activity in growing oocytes was coupled to Kit activation, a TRAP assay was performed in isolated oocytes freshly collected or cultured for 24 hours in the presence or absence of 100 ng/ml of Kitl. Growing oocytes collected from 10 dpn females were chosen, since at this age they respond to Kitl by increasing their diameter (Packer et al., 1994) but are unable to undergo spontaneous meiotic resumption (GVBD). During this period of culture, Kitl did not influence growing oocyte viability, and cell survival in the two groups was comparable (data not shown). Telomerase activity was present in freshly isolated oocytes, and its levels were stably maintained during 24 hours of culture (Fig. 6). The enzyme activity was not influenced by the addition of Kitl, and all the oocytes were at the GV stage (data not shown).

Discussion

It has been recently reported that the sixth generation of telomerase-null mice show an 80% reduction in testis weight compared with age-matched controls and a complete absence of spermatogenic cells within the tubules (Lee et al., 1998). Furthermore, by the third to fifth generation a marked increase in apoptosis as well as a decrease in proliferation was observed in spermatogonia of the mutant mice, suggesting that telomere shortening might be involved in the regulation of both survival and proliferation of male germ cells in vivo. Kitl is able to induce DNA synthesis in Kit-expressing type A spermatogonia (Rossi et al., 1993; Feng et al., 2000) and to act as an antiapoptotic factor in these cells (Packer et al., 1996; Dirami et al., 1999). In this paper we show that freshly isolated spermatogonia, which are actively proliferating, express high levels of telomerase activity but after 24 hours of culture in the absence of Kitl both their mitotic index and telomerase levels strongly decrease. When spermatogonia are starved in culture and then Kitl added, a net increase in telomerase activity can be observed, suggesting that Kitl is not a maintenance factor but rather an inducing factor of telomerase activity. We recently found that other growth factors such as GDNF (glial derived neurotrophic factor) and Neurturin can induce an increase of 3H-thymidine incorporation in spermatogonia (Viglietto et al., 2000), but the addition of these factors to spermatogonia cultures did not induce telomerase activity with respect to the control (data not shown). Addition of Kitl is able to upregulate both telomerase activity and TERT and TR transcripts compared with the control, which are consistent with an increase of the mitotic index in these cells. Telomerase activity has been shown to be induced by antigen receptor stimulation in early hematopoietic precursor cells and in fully differentiated T and B lymphocytes (Hyama et al., 1995) or by FGF2 in neural precursor cells (Haik et al., 2000). Furthermore mTR has been shown to be dramatically upregulated in mouse fibroblasts following immortalization...
(Blasco et al., 1995). However to date no growth factors have been shown to upregulate both telomerase activity and mTERT and mtTR expression upon induction of proliferation and/or survival on its target cell.

Recently, it has been shown that the activation of one of the PI3K downstream targets, the protein-kinase Akt, enhances human telomerase activity through the phosphorylation of the TERT subunit (Kang et al., 1999). In addition, induction of telomerase activity and proliferation by antigen stimulation in B lymphocytes can be suppressed by the PI3K inhibitor whortmannin (Igarashi and Sakaguchi, 1997). The possibility that Kitl might influence telomerase activity through Kit-mediated PI3K pathway activation in mitotic spermatagonia was evaluated. Treatment of the spermatagonia cultures with the PI3K inhibitor LY42000 did not modify the basal levels of telomerase activity, instead it blocked the increase of enzyme activity induced by Kitl as well as proliferation, indicating that Kit activation of the PI3K downstream signaling can regulate telomerase activity. It is interesting to note that the targeted mutation of the PI3K docking site in the Kit receptor completely blocks spermatagonia proliferation 8 days post-natum (Blume-Jensen et al., 2000; Kissel et al., 2000).

Genetic evidence has shown that mutations in the Steel locus (Sl) encoding Kitl causes multiple stem cell defects in the homozygous mice. On of these effects – germ cell deficiency – is always present during embryonic development. It has in fact been demonstrated that Kitl plays an important role in the establishment of the mouse germline during embryogenesis (Godin et al., 1991; Dolci et al., 1991). Here we show that telomerase activity is also present in the precursors of spermatagonia, that is, in proliferating primordial germ cells, and that such activity is induced by Kitl treatment. At the entry in G1 mitotic arrest, Kitl is downregulated and is no longer induced by Kitl in prospermatogonia. Germline stem cells play a critical role in the reproductive process. Their divisions rely on the regulatory control of several extrinsic signals, including Kitl, which plays a crucial role in germ cell development. Kitl-induced upregulation of telomerase correlates with the stimulation of germ cell proliferation, which is the basis of the self-renewing ability of these cells. This effect appears to be germline specific, since PGCs also upregulate telomerase activity upon Kitl stimulation, whereas primitive human hematopoietic stem cells, which are responsive to Kitl, do not increase telomerase activity after Kitl treatment (Yui et al., 1998). Kitl induction of telomerase activity is also developmentally regulated. In fact, Kit-expressing growing oocytes, which are blocked in the first meiotic prophase, do not show any increase in telomerase activity upon Kitl treatment, and Kit activation does not lead to meiotic progression, suggesting that Kitl-activated signal transduction and telomerase are not coupled in meiotic cells. The PI3K-Akt pathway is probably not required or is redundant in these cells, since targeted mutation of the specific PI3K-docking site of Kit receptor does not affect (Blume-Jensen et al., 2000), or only partly affects (Kisser et al., 2000) female germ cells, although it blocks spermatagonia proliferation. The results we obtained show a strong correlation between mitosis and telomerase activity in the male germline and identify telomerase as a molecular target of a PI3K-dependent pathway induced by Kitl activation in mitotic germ cells from neonatal testis. It will be interesting to investigate whether a PI3K-dependent downstream effector can also regulate telomerase at the expression level. To study this, regulatory sequences have been recently identified in the mouse telomerase gene (Greenberg et al., 1999), and it has been shown that, at least in the human gene, c-myc binds to two regulatory regions located within 300 bp 5’ upstream to the ORF (Greenberg et al., 1999) and induces TERT expression (Wuang et al., 1998; Wu et al., 1999). We are grateful to M. De Felici, P. Grimaldi, E. A. Jannini and P. Rossi for helpful comments on the manuscript. This work has been supported by CNR strategic project ‘Cell cycle and apoptosis’ grant no. 97.04909.ST74 and by MURST National project on ‘Development and differentiation of germ cells’.

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