Rapid Communication

Membrane-Bound Steel Factor Induces More Persistent Tyrosine Kinase Activation and Longer Life Span of c-kit Gene-Encoded Protein Than Its Soluble Form

By Keisuke Miyazawa, David A. Williams, Akihiko Gotoh, Jiroh Nishimaki, Hal E. Broxmeyer, and Keisuke Toyama

Alternative splicing of exon 6 results in the production of two isoforms of Steel factor (SLF): the membrane-bound and soluble forms. To investigate differences in the kinetics of c-kit tyrosine kinase activated by these two isoforms, we used a stromal cell line (SI/SF) established from SI/SI homozygous murine embryo fetal liver and its stable transfectants containing either hSCP248 cDNA (including exon 6; secreted form) or hSCP205 cDNA (lacking exon 6; membrane-bound form) as the source of each isoform. Interaction of factor dependent myeloid cell line M07e with stromal cells producing either isoform resulted in activated c-kit tyrosine kinase and induction of the same series of tyrosine phosphorylated cellular proteins in M07e cells. However, SI'-h220 (membrane-bound form) induced more persistent activation of c-kit kinase than SI'-h248 (soluble form) did. Flow cytometric analysis and pulse-chase studies using [35S]methionine showed that SI'-h248 induced rapid downmodulation of cell-surface c-kit expression and its protein degradation in M07e cells, whereas SI'-h220 induced more prolonged life span of c-kit protein. Addition of soluble recombinant human SLF to SI'-h220 cultures enhanced reduction of cell-surface c-kit expression and its protein degradation. Because the kinetics of c-kit inactivation strikingly fits with the protein degradation rates of c-kit under the conditions described above, rapid proteolysis of c-kit protein induced by soluble SLF stimulation may function as a "turn-off switch" for activated c-kit kinase.

© 1995 by The American Society of Hematology.

Signal Transduction initiated by the interactions of growth factors with specific receptors is an important mechanism of regulating normal cell growth and differentiation. The c-kit proto-oncogene encodes a 145-kD membrane-spanning receptor that is structurally a member of the platelet-derived growth factor (PDGF) receptor family and possesses intrinsic tyrosine kinase activity. The gene for Steel factor (SLF), the ligand for the receptor protein encoded by c-kit, has been cloned and shown to be expressed by bone marrow (BM) stromal cells. The soluble recombinant SLF is a potent stimulatory cytokine that synergizes with a number of cytokines to stimulate growth of hematopoietic progenitors in vitro and blood cell production in vivo in animals. With regard to the initial signaling event leading to a cellular response to SLF, some studies suggest that interaction of SLF with the extracellular domain of c-kit-encoded protein induces receptor dimerization followed by receptor transphosphorylation. This leads to enhanced tyrosine kinase activity and the formation of phosphorytrosine residues that serve as a coded template to direct substrate association and subsequent phosphorylation.

The SI gene encodes a primary translation product of 248 amino acids with a leader sequence and extracellular, transmembrane, and cytoplasmic domains. The resulting protein contains a proteolytic cleavage site encoded by exon 6 sequences (between amino acids 149 and 177) and posttranslational processing at this site leads to the secretion of a biologically active protein (soluble SLF) of 165 amino acids. An alternative spliced cDNA codes for a smaller polypeptide of 220 amino acids that lacks exon 6 sequences including the proteolytic cleavage site and, hence, results in a membrane-bound protein. Several lines of evidence support the physiologic importance of the membrane-bound form of SLF in vivo: The viable SI-Dickie mutant in which the genomic regions encoding the transmembrane and cytoplasmic domains are deleted can only produce the secreted form of SLF, but shows all the pleiotropic defects seen in SI/SI mutants. A study of mast cell attachment to fibroblasts derived from SI/SI mice showed that the extracellular domain of the membrane-bound SLF was required to mediate this attachment. Adhesion of human megakaryocytes to BM stromal fibroblasts has also been shown to mediate in part the interaction between membrane-bound SLF and c-kit protein. In addition, using stable gene-transfected stromal cell lines that selectively produce either the membrane-bound or the secreted form of SLF, it has been shown that the membrane-bound form of SLF supports hematopoiesis in a long-term culture system longer than secreted protein. However, the mechanism by which these biologic differences are induced by these isoforms remains to be clarified and the physiologic role(s) of each isoforms are not yet known.

We and others have previously reported that treatment of the factor-dependent myeloid cell line M07e with soluble recombinant (r)SLF leads to transient tyrosine phosphorylation of cellular proteins including the 145-kD c-kit gene-encoded protein. In addition, this c-kit protein was rapidly polyubiquitinated, internalized, and degraded after treatment...

From the First Department of Internal Medicine (Hematology and Oncology), Tokyo Medical College, Tokyo, Japan; Howard Hughes Medical Institute, Herman B Wells Center for Pediatric Research, Riley Hospital for Children; Walther Oncology Center, and Indiana University School of Medicine, Indianapolis.

Submitted July 22, 1994; accepted November 2, 1994.
Supported by a Grant-in-Aid for Encouragement of Young Scientists, from the Ministry of Education, Science and Culture of Japan to K.M. and by US Public Health Service Grant RO1 HL46528 to D.A.W., and R37CA36464, RO1 HL46549, and RO1 HL49202 to H.E.B. from the National Cancer Institute and National Institutes of Health.

Address reprint requests to Keisuke Miyazawa, MD, First Department of Internal Medicine, Tokyo Medical College, 6-7-1, Nishishinjuku, Shinjuku-ku, Tokyo 160, Japan.

The publication costs of this article were defrayed in part by charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1995 by The American Society of Hematology.

Blood, Vol 85, No 3 (February 1), 1995; pp 641-649
ment with soluble rSLF stimulation. This suggested that ligand stimulation shortens the life span of c-kit protein and this may function as a negative regulatory feedback loop for activated c-kit protein. Therefore, we compared the effect of the secreted and membrane-bound forms of SLF regarding the kinetics of c-kit kinase and the degradation rate of c-kit protein. These studies were done with M07e cells because in many ways this cell line responds to SLF as do normal cells and also because due to the very low frequency of hematopoietic stem and progenitor cells in the main tissue source of these cells, BM, and it is not yet possible to obtain purified stem and progenitor cells to do these studies. The results showed that the ligand-induced active state of c-kit kinase persisted longer with the membrane-bound SLF than with the soluble form in M07e cells. Additionally, the length of the c-kit protein life span correlated well with the kinetics of c-kit kinase activity after stimulation with each isoform of SLF and the half-life of c-kit protein was longer after stimulation with the membrane-bound isoform. These data suggest that ligand-induced proteolysis of the c-kit protein may function as a regulatory mechanism of c-kit kinase. The data presented may also explain at least some of the biologic differences between the membrane-bound and soluble forms of SLF.

MATERIALS AND METHODS

Cytokines, antibodies, and reagents. Highly purified recombinant human (rhu) SLF and rhu granulocyte macrophage colony-stimulating factor (GM-CSF; 4 × 10^7 U/mL) were kindly provided by Dr Douglas E. Williams (Immunex Corp, Seattle, WA). Mouse anti-c-kit monoclonal antibody (MoAb), YB5.B8, which recognizes the extracellular epitope of the human c-kit gene product, was a gift from Dr Leonie K. Ashman (Hanson Center for Cancer Research, Adelaide, South Australia). Rabbit anti-c-kit polyclonal antibody (4G10) and agarose-conjugated 4G10 were purchased from Upstate Biotechnology Inc (Lake Placid, NY). Alkaline phosphatase conjugated goat-antirabbit IgG (H+L) and goat-antimouse IgG (H+L) were obtained from Zymed Laboratories, Inc (San Francisco, CA).

Cell lines. The human growth factor-dependent cell line, M07e, was a gift from Aggie Ciarletta (Genetic Institute, Boston, MA). This cell line responds to human GM-CSF and interleukin-3 (IL-3) from Dr Leonie K. Ashman (Hanson Center for Cancer Research, Uniondale, NY). Mouse antiphosphotyrosine MoAb (4G10) was obtained by heterozygote crosses. The generation of S1/S1-h220 or soluble SLF (S1/S1-h248) has been previously described. The biologic characteristics of this cell line and culture conditions have been previously described. The human growth factor-dependent cell line, M07e. This cell line responds to human GM-CSF and interleukin-3 (IL-3) from Dr Leonie K. Ashman (Hanson Center for Cancer Research, Uniondale, NY). The human growth factor-dependent cell line, M07e. This cell line responds to human GM-CSF and interleukin-3 (IL-3) from Dr Leonie K. Ashman (Hanson Center for Cancer Research, Uniondale, NY). The human growth factor-dependent cell line, M07e.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were performed as previously described. Briefly, cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were electrophoretically transferred onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking residual binding sites on the transfer membrane by incubation with TBST (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% glycerol, 1% N antibiotic, 10 mmol/L EDTA, 10 μg/mL leupeptin, 100 mmol/L sodium fluoride, 2 mmol/L sodium orthovanadate) at 4°C for 30 minutes. Cell lysates were clarified by centrifuging for 30 minutes at 10,000g at 4°C. Total protein content of the lysate was determined by a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). Cell lysates were used for immunoprecipitation and immunoblotting.

Metabolic labeling. For metabolic labeling with [35S]methionine, exponentially growing M07e cells were washed and resuspended at 10^6 cells/mL in methionine-free RPMI-1640 medium containing 5% FBS and incubated for 1 hour. The cells were then resuspended (10^6 cells/mL) in methionine-free RPMI-1640 medium containing 100 μCi/mL [35S]methionine (specific activity >800 Ci/mmol; Amersham Corp, Buckinghamshire, UK), 10% FBS, and 100 U/mL rhuGM-CSF and incubated for 3 hours at 37°C. After metabolic labeling, the cells were washed three times and resuspended in complete medium containing a 10-fold molar excess of unlabeled methionine and incubated for 1 hour to wash out free [35S]methionine. For metabolic labeling with [32P]orthophosphate, M07e cells at 10^6 cells/mL were incubated in phosphate-free RPMI-1640 medium containing 0.5% BSA for 2 hours. Then cells were resuspended at 5 × 10^6 cells/mL in phosphate-free medium equilibrated with carrier-free [32P]orthophosphate (Amersham) at 1.0 μCi/mL for 90 minutes at 37°C, and washed three times with FBS. The radiolabeled cells were loaded onto stromal cell layers that expressed either isoform of rhuSLF, as described above.

Immunocomplex kinase assays. Immunoprecipitation with anti-c-kit antibody was performed as described above. The immunoprecipitates were washed three times with lysis buffer and three times with kinase buffer (50 mmol/L Tris-HCl, pH 7.4, 25 mmol/L β-glycerophosphate, 2 mmol/L sodium orthovanadate, 1 mmol/L di-
MEMBRANE-BOUND AND SOLUBLE FORMS OF STEEL FACTOR

thiothreitol, 10 mmol/L MgCl2. Ten microcurie of [32P-γ] ATP (Amersham) was added for each assay, and incubated for 10 minutes at room temperature. The reaction was terminated by addition of SDS-PAGE sample buffer containing 2-mercaptoethanol and boiled for 5 minutes. The samples were analyzed by 7.5% SDS-PAGE and transferred onto Immobilon-P membrane. The blots were subsequently incubated in 1 mol/L KOH for 1 hour at 55°C to selectively remove the radioactive label from proteins phosphorylated on serine and threonine residues.

Flow cytometric analysis. After coculture with stromal cell layers for various periods of time, M07e cells were collected, and washed twice with PBS containing 5% FBS. Cells, 1 x 10⁷ suspended in 0.5 mL PBS containing 5% FBS were incubated with anti-c-kit MoAb, YBS.B8 (1 µg) or with isotype-matched murine IgG, (1 µg; Zymed) for 30 minutes at 4°C. Samples were then washed twice and incubated with fluorescein isothiocyanate (FITC)-conjugated sheep-anti-mouse IgG secondary antibody (20 µg; Becton-Dickinson, Sunnyvale, CA) for 20 minutes at 4°C. Immunofluorescence was analyzed by flow cytometry using a FACScan (Becton-Dickinson) and a Consort 30 (Becton-Dickinson) FACS data analysis program.

RESULTS

S1/S1' transfectants producing either the membrane-bound or the soluble form of SLF support growth of factor-dependent M07e cells. S1/S1' stromal cell line lacking production of SLF and its stable gene transfectants producing either the membrane-bound SLF (S1'-h220) or the secreted form of SLF (S1'-h248) were treated with mitomycin C, thoroughly washed, then cultured for 48 hours as described in Materials and Methods. Thereafter, factor-starved M07e cells were loaded onto these mitomycin C–treated subconfluent stromal cell layers and cocultured for another 48 hours. As shown in Fig 1, both S1'-h220 and S1'-h248 cells supported growth of M07e cells. After 48-hour coculture with S1/S1' cells that do not produce SLF, over 80% of M07e cells were not viable as determined by trypan blue dye exclusion (data not shown). It is noteworthy that in S1'-h220 cultures producing the membrane-bound SLF, growing M07e cells were seen adjacent to the cell-surface of S1'-h220 cells, whereas in S1'-h248 cultures secreting the soluble form of SLF, most of growing cells were observed in the open spaces left by S1'-h248 cells and only some of them were seen associated with these stromal cells.

Induction of protein tyrosine phosphorylation in M07e cells by interaction with either S1'-h220 or S1'-h248 stromal cell layers. After treatment with mitomycin C, the confluent stromal cells were further incubated for 48 hours. Factor-starved M07e cells were loaded on these stromal layers and cocultured for various periods of time. Since the recovery of attached M07e cells from the stromal cell cultures by pipetting induced cellular protein phosphorylation in M07e cells, which resulted in high background in immunoblots (data not shown), we removed the supernatants and directly added lysis buffer into the cultures to make whole-cell lysates. The soluble cellular proteins consisting of M07e and stromal cells were analyzed by immunoblotting with anti-phosphotyrosine MoAb. Figure 2A shows induction of protein tyrosine phosphorylation in M07e cells after 15-minute coculture with either S1'-h220 or S1'-h248 cells or treatment with 50 ng/mL soluble rhuSLF. One prominent band with a molecular weight of about 145 kD has been reported to represent c-kit–encoded protein due to ligand-induced receptor transphosphorylation in M07e cells.24 Interaction of M07e cells with S1/S1' cells did not induce any change of cellular protein tyrosine phosphorylation in M07e cells compared with the cells treated with control medium (Fig 2A, lanes 2 and 7). To further confirm that these tyrosine phosphorylated protein bands were exactly induced in M07e cells but not in the mitomycin C–treated stromal cell layers, [32P]radiolabeled M07e cells were loaded on transfected stromal cells and cocultured for 15 minutes. Then cells were lysed and tyrosine phosphorylated cellular proteins were collected with agarose-conjugated 4G10, separated by SDS-PAGE, and visualized by autoradiography (Fig 2B). The same series of tyrosine phosphorylated bands were observed among S1'-h220–, S1'-h248–, and rhuSLF–stimulated cells. In addition, only a few weak phosphorylated bands were detected in cell lysates of the stromal cells after coculture with M07e cells (Fig 2C). Therefore, most of phosphorylated protein bands in Fig 2A, lanes 4 and 6 represent cellular phosphorylated proteins derived from M07e cells after interaction with M07e cells (Fig 2C). Therefore, most of phosphorylated protein bands in Fig 2A, lanes 4 and 6 represent cellular phosphorylated proteins derived from M07e cells after interaction with M07e cells. These data also suggest that there are no significant differences in quality of signals produced by the membrane-associated and the soluble forms of SLF. At lower molecular weight proteins of less than 50 kD, 10% SDS-PAGE showed no difference regarding protein tyrosine phosphorylation pattern between S1'-h220 and S1'-h248 stimulation (data not shown).

In contrast to the lack of difference in the quality of signals induced by either SLF isoform, the kinetics of cellular protein phosphorylation in M07e cells was different after stimulation with the membrane-bound or the soluble SLF. As shown in Fig 3, interaction with S1'-h248 cells induced transient tyrosine phosphorylation of cellular proteins in M07e cells, and the protein phosphorylation level returned close to that observed in the unstimulated state within 60 minutes. This result was consistent with those of our previous report in which we used highly purified recombinant soluble murine SLF as a c-kit activator in M07e cells.19 In contrast, after 60 minutes of interaction with S1'-h220, cellular protein phosphorylation was still high and remained elevated at 120 minutes.

The soluble SLF enhanced downmodulation of cell-surface c-kit expression more than the membrane-bound form. We have previously reported that stimulation with recombinant soluble SLF at 37°C induced polyubiquitination of c-kit–encoded protein and subsequent internalization of the ligand-receptor complex resulting in rapid proteolysis of the c-kit protein.25 Therefore, we presumed that the difference in kinetics of tyrosine phosphorylation shown in Fig 3 was caused by enhanced degradation of the c-kit protein after internalization of the ligand-receptor complex when M07e cells were stimulated with soluble SLF. In this view the membrane-bound SLF could not be internalized. To test this hypothesis, we first examined cell-surface c-kit expression in M07e cells by flow cytometry after interaction with the soluble and the membrane-bound SLF. As shown in Fig 4, coculture with S1'-h248 stromal cells for 15 minutes induced downmodulation of cell-surface c-kit protein expression in

From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
MO7e cells compared with untreated cells. No downmodulation of c-kit was detected after coculture with SI/SI' stromal cells. Interaction with SI'-h220 stromal cells resulted in a slight but significant reduction of c-kit expression in MO7e cells compared with untreated cells. However, addition of 15 ng/mL soluble rhuSLF to SI'-h220 cultures markedly enhanced c-kit downmodulation in MO7e cells, to levels similar to cells cocultured with SI'-h248 stromal cells, which are known to produce an equivalent amount of soluble rhuSLF. These data suggest that the soluble form of SLF plays an important role in cell-surface c-kit expression.

**Kinetics of c-kit kinase inactivation and ligand-induced c-kit degradation rate after interaction with the membrane-bound and soluble forms of SLF.** To determine the degradation rate of c-kit–encoded protein after interaction with each SLF-isofrom, c-kit protein in MO7e cells was first metabolically labeled with [35S]methionine then chased during coculture with the confluent layers of each SI/SI' transfectant for various periods of time. After cell lysates were immunoprecipitated with anti-c-kit antibody and separated by SDS-PAGE, autoradiography was performed. Interaction with SI'-h248 cells induced rapid reduction of radiolabeled c-kit protein in MO7e cells (Fig 5, B and C). In contrast, the life span of c-kit protein was much more longer after interaction with SI'-h220 cells. Similar to its effect on cell-surface c-kit expression (Fig 4), addition of soluble rhuSLF accelerated c-kit degradation in MO7e cells cocultured with the SI'-h220 cell layer as much as in those cocultured with SI'-h248 cells. A more pronounced c-kit degradation was observed when the concentration of soluble rhuSLF was increased to 50 ng/mL in SI'-h220 cultures (Fig 5C). It is noteworthy that the kinetics of c-kit degradation under these conditions strikingly fit with both the tyrosine phosphorylation levels and the immune-complex kinase activities of c-kit protein after interaction with each SLF isoform (Fig 5, A and B and Fig 6). In MO7e cells stimulated with SI'-h248 cells, tyrosine phosphorylation of the c-kit protein was transiently activated and it returned to the unstimulated state within 90 minutes, whereas it remained active even after 120 minutes of interaction with SI'-h220 cells. Addition of 50 ng/mL soluble rhuSLF to SI'-h220 cultures resulted in enhanced inactivation of c-kit kinase of MO7e cells at 60-minute incubation, though tyrosine phosphorylation of c-kit protein was more enhanced at the initial period of incubation (Fig 5A). Taken together, these data show that the c-kit kinase remained active significantly longer after stimulation with the mem-
brane-associated SLF than when stimulated with the soluble form. This appears to be caused, at least in part, by the longer life span of c-kit protein after stimulation with the membrane-bound SLF.

**DISCUSSION**

In this study, we have shown that activation of c-kit tyrosine kinase from MO7e cells persists for a longer period of time when MO7e cells are interacting with membrane-associated SLF compared with soluble SLF. Soluble rhuSLF added to the stromal cell cultures that produce the membrane-bound SLF accelerated inactivation of c-kit tyrosine kinase. This suggests that the difference in the kinetics of c-kit kinase is not caused by the amount of SLF produced by each stromal cell line, but that the form of the ligand is more crucial for this inactivation process. We and others have
reported that stimulation of the cells expressing c-kit receptor with soluble SLF induces rapid downmodulation of cell-surface c-kit expression and degradation of metabolically radiolabeled c-kit protein.\textsuperscript{25,29} We have also reported that soluble rh\textsuperscript{125}I[SLF was promptly internalized at 37°C after ligand-receptor binding in MO7e cells.\textsuperscript{25} Degradation after internalization of the ligand-receptor complex has been reported in many other receptor systems including the PDGF and CSF-1 receptors that are structurally related to c-kit.\textsuperscript{30,33} Therefore, we would expect that the regulation of c-kit inactivation involved, at least in part, the proteolysis of c-kit protein mediated by accelerated internalization of the ligand-receptor complex after soluble ligand stimulation. In contrast, because the immobilized membrane-bound ligand-receptor complex presumably cannot be internalized and degraded by this pathway, the activation of c-kit by the membrane-bound SLF appears to be prolonged. Prolonged activation of c-kit might be expected to continue producing efficient signals for cell proliferation and differentiation. This idea was supported by the lines of evidence presented in this report: First, cell-surface expression of c-kit was downmodulated after 15-minute interaction with the soluble SLF, whereas downmodulation was much less after interaction with the membrane-bound form (Fig 3). Second, the radiolabeled c-kit protein was rapidly degraded after stimulation with soluble SLF, whereas its half-life was much longer after interaction with membrane-bound SLF. Moreover, soluble rhSLF added to the stromal cultures that produce membrane-bound SLF enhanced c-kit degradation to the same extent observed in the case of the stromal cell cultures that produce soluble SLF (Fig 5, B and C). Third, the kinetics of c-kit inactivation after stimulation with either SLF isofrom correlated well with the c-kit protein degradation rate under either condition (Fig 5, A and B and Fig 6).

The potential role for protein tyrosine phosphatases in growth regulation is becoming evident. Recently one novel protein tyrosine phosphatase, termed hematopoietic cell phosphatase (HCP) or PTP1C, has been identified.\textsuperscript{33,34} PTP1C encodes a cytoplasmic phosphatase that contains two SH2 domains and has been shown to transiently associate with ligand-activated c-kit protein through the SH2 domain in vivo and dephosphorylate the ligand-induced autophosphorylated c-kit protein in vitro.\textsuperscript{35} implying that this phosphatase might be involved in the negative feedback regulation of ligand-activated c-kit receptor. As shown in Fig 5A, a slight but significant reduction of tyrosine phosphorylation level of c-kit after a 90- to 120-minute interaction with the membrane-bound SLF was observed, whereas the amount of cellular protein was almost consistent until up to a 120-minute incubation. This suggests that some protein tyrosine

Fig 5. Kinetics of the c-kit protein degradation rate in MO7e cells after interaction with stromal cells producing either the membrane-bound or the soluble forms of SLF. (A) MO7e cells were cocultured with SI'h248 cells, SI'h220 cells, or SI'-h220 cells in the presence of 50 ng/mL soluble rhSLF for various periods of time at 37°C. Then the cells were processed for immunoprecipitation using anti-c-kit MoAb. The c-kit immunoprecipitates were separated by SDS-PAGE and probed with antiphosphotyrosine MoAb. (B) The c-kit gene product was labeled for 4 hours with \textsuperscript{35}S methionine and then incubated with excess cold methionine for 30 minutes to wash out free-\textsuperscript{35}S methionine. Thereafter, cells were loaded onto the stromal layers and incubated as described above. The c-kit immunoprecipitates were separated by SDS-PAGE and detected by fluorography. (C) The immunoprecipitates of \textsuperscript{35}S methionine-labeled c-kit protein were separated by SDS-PAGE and the proteins were transferred onto Immobilon-P membrane. Each lane in the molecular weight region that was greater than 100 kD was excised and radioactivity of each lane was counted. (D) MO7e cells cocultured with SI'-h220 cells (membrane-bound SLF); (E) cells cocultured with SI'-h220 cells plus 15 ng/mL soluble rhSLF; (F) cells cocultured with SI'-h220 cells plus 50 ng/mL soluble rhSLF; (G) cells cocultured with SI'-h248 cells (soluble SLF); (H) cocultured with SI'/SI' cells that do not produce SLF. The data shown here are reproducible results from one of three separate experiments.
MEMBRANE-BOUND AND SOLUBLE FORMS OF STEEL FACTOR

A

<table>
<thead>
<tr>
<th>h248</th>
<th>h220</th>
<th>h220 + SLF</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
</table>

B

<table>
<thead>
<tr>
<th>h248</th>
<th>h220</th>
<th>h220 + SLF</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th></th>
</tr>
</thead>
</table>

C

<table>
<thead>
<tr>
<th>% Radioactivity of c-kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
</table>

- □ SSI/SSI^1
- ○ h220
- △ h220 + SLF (15)
- h248
- ▲ h220 + SLF (50)
immune-complexes were separated by 7.5% SDS-PAGE and incubated with anti-c-kit antibody. The immune-complexes were collected on protein-A-Sepharose beads, washed, and immunoprecipitated with anti-c-kit antibody. The immune-complexes were separated by 7.5% SDS-PAGE and proteins were transferred onto Immobilon-P membrane. After treatment the membranes in 1 mol/L KOH for 1 hour at 55°C. [32P] incorporation into c-kit protein was visualized by autoradiography. This is a reproducible result from one of three separate experiments.

The biologic significance of the differences in inactivation kinetics and ligand-induced c-kit degradation rate between the two SLF isoforms remains an open question, especially because we have not observed gross differences between membrane-bound and soluble SLF on proliferation of MO7e cells as evaluated by [3H]thymidine incorporation (data not shown). However, based on the data presented here and information presented below, it can be speculated that the membrane-bound SLF might have more potent or prolonged effects on effector cells including hematopoietic progenitors due at least in part to induction of prolonged tyrosine phosphorylation. The membrane-associated SLF is superior to the soluble form in supporting hematopoiesis in a long-term culture system. In addition, in the viable SI-Dickie mutant mouse which has a deletion of the SI gene that removes the transmembrane and intracellular domains and can only produce the secreted form of SLF, development of hematopoietic cells, melanocytes, and germ cells is impaired. As shown in Fig 1, MO7e cells proliferated only when directly interacting with SIh220 cells, whereas direct contact was not required for its proliferation when cocultured with SIh248 cells. Because SLF has pleiotropic effects on hematopoietic progenitors, further studies to determine biologic differences between the two isoforms including cell differentiation and cell-cycle state should be pursued. Unfortunately, the capacity to evaluate effects of membrane-bound and soluble SLF on receptor mediated and intracellular events including phosphorylation patterns of primary normal hematopoietic stem and progenitor cells must await advances in technology that will allow one to isolate enough of these rare cells in purified form to pursue such studies.

ACKNOWLEDGMENT

We thank Dr. Douglas E. Williams of Immunex Corporation for providing rhu Steel factor and rhuGM-CSF, and Dr. Leonie K. Ashman of Hanson Center for Cancer Research for the gift of anti-c-kit MoAb YB5.B8.

REFERENCES


MEMBRANE-BOUND AND SOLUBLE FORMS OF STEEL FACTOR


Membrane-bound Steel factor induces more persistent tyrosine kinase activation and longer life span of c-kit gene-encoded protein than its soluble form

K Miyazawa, DA Williams, A Gotoh, J Nishimaki, HE Broxmeyer and K Toyama