Biochemical and Genetic Characterization of Multiple Splice Variants of the Flt3 Ligand

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We have performed a comprehensive analysis of cell lines and tissues to compare and contrast the expression patterns of Flt3 ligand (FL), c-Kit ligand (KL), and macrophage colony-stimulating factor as well as their receptors, Flt3, c-Kit, and c-Fms. The message for FL is unusually ubiquitous, whereas that of its receptor is quite restricted, apparently limiting the function of the ligand to fetal development and early hematopoiesis. We have also sequenced a mouse FL genomic clone, revealing how the three splice variant FL mRNAs that we have isolated arise. The chromosomal location of the FL gene has been mapped, by in situ hybridization, to chromosome 7 in mouse and chromosome 19 in human. Natural FL protein has been purified from a stromal cell line and shown to be a 65 kD nonsulfide-linked homodimeric glycoprotein comprised of 30 kD subunits, each containing 12 kD of N- and O-linked sugars. Pulse-chase experiments show that one of the splice variants (T110) is responsible for producing the bulk of soluble FL, but only after it has first been expressed at the cell surface as a membrane-bound form. The other splice-variant forms produce molecules that are either obligatorily soluble (T169) or membrane-bound but released only very slowly (T118). Finally, even though most cell lines express some amount of FL mRNA, we found that very little FL protein is actually made, with T cells and stromal cells being the major producers. The data suggests that FL plays its roles over very short distances, perhaps requiring cell-cell contact. © 1996 by The American Society of Hematology.

MATERIALS AND METHODS

Reverse transcription (RT) and polymerase chain reaction (PCR). Total RNA was isolated from cell lines or tissues using RNAzol (TelTest, Inc, Friendswood, TX), and RT was performed as previously described.13 PCR primers used for c-Kit, c-Fms, Flt3, KL, and M-CSF have been published previously.15 Primers for FL were as follows: sense 5'ACACCTGACTTGA 3', location 180 base pairs (bp); antisense 5'ATCTTTAAGGATTGTC 3', location 267 bp. These primers result in an 87 bp product, and this primer combination spans intron 1. One hundred nanograms of cDNA was used per PCR reaction, and one half of each product was run on agarose gels and visualized by ethidium bromide staining.

cDNA screening. FL mRNA forms T110 and T118 were isolated from a TA4 thymic stromal cell cDNA library as previously described.1 Form T526 and T169 were isolated at a similar frequency (1 per 400,000 clones) from the same library. Form H26 was isolated from an HT2 T-cell library, using the method described previously.1

Genomic sequencing. A genomic clone of FL was isolated from a lambda mouse YC101 genomic library (Clontech, Palo Alto, CA) by hybridization with a 643 bp fragment from the FL cDNA 5' end under stringent washing conditions (70°C, 0.1 × SSC, 30 minutes). Specific sets of oligonucleotide primers were used to amplify regions of this genomic clone by PCR, both to determine the size and the location of the putative introns and subsequently to clone them by the TA cloning method (invitrogen, San Diego, CA). These genomic subclones were then sequenced in their entirety by double-stranded dyeoxy sequencing (Sequenase kit; US Biochemical, Cleveland, OH). The complete sequence of the mouse FL gene has been deposited in Genbank (accession number Bankit27618 U44024).

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Chromosomal location. In situ hybridizations were performed according to previously described procedures. In the mouse, in situ hybridization was performed using metaphase spreads from a WMP male, in which all the autosomes except 19 were in the form of metacentric Robertsonian translocations. Concaveanin A-stimulated lymphocytes were cultured at 37°C for 72 hours. For the chromosomal localization of FL in the human genome, in situ hybridization was performed on chromosome preparations obtained from phytohemagglutinin-stimulated human lymphocytes cultured for 72 hours. In both cases, 5-bromodeoxyuridine was added for the final 6 hours of culture (60 mg/mL) to ensure a chromosomal R-banding of good quality. Plasmids p526#4, of mouse origin, containing an insert of 0.8 kb in vector pME18S,117 and phS109, of human origin, containing an insert of 0.9 kb in pME18S, were tritium-labeled by nick-translation to a specific activity of 1.7 × 10^6 dpm. mg⁻¹ and 1.2 × 10^6 dpm. mg⁻¹, respectively. The radiolabeled probes were hybridized to metaphase spreads at a final concentration of approximately 100 ng and 25 ng per mL of hybridization solution.

After coating with nuclear track emulsion (Kodak NIB2; Eastman Kodak, Rochester, NY), the slides were exposed for 7 and 20 days, respectively, at 4°C, then developed. To avoid any slipping of silver grains during the banding procedure, chromosome spreads were first fixed with buffered Giemsa solution and then dried. R-banding was then performed by the fluorochrome-photolysis-Giemsa method and metaphases rephotographed before analysis.

Construction of FL mutant forms and FL/KL chimera. Construction of each mutant form was accomplished using specifically designed PCR primers, either to subclone the appropriate shortened segment of the FL gene in the case of T110S, or to introduce the desired mutation in the case of T110X1, X2, X1/X2, D1, and T169+1. PCR reactions with the Pfu enzyme (Perkin Elmer-Roche, Branchburg, NJ) were conducted as described. Specifically, a nonmutated oligo at the 5’ end of the FL coding region was used with an oligo containing the mutation to be introduced. The product of this amplification was then mixed with an overlapping fragment generated by PCR with nonmutant oligos, extending to the 3’ end of the coding region. The resulting material was cloned into the expression vector pME18S, and fully sequenced in both strands to confirm the existence of only the desired change. The FL/KL chimera was constructed by PCR amplification of the precise cytogenetic region of FL, including a restriction site at the 3’ end of this region, and ligation to a PCR amplified precise region of the tether and downstream regions of FL with the same restriction site introduced at the 5’ end, resulting in a precise, in-frame chimeric protein.

COS transfections. Subconfluent COS cells were transfected by electroporation (1 × 10^6 cells/mL, 750 µg/0.4 cm cuvette; 0.2 V, 960 µF, 20 µg DNA/cuvette) and plated in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum (FCS), 1% penicillin-streptomycin in either 35 mm dishes for radiolabeling or 100 mm dishes containing 0.8 kb in vector pME18S,117 and phS109, of human origin, containing an insert of 0.9 kb in pME18S, were tritium-labeled by nick-translation to a specific activity of 1.7 × 10^6 dpm. mg⁻¹ and 1.2 × 10^6 dpm. mg⁻¹, respectively. The radiolabeled probes were hybridized to metaphase spreads at a final concentration of approximately 100 ng and 25 ng per mL of hybridization solution.

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Supernatant production. COS cells were washed twice with phosphate-buffered saline (PBS) one day after transfection and then covered with serum-free medium containing 1% HLI protein supplement (30 µg/mL) (Ventrex Laboratories, Portland, ME). Supernatants were collected after the three days of conditioning and concentrated 100-fold using Centricon-10 concentrators (Amicon, Beverly, MA). Supernatants from stromal cell lines were obtained similarly after 3 days of conditioning as those of nonadherent cell lines, suspended at 2 × 10^6 cells/mL.

Lysate production. Cells were shifted to fresh complete medium one day after transfection and in two more days were washed once with PBS, harvested using 2 mmol/L EDTA/PBS for 15 minutes at 37°C, and then washed twice with PBS. COS cells or other cell lines were resuspended at 2 × 10^6 cells/mL (a 100 mm plate contains about 5 × 10^6 COS cells) and sonicated at intensity 3 (out of 10), 80% duty cycle, for 15 one second pulses on a Branson Sonifier 450 (Branson, Danbury, CT). Cell debris was centrifuged for 5 minutes at 15,000 rpm, and the lysates were sterilized by filtration.

Bioassays. Supernatants and lysates were tested for FL bioactivity using Ba/F3 cells stably transfected with Flt3 (Baft cells), as described previously. Briefly, samples were titrated in parallel onto Baft and Ba/F3 cells, incubated overnight at 37°C, and assessed for viability using MTT. Signal obtained with Ba/F3 cells was then subtracted from the Baft signal, resulting in FL-specific activity. Based on a specific activity for FL of 3 × 10^6 U/mg, this assay can detect approximately 100 pg/mL FL. Supernatants containing low levels of FL were typically concentrated 100-fold, thus effectively lowering our threshold of sensitivity to 1 pg/mL.

Fluorescence-activated cell sorter (FACS) staining. Three days after transfection COS7 cells were harvested with 1 mmol/L EDTA, washed twice with PBS and resuspended at 1 × 10^7 cells/mL in staining buffer (Hanks’ buffered saline solution, 2% FCS, 0.1% NaN₃, 20 mmol/L HEPES, pH 7.5). 1 × 10^6 cells were stained with Flt3-IgG soluble receptor fusion protein (expressed in baculovirus) at 16 µg/mL for 45 minutes on ice, washed in staining buffer, and probed with antihuman Ig-fluorescein isothiocyanate (Vector Labs, Burlingame, CA) at 5 µg/mL for 45 minutes on ice. Control cells were incubated with secondary antibody alone. All cells were washed again with staining buffer, resuspended in propidium iodide at 10 µg/mL, and cell fluorescence was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Pulse radiolabeling. One day after transfection of the COS cells, supernatants were aspirated and complete medium added. Three days after transfection, 5 × 10^6 cells in a 35-mm dish were washed once with 0.5 µL cytosine-free medium and then starved for 1 hour with 0.5 µL cytosine-free medium containing dialyzed FCS. 1S-cysteine (0.5 µCi in 50 µL) was then added and the labeling time course started. After 30 minutes of labeling the supernatant was discarded and fresh complete medium was added without washing.

Precipitation from labeled supernatants and lysates. Cells were harvested by removing the supernatant, adding 0.5 mL lysis buffer (0.1% sodium dodecyl sulphate (SDS), 2 mmol/L EDTA, 0.5% NP-40, 174 µg/mL phenyl methyl sulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin) per 35-mm plate and lysing cells on ice for 45 minutes. Lysates were centrifuged 10 minutes at 12,000 rpm to remove debris. Supernatants and lysates were tumbled overnight with 30 µL protein A beads preloaded with 5 µg Flt3-IgG soluble receptor fusion protein. Beads were then washed 3 times with lysis buffer, bound proteins were solubilized with Laemmli sample buffer and run on 16% SDS-polyacrylamide gels (Novex, San Diego, CA). The gels were then stained, dried and analyzed using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Northern analysis. Total RNA was isolated from cell lines using RNAzol (TelTest, Inc), then poly A⁺ selected using Oligotex beads (Qiagen Inc, Chatsworth, CA). RNA was quantitated by measuring the absorbance at 260 nm. RNA was run in formaldehyde gels and transferred to nylon membranes by standard methods. DNA probe was generated by PCR from T110 cDNA, and labeled with 32P dCTP using the Prime-it II kit (Stratagene, La Jolla, CA). RNA blots were hybridized and washed at 65°C as described.

RESULTS

Cellular and tissue distribution of FL mRNA. Initial characterization by RNA blot analysis revealed widespread expression of FL mRNA, including high message levels in stromal cells and T cells as well as in tissues such as spleen...
and lung. To investigate further the expression of FL in a wide variety of cell lines and developing hematopoietic tissues, PCR analyses were conducted using primers that amplify a region of the FL message near the 5' end, a region within the cytokine domain which is common to all of the known cDNA forms of FL. At the level of sensitivity afforded by PCR, the FL message was detectable in all cell types and tissues examined (Fig 1). Southern blotting of this gel and probing with an internal primer confirmed the pattern of bands observed on the ethidium bromide (EtBr)-stained gel (data not shown).

We also examined the expression patterns of the related molecules M-CSF and KL. In contrast to the widespread expression of the FL mRNA, the expression of both M-CSF and KL mRNAs was much more restricted, with a PCR product for KL seen only in stromal cell lines and a PCR product for M-CSF only in stromal cells and T cells (Fig 1). KL and M-CSF mRNAs were highly expressed in developing embryoid bodies, fetal liver, and brain.

PCR analysis also revealed restricted expression patterns for the receptors, Flt3, c-Fms, and c-Kit. Flt3 mRNA was only detected in some pre-B cell lines and in developing...
embryoid bodies, day 14 fetal liver, and fetal, and newborn brain (Fig 1). c-Kit mRNA was detected in some cell lines of early myeloid lineage and in one T cell line, HT2, but absent in most stromal and lymphoid cell lines examined. c-Fms mRNA was seen in stromal cells, macrophages and pre-B cells, but absent in T- and B-cell lines. Southern blotting and probing with an internal probe did not reveal any additional bands and confirmed the patterns observed on the EtBr-stained gels (data not shown). As previously shown, mRNA for all three receptors is detectable in developing embryoid bodies, fetal liver, and brain.15

Although by no means a quantitative study, this analysis revealed that FL had a widespread pattern of mRNA expression, in striking contrast to the restricted mRNA expression patterns observed with the closely related ligands KL and M-CSF, as well as with all three receptors. This analysis also provided the first evidence that the presence of detectable mRNA for FL does not necessarily correlate with the production of protein; indeed, Ba/F3 cells make appreciable levels of FL mRNA but clearly do not make enough FL protein to cause Ba/f3 cells (Ba/F3 cells stably transfected with Flt3) to be autostimulatory. These cells die within 24 hours in the absence of interleukin-3 or exogenously added FL.1

**FL mRNA splice variants.** Mouse FL is encoded by a set of at least five mRNA forms as detected by cDNA cloning, which appear to arise by alternative splicing and predict three different polypeptides (Fig 2). All cDNA forms that we and others have isolated share the first 163 codons of open reading frame encoding the FL helical cytokine domain, then diverge to encode distinct C-termini.1,12,22 Three cDNAs, T110, T526, and H23, are identical in coding sequence but diverge in the 3' untranslated region. These forms contain a tether region linking the cytokine domain to a transmembrane domain (TM), a cytoplasmic tail of 21 amino acids, followed by different untranslated regions (data not shown). T110 and T526 were isolated from a TA4 thymic stromal cell cDNA library and H23 was isolated from an HT2 T cell line cDNA library, each at a similar frequency of one clone per \(4 \times 10^5\) clones screened. Because the coding regions of these three forms are identical, we will henceforth refer to this coding region as the T110 form, even though it represents at least three different cDNAs. A similar clone, named 6C, has been reported by Lyman et al.22

The T118 cDNA form of FL, isolated from the TA4 cDNA library at a similar frequency to the T110 forms, predicts a 57 amino acid hydrophobic C-terminus at the divergence point following the helical cytokine domain, suggesting that it too may be membrane-associated. This appears to be similar to clone 5H, recently reported by Lyman et al.22 Another cDNA form, T169, was isolated from the TA4 library at a much lower frequency than the T110 and T118 forms; only once in \(-5 \times 10^5\) clones screened. This cDNA has the same structure in the coding region as T110 except that it contains an 83 bp insert between the cytokine domain and the tether region. This insert contains stop codons in all three frames and would thus result in a truncated soluble form of the protein, including the common cytokine domain plus nine additional amino acids that are unique to this splice variant.

PCR analysis to discern between the various cDNA forms of FL revealed that all forms were present in a wide variety of cell types tested (data not shown), although the relative ratios at which each form is represented were not determined.

**Genomic organization and chromosomal location of FL.** The signal peptide of mouse FL is encoded by part of exon 1 and part of exon 2. The cytokine domain of mouse FL is encoded by five exons which roughly correspond to the helical domains predicted by the putative structure of FL, with helix A encoded on exon 2, helices B and C on exons 3 and 4, and helix D on exon 5 (Fig 2). Exon 5 also encodes the T118 divergent C-terminus directly after the portion of exon encoding helix D. Further characterization of the T118 cDNA revealed that this form contains downstream sequences which include the T169 insert region (exon 6) and

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**Fig 2.** Schematic diagram of cDNA structure of three forms of FL mRNA, which arise by alternative splicing. The intron/exon organization of a mouse genomic clone encompassing the FL gene and the structures of the three FL splice variant cDNAs discussed in this report are shown. Also shown are cartoons representing the probable structure of each protein product, its relationship to the cell membrane, and the probable locations of proteolytic processing sites. The number of nucleotides in each exon and intron are indicated. The number of amino acids comprising the open reading frames (ORFs) of each cDNA include the 27 amino acid signal sequence that is common to all three splice variants.
the tether, transmembrane, and cytoplasmic regions (exons 7 and 8), and thus may constitute a relatively well represented stable splicing intermediate. Similarly, the T169 cDNA form also arises by alternative splicing from the end of the cytokine domain to the 83 bp exon 6 position and then directly to the tether and TM region (exon 7). Two additional forms of FL cDNA that include exon 6 have recently been reported, E6 and E6D16, which appear to be yet other stable splicing variant forms. The FL genomic region we have characterized is \(-4.8\) kb in length. Our results are similar to recent report of the murine genomic organization of FL except we have found there to be no intron between exon 5 and exon 6, because this region is represented in the cDNA form T118 (region 5' in Fig 2).

FL closely resembles KL, both at the level of genomic organization and the resultant forms of mRNA. KL protein is encoded by two splice variant mRNAs, KL-1 and KL-2, where KL-2 encodes a cell-bound cytokine linked to the membrane via a short (13 amino acid) tether that is slowly cleaved by proteases to liberate soluble KL. KL-1 is identical to KL-2 except for the addition of a 28 amino acid insert (encoded by exon 6) that contains a more rapidly cleaved processing site.

To localize the chromosomal location of the mouse FL gene, in situ hybridization with a mouse FL probe was performed as described in Materials and Methods. In 100 metaphase cells examined, 185 silver grains were associated with chromosome 7. The distribution of grains was not random: 77.5% were localized to the B2-C region of this chromosome, thus mapping the FL gene to the 7B2-C region of the mouse genome. Using a human FL probe, in 100 metaphase cells examined after in situ hybridization, 182 silver grains were associated with chromosomes, 46 of which (25.3%) were located on chromosome 19. Again the distribution of grains was not random: 78.3% were localized to the q13.3-q13.4 region of the long arm of chromosome 19, thus mapping the human FL gene to the 19q13.3-q13.4 region of the human genome. These chromosomal locations confirm a recently published report localizing the FL gene by a different method.

The localization of orthologous FL genes to human chromosome 19q13 and mouse chromosome 7B2-C corresponds to a known linkage group conserved between chromosome 19 in man and chromosome 7 in mouse. This syntenic group contains a great number of other known genes, although no obvious spontaneously occurring mutations have been identified in this region of the mouse genome that could correspond to the FL locus. Many of the genes mapping to region q13 of human chromosome 19, however, have been shown to derive en bloc from a trans-duplication event.

Whether a related FL gene exists at 11q23 is not known.

**Biochemical characterization of native FL protein.** Natural soluble FL was purified from the supernatants of a thymic stromal cell line (TA4), and the concentration of this molecule in stromal cell-conditioned media was very low (<50 pg/mL) compared to that reported for KL (>1,000 pg/mL).

The biological activity of natural FL migrated at a molecular weight of about 65 kD on gel filtration chromatography, with a trace of activity also seen at 34 kD (Fig 3A). This suggests an equilibrium between monomeric and dimeric forms, a fact substantiated by two-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (nonreduced followed by reduced) in which the protein was located on the diagonal, showing it to be a nondisulfide linked homodimer (Fig 3B). Two-dimensional gels, in which the first dimension is isoelectric focusing and the second is SDS-PAGE, showed that natural FL is comprised of a set of anionic species whose isoelectric points span more than 1 pH unit (Fig 3C). This indicates that FL is a glycoprotein containing about 12 kD (per subunit) of both N-linked and O-linked sugars, including sialic acids (Fig 3D).

**Expression and processing mechanisms of FL mRNA forms.** Because FL was originally purified from conditioned media, it is clear that some FL is made as a soluble molecule. The T169 form contains an in-frame stop codon upstream of the transmembrane region, representing a naturally occurring soluble form of FL. However, like KL and M-CSF, the predominant cDNA forms of FL that we isolated, T110 and T118, both predict membrane-associated proteins that must undergo processing to yield soluble activity (Fig 2).

To investigate the biosynthesis of FL from the splicing variants, COS cells were transiently transfected with each cDNA form, and assays were conducted to examine the production of both soluble and membrane-associated FL activity. FL activity was found in both the cell supernatants and cellular lysates of COS cells transfected with all three cDNA forms of FL: T110, T118, and T169 (Fig 4A). T110 consistently produced at least 10-fold higher activity in cell supernatants than T169, and 100-fold higher activity than T118. In cellular lysates T110 again typically produced far greater biological activity than did T118 or T169. Because T169 is an obligatorily soluble form of FL it is probable that the activity it produces in lysates represents molecules caught along the secretory pathway. The status of T118 in this regard remains unclear since its predicted structure is rather unusual, making its resultant localization less obvious.

The presence of FL proteins on the surfaces of the COS transfectants was also measured by FACS analysis using a soluble Ig fusion chimera of Flt3 (Fig 4B) and by a fixed-cell bioassay (data not shown). These data corroborate the observation that membrane-bound FL is produced by either T110 or T118, and showed that these forms are biologically active on the membranes of transfected cells.

To examine the biosynthesis of both soluble and membrane-associated FL protein, metabolic labeling experiments were conducted on transient COS transfectants expressing the various forms of FL cDNAs, using Flt3-Ig fusion protein to precipitate FL from supernatants or cellular lysates (Fig 4C). A 36 kD membrane-bound species was detectable in lysates from cells transfected with the T110 form. A faint band of 24 kD was detected in the lysates from cells transfected with the T169 form; this band was the same size as the mature, soluble form present in supernatants of these
cells and presumably represents material associated with cell membranes during the process of secretion. Although biological activity could be detected in the cellular lysates of TI18 transfectants, the precipitable radiolabeled material resulting from this form was barely at the limit of detection. The TI18 protein was successfully observed when the input number of cells and the amount of radiolabel were both increased 10-fold (Fig 4C, lanes with asterisks). The cell-bound molecules encoded by TI18 appear to include an underglycosylated (sharp) form at 29 to 30 kD and a smaller glycosylated molecule at 24 kD. Precipitation from supernatants of cells transfected with the TI10 form revealed a 30 kD band that corresponds to the mature, fully-glycosylated natural FL that was originally purified from stromal cell supernatants. A band at 24 kD was detectable in supernatants of cells transfected with the TI10 form of FL mRNA. To examine the kinetics of FL biosynthesis, surface expression, and processing, a high resolution pulse-chase experiment was performed with COS cells transfected with the TI10 cDNA (Fig 5). Lysates of these transfectants showed membrane-associated species corresponding to a transient 32 kD under-glycosylated intermediate, strongest at 75 minutes, followed by a longer-lived 36 kD fully glycosylated molecule that probably represents the surface form of FL. Soluble FL that had been processed from the cell surface appeared in the supernatants as a 30 kD glycoprotein starting at 2-2.5 hours, with accumulation still rising at 24 to 48 hours after the pulse.

The molecular relatedness of FL and KL is striking in that the TI10 mRNA form results from precisely the same pattern of splicing events as does KL2. Similarly, TI69 is the structural equivalent of KL1 at the mRNA level, in that it includes exon 6. RNA blots have shown that FL mRNA is well-represented in stromal cells and T cells, and given their obvious structural relationship it is unclear why cells expressing both FL and KL message appear to produce such comparatively small amounts of soluble FL protein. Is FL processed from the cell surface by a mechanism and at a rate similar to those of KL? Because TI69 and TI18 produce soluble activity very poorly in COS cells (Fig 4), and because the TI69-encoded soluble product is much smaller than naturally occurring soluble FL, it is probable that TI10 is the natural source of most soluble FL. TI10 does not contain the exon 6-encoded processing site that makes KL1 so much more efficient than KL2 in producing soluble cytokine. But the tether of TI10 is twice as long as that of KL2 (Fig 6A),

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perhaps making it inherently more accessible to a processing protease without requiring an exon 6-type addition. To compare the rates at which the T110 and KL-1 tethers are cleaved, we performed pulse-chase experiments in COS cells transfected with either T110 or a chimeric cDNA (FL/KL) composed of the FL cytokine domain fused to the exon 6 processing sequences and entire tether, transmembrane and cytoplasmic regions of KL-1 (Fig 6A). We also assessed the sensitivity of this processing event to phorbol 12-myristate 13-acetate (PMA) stimulation, since several membrane-bound cytokines, including KL-1 and KL-2, have been shown to be much more rapidly processed following such treatment.\textsuperscript{24,30} Cell supernatants were precipitated with the Flt3-Ig protein, as above. As seen in Fig 6B, both the kinetics of soluble FL production and the response to PMA were identical between T110 and the FL/KL chimera, suggesting that once it is expressed on the cell surface FL can be released into the supernatant as rapidly as KL. Therefore the enzymatic mechanism of cleavage for surface FL is no less efficient than that for KL.

To locate the site/s of proteolytic cleavage in the T110 tether we constructed three plasmids encoding mutant proteins, each of which deleted or replaced amino acids that we predicted to encode putative proteolytic cleavage sites based on their sequence similarity (consecutive small amino acid side chains) and positions relative to the transmembrane region, as compared to previously mapped cleavage sites in the KL-1 and KL-2 tethers.\textsuperscript{24,30} Figure 6A shows the sites that were changed or deleted in the FL tether as well as the analogous positions in the KL protein. Changing or deleting either one of these sites did not impair the production of soluble FL, although changes in the X1/D1 site resulted in a much lower molecular weight product, presumably due to the removal of an O-linked glycosylation site (Fig 6C).

Finally, because the FL exon 6 contains in-frame stop codons and renders soluble the T169-encoded product, we wondered whether this exon could be repaired, thus producing a membrane-bound form of FL that contains an added processing insert and is truly analogous to KL-1. Thus we removed the in-frame stops in T169 by arbitrarily correcting an apparent frameshift mutation. This corrected form, termed T169 +1, yielded both soluble and membrane-associated FL molecules, both of which were increased in molecular weight due to the additional 28 amino acids encoded by the exon 6 insert, thus showing that the corrected form did not generate additional cleavage sites (Fig 6C).

One observation that remains unexplained but may relate to the control of the rate of FL secretion, is the fact that the level of precipitable FL in COS lysates and supernatants increased dramatically, relative to T110, with many of the mutant FL forms, even though some of these forms differ from T110 by only two amino acids. Also of interest is the
fact that T169 was produced so much more poorly than was an engineered soluble mutant protein form, T110S. Both of these are obligatorily soluble forms consisting of the cytokine domain plus either a nine amino acid tail, in T169, or the entire tether, in T110S. It should also be stated that these observations were very reproducible, and in all cases these increases in precipitable protein were always accompanied by similar increases in biological activity in the COS supernatants (data not shown). Figure 6A shows the sequences of the respective tethers of all of the FL forms tested, and Fig 6C shows the relative amounts of precipitable material from their parallel transfections. This data suggests that the rate of FL secretion is negatively regulated, either pretranslationally by the mRNA sequence encoding the tether or posttranslationally by the sequence and/or length of the tether itself.

**FL production in natural cell lines.** To assess the biological relevance of the FL molecule we sought to determine what types of cells produce FL activity. PCR analysis of mRNA expression had clearly shown that virtually all cell types examined produce stable FL messenger RNA (Fig 1). However, the low level of active FL protein produced by stromal cells\(^1\) and transfected COS cells suggested that the amount of FL protein secreted by a cell does not necessarily correlate directly to the level FL mRNA expressed by that cell.

Figure 7 shows the relative amounts of FL mRNA found in equal quantities of poly A\(^+\) RNA purified from various types of cells. Table 1 shows the amount of biologically active FL present in the supernatants of these same cells. All of the cells contain FL mRNA, and accordingly, all of the cells produce some FL bioactivity, with T cells being the best producers. The levels of production are exceedingly low, however, with even the best producer (T-cell hybridoma 4.B2) making FL at a rate that is about 50-fold lower than that at which stromal cells can produce KL.\(^{29}\) It is also of interest that the two non-T-cell lines that contain the most FL mRNA, Ba/F3 and A20, produce exceedingly little FL protein, showing that the level of FL mRNA does not directly correlate to the level of FL secretion.

It is formally possible that more FL protein is present than has been measured in these studies, and that methods of detection using Flt3 itself (either bioassays or precipitations and labelings with soluble forms of the receptor) have only recognized the biologically active dimeric form of the cytokine. Monoclonal antibodies recognizing the FL protein would clarify this point but are presently unavailable.

**DISCUSSION**

The isolation and characterization of the Flt3/Flk2 tyrosine kinase receptor revealed a very limited expression pattern, detectable in primitive populations of hematopoietic cells, thymus, placenta and brain.\(^{18,30}\) The described biological activities of the Flt3 ligand, FL, suggest a role for this cytokine in the proliferation and expansion of primitive hematopoietic progenitors, B-cell progenitors, dendritic cells,\(^{5,20}\) and perhaps also thymocytes,\(^{1,3,5}\) findings that are consistent with the limited expression pattern of the receptor. In this report, we present a PCR expression analysis that compares the relative expression patterns of FL, KL, M-CSF, and their respective receptors. This data reveals that FL mRNA is widely expressed, in contrast to the messages for KL, M-CSF, Flt3, c-Fms, and c-Kit, whose expression patterns are quite restricted; these mRNAs are undetectable in many cell types even at the level of sensitivity afforded by PCR analysis and Southern hybridization.

In our initial characterization of the FL molecule we observed that the level of FL mRNA in stromal cells was readily detectable by RNA blot analysis, yet the amount of protein produced by these cells was exceedingly low, on the order of 10 \(\mu\)g of FL protein per 200 L of conditioned medium.\(^1\) It is now clear that Ba/F3 cells express significant levels of FL mRNA, and yet Ba/F3 cells do not produce enough FL bioactivity to permit autocrine growth. These observations lead us to conclude that production of FL protein is not fundamentally controlled at the transcriptional level, but instead that translational and/or processing regulatory points govern the secretion of biologically relevant protein.

Although the overall expression of FL mRNA is apparently not transcriptionally regulated, the production of various forms of FL is clearly regulated by alternative splicing. In fact, comparative analyses of the cDNA and genomic sequences of FL and KL show that these molecules are
extremely analogous in their organizations. We have presented the cDNA structures of three distinct forms of FL that encode both membrane-bound and soluble forms of FL protein. Several growth factors have been shown to function as membrane-bound ligands in binding to receptors on cells in close proximity, resulting in limited microenvironmental effects termed juxtacrine stimulation. Indeed, mice with the Steel mutation, which encodes an obligatorily soluble form of KL, suffer from severe deficiencies in hematopoietic, germ cell, and melanocyte development, dramatically show-
ing the critical role of the membrane-bound forms of KL. It seems probable that FL also acts as both a membrane-bound and a soluble factor, although the low level at which soluble FL is observed in conditioned media suggests that the former role may be the more critical.

The isolation of the T169 mRNA form of FL, that contains an 83 bp insert region, led us to consider whether a processing insert is present in any mRNA forms of FL, analogous to the processing inserts present in the mRNAs for both KL and M-CSF. Isolation and complete sequencing of the FL gene revealed that the 83 bp region present in T169 contains stop codons in all three frames, resulting in a naturally-occurring soluble form, and ruled out the possibility that the T169 cDNA that we isolated had undergone mutation. Our knowledge of the precise sequence of the entire FL genomic region also reveals that the mRNA forms recently described by other investigators also must arise by alternative splicing, using yet other splice donor/acceptor sites in this same region at the 3' end of the helical cytokine domain.

COS transfection experiments allowed us to examine the relative activity of the three mRNA forms of FL that we identified, and to investigate their biosynthesis and processing kinetics. Although T169 is truncated by a stop codon present in the 83 bp insert and would thus be produced solely as a soluble molecule, the relative activity of this form is 10-fold lower than the soluble form produced by membrane processing of the T110 form. Analysis of COS transfectants showed roughly equal steady state RNA levels in cells transfected with each of these three forms (data not shown). We cannot exclude the possibility that the T169 form is not translated as well as T110. Alternatively, the divergent nine amino acid tail of this form might result in a protein that forms dimers less readily and therefore is less active, although the decreased activity obtained with T169 cDNA also appears to correlate with decreased levels of precipitable protein.

The T118 form contains a long hydrophobic region, which presumably is embedded in the membrane and is followed by a very small cytoplasmic domain. Transfection with this form produces sufficient FL protein to be detectable on the cell surface by FACS staining and, at great effort, by autoradiography in precipitations from metabolically labeled COS lysates. It also produces detectable biological activity in concentrated COS supernatants when they are analyzed in the more sensitive Bafl bioassay. Therefore the T118-encoded polypeptide is also clearly processed from the cell surface in some manner, although the mechanism of such processing is yet to be elucidated.

The question remains, why is so little FL protein produced by cell lines that contain reasonable levels of FL mRNA or even by COS cells transfected with large amounts of the splice variant FL cDNAs? The data presented in this paper show that FL can be synthesized, moved to the cell surface, and processed to liberate soluble cytokine as rapidly as is KL-1, with its extra processing insert. The amount of FL protein actually produced with such rapid kinetics, however, is quite low, although almost any change to the sequence encoding the FL tether results in a significant increase in the level of precipitable FL protein that accumulates in the membrane and ultimately in the supernatant (Fig 6C). Although we have not explained this phenomenon, it is apparent that control of the production of useful FL protein takes place downstream of transcription, perhaps due to translational control and/or protein degradation.

Because FL is first made as a membrane-bound molecule and very little soluble protein ever accumulates around producer cells, it is probable that FL acts primarily through cell-cell contact. This seems consistent with the emerging biological profile of FL showing that it is most active on extremely primitive hematopoietic progenitors, cells that reside in tight association with stroma within the hematopoietic microenvironment of the bone marrow.

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Table 1. FL Protein Detected in the Supernatants of Mouse Cell Lines Using the Bafl Bioassay and Calculated Using a Specific Activity of 3 x 10^6 U/mg for FL

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Type</th>
<th>Soluble FL Protein (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba/F3</td>
<td>pro B cell</td>
<td>6</td>
</tr>
<tr>
<td>TA4</td>
<td>stroma</td>
<td>21</td>
</tr>
<tr>
<td>30R</td>
<td>stroma</td>
<td>15</td>
</tr>
<tr>
<td>4.B2</td>
<td>T-cell hybrid</td>
<td>105</td>
</tr>
<tr>
<td>A3.2</td>
<td>T-cell hybrid</td>
<td>90</td>
</tr>
<tr>
<td>CDC25</td>
<td>T-cell line</td>
<td>6</td>
</tr>
<tr>
<td>KD83</td>
<td>plasmacytoma</td>
<td>3</td>
</tr>
<tr>
<td>B9</td>
<td>plasmacytoma</td>
<td>6</td>
</tr>
<tr>
<td>A20</td>
<td>B lymphoma</td>
<td>12</td>
</tr>
</tbody>
</table>

Serum-free supernatants were conditioned for 72 hours and concentrated 100x before assay. Expressed values are for 1x supernatants.
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Biochemical and genetic characterization of multiple splice variants of the Flt3 ligand

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