Transmembrane Kit Ligand Cleavage Does Not Require a Signal in the Cytoplasmic Domain and Occurs at a Site Dependent on Spacing from the Membrane

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The kit ligand (KL) is one of several growth factors that are active as transmembrane molecules and can also be proteolytically cleaved to yield soluble forms. We have investigated the signals and structural determinants that control the cleavage of KL. Presentation at the membrane appears to be critical, because no cleavage occurs in variants that lack a transmembrane domain. Signals in the cytoplasmic domain do not seem to be required, because cleavage was not blocked by removal of the C-terminal valine residue, deletion of the whole cytoplasmic tail, or the replacement of the cytoplasmic tail that occurs in the SJ^{17H} mutation. KL thus appears to differ from transforming growth factor- α , which apparently requires a C-terminal valine as a signal for cleavage. Although proteolysis must be tightly restricted to the correct cell surface proteins and sites within each protein, cleavage of KL does not seem to be determined entirely by a requirement for a specific substrate sequence. However, the effects of deletion or insertion variants of KL suggest that cleavage may be limited to sites within a specific range of distances from the cell membrane.

INTRODUCTION

Polypeptide growth factors play important roles in intercellular communication and can act either as diffusible molecules or by direct cell-cell contact. In several cases, growth factors or their receptors are produced as transmembrane molecules that can subsequently be cleaved to yield soluble forms (reviewed by Ehlers and Riordan, 1991; Gordon, 1991; Massague and Pandiella, 1993). The specific biological functions of this cleavage process are generally not well understood, but cleavage of growth factors may serve to regulate local cell-cell interactions or to produce factors that act at a longer range, whereas cleavage of receptors may produce soluble binding proteins that can antagonize the action of their ligands. Abnormalities of the cleavage process may be involved in disease. For example, Alzheimer's disease is associated with aberrant cleavage of the β -amyloid precursor (Price *et al.*, 1993), and cutaneous mastocytosis is associated with an alteration in the balance of soluble and cell-bound kit ligand (KL) (Longley *et al.*, 1993).

The growth factors produced as transmembrane molecules that can subsequently be cleaved include KL (also known as stem cell factor, mast cell growth factor, or steel factor), the related molecule colony stimulating factor-1 (CSF-1 or M-CSF), tumor necrosis factor- α (TNF- α), and members of the epidermal growth factor (EGF) family including EGF and transforming growth factor- α (TGF- α) (Massague and Pandiella, 1993). Both the soluble and the transmembrane forms of these growth factors are apparently capable of stimulating receptor-bearing cells, indicating that the transmembrane forms are not simply inactive precursors (Massague and Pandiella, 1993). In addition, the transmembrane forms of TGF- α and KL have been found to act as cell adhesion molecules (Anklesaria *et al.*, 1990; Flanagan *et al.*, 1991; Kaneko *et al.*, 1991) and soluble or transmembrane KL may have roles in the guidance of cell migrations during development (Flanagan and Leder, 1990; Matsui *et al.*, 1990; Kaneko *et al.*, 1991; Keshet *et al.*, 1991; Meininger *et al.*, 1992).

Although the proteolytic cleavage of transmembrane growth factors is evidently widespread, little is known

about the mechanisms, control, or precise biological role of the cleavage of these molecules. For TGF- α , KL, and CSF-1, the cleavage is known to be a regulated process that can be activated by growth factors or stimulators of protein kinase C (Pandiella and Massague, 1991; Stein and Rettenmier, 1991; Huang *et al.*, 1992). The amino acid sequences at the cleavage sites are highly diverse (Massague and Pandiella, 1993), suggesting either that multiple proteases may be involved or that the site specificity of the protease(s) may be dictated at least in part by factors other than the amino acid sequence of the cleavage site. Surprisingly, in the case of TGF- α , one of these factors appears to be a signal located in the cytoplasmic domain, because a C-terminal valine residue was found to be essential for cleavage in transfected cells (Bosenberg *et al.*, 1992). A C-terminal valine residue is also present in KL and CSF-1, raising the possibility that this signal might be a general requirement for cleavage of all three peptides (Bosenberg *et al.*, 1992).

The KL polypeptide is initially produced in at least two forms that arise by tissue-specific alternative splicing: KL-M1 (248 amino acids) and KL-M2 (220 amino acids) (Anderson *et al.*, 1990; Huang *et al.*, 1990; Martin *et al.*, 1990; Flanagan *et al.*, 1991). Both of these polypeptides are transmembrane molecules with a 36 amino acid cytoplasmic tail. They differ in that the KL-M2 mRNA has an alternative splice that removes exon 6, which encodes a 28 amino acid region just outside the transmembrane domain. This region includes the KL-M1 proteolysis site, and its removal results in a KL-M2 peptide that is less readily cleaved and is thus preferentially retained at the cell surface (Flanagan *et al.*, 1991; Huang *et al.*, 1992).

Much information on the biological action of KL has come from the observation that both KL and its receptor, the product of the *c-kit* proto-oncogene, are encoded at well characterized genetic loci in the mouse, each with multiple mutant alleles. These are the steel (*Sl*) and dominant white spotting (*W*) loci, respectively (Chabot *et al.*, 1988; Geissler *et al.*, 1988; Nocka *et al.*, 1989; Copeland *et al.*, 1990; Huang *et al.*, 1990; Zsebo *et al.*, 1990a; Flanagan *et al.*, 1991). The mutant phenotypes of these two loci are similar and affect at least three separate sets of progenitor cells during embryonic development: primordial germ cells, neural crest progenitor cells, and early progenitor cells of the hematopoietic system. The result is that mice homozygous for severely impaired alleles are typically sterile and deaf and have unpigmented coats and deficiencies in multiple hematopoietic lineages (Russell, 1979; Silvers, 1979).

Two of the mutant alleles of the *Sl* locus, *Sl^d* and *Sl^{17H}*, are of particular interest with regard to the expression and function of the transmembrane form of KL. The *Sl^d* allele has a deletion that almost precisely removes the transmembrane and cytoplasmic domains of the polypeptide (Brannan *et al.*, 1991; Flanagan *et al.*, 1991).

The result is production of a soluble polypeptide that retains growth stimulatory activity in vitro but is not associated with the cell surface (Flanagan *et al.*, 1991). Because the *Sl^d* mutant phenotype is nearly as severe as that of null alleles with a complete deletion of KL, the expression of KL as a transmembrane molecule appears to be critical for its normal function.

The *Sl^{17H}* mutation leaves the extracellular and transmembrane domains of KL intact but results in a replacement of the normal cytoplasmic domain by a different sequence of similar length (Brannan *et al.*, 1992). The phenotype of the *Sl^{17H}* mutation is noteworthy for a particularly severe effect on the development of male germ cells, though other lineages are also affected. The nature of the *Sl^{17H}* mutation, as well as the strong sequence conservation of the cytoplasmic tail in comparisons between several species, suggest that the cytoplasmic domain of KL has an important biological function. Because the cytoplasmic domain of TGF- α is required for cleavage of the extracellular domain, one possibility would be that loss of the C-terminal valine residue or other cytoplasmic residues in the *Sl^{17H}* mutant KL might block proteolytic cleavage of the transmembrane form.

Here we have investigated the determinants of the proteolytic cleavage of transmembrane KL. In COS cells or mouse fibroblasts, cleavage is not dependent on signals in the cytoplasmic domain, because it was not blocked by the *Sl^{17H}* mutation, nor by removal of the C-terminal valine residue or deletion of the entire cytoplasmic domain past the positively charged stop transfer sequence. An analysis of requirements in the transmembrane and extracellular regions of the molecule indicates that cleavage does not seem to have a precise substrate sequence specificity but that it does require expression as a transmembrane molecule, and that cleavage site selection is dependent on spacing from the cell membrane.

MATERIALS AND METHODS

DNA Constructs and Cell Lines

All experiments were performed with KL sequences derived from the mouse. Plasmids KL-M1, KL-M2, KL-S, and KL-*Sl^d* have been described elsewhere (Flanagan *et al.*, 1991). Other KL cDNAs were generated by polymerase chain reaction using KL-M1 as a template and were cloned between the *Bam*HI and *Pst*I sites of pcDNA1 (Invitrogen, San Diego, CA). The modified regions of all new constructs were checked by nucleotide sequencing.

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum. COS cells were transiently transfected by the diethylaminoethyl (DEAE) dextran method essentially as described (Flanagan *et al.*, 1991). Cells at approximately one-third confluence in six-well plates were transfected in 1 ml of DMEM containing 10% Nuserum (Collaborative Research, Lexington, MA). DEAE-dextran (molecular weight 500 000) (Pharmacia, Piscataway, NJ) and chloroquine phosphate were added as a single 25 $\times$ stock to give final concentrations of 400 μ g/ml and 100 μ M, respectively. DNA was then added to give a concentration of 2 μ g/ml. After 4 h at 37°C,

the cells were treated for 2 min with 10% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS), which was then replaced with complete medium. MOP-8 cells were transiently transfected with LipofectAMINE (GIBCO-BRL, Grand Island, NY) as described by the manufacturer for NIH-3T3 cells.

Assessment of AP-KIT Soluble Receptor Binding to Cell Surfaces

Detection and characterization of KL polypeptides was done using the c-kit receptor extracellular domain fused to alkaline phosphatase (AP-KIT) essentially as described previously (Flanagan *et al.*, 1991). For quantitative detection of cell surface KL, COS cells 3 d after transfection were washed with HBHA buffer (Hank's balanced salt solution with 0.5 mg/ml bovine serum albumin [BSA] [Sigma, St. Louis, MO], 0.1% NaN₃, 20 mM N-2-hydroxyethylpiperazine-N¹-2-ethanesulfonic acid [HEPES] pH 7.0). The plates were then incubated at room temperature with supernatant from clone 152.7, a line of transfected NIH-3T3 cells, containing AP-KIT (1250 OD/h/ml AP activity) and also containing 0.1% NaN₃ and 20 mM HEPES pH 7.0. After 90 min the cells were rinsed six times with HBHA, taking care to avoid detachment of cells from the plate, and lysed in 1 ml of Triton-tris (hydroxymethyl) amino methane (Tris) buffer (1% Triton X-100, 10 mM Tris-HCl pH 8.0). After vortexing, the nuclei were spun out in a microfuge for 5 min, and the supernatants were heated for 10 min at 65°C to inactivate endogenous cellular phosphatases and were then assayed for alkaline phosphatase activity essentially as described (Flanagan *et al.*, 1991) by measuring the OD₄₀₅ after incubating at room temperature with 1 M diethanolamine pH 9.8, 0.5 mM MgCl₂, 0.5 mg/ml BSA, and 12 mM p-nitrophenyl phosphate (Sigma), all prepared as a single 2× stock solution and stored at -20°C. Alkaline phosphatase activities are expressed here as OD units per hour, indicating the rate of hydrolysis of the chromogenic substrate under the conditions used.

For in situ analysis, binding and washing were done exactly as described above for the quantitative assay. The cells were then fixed for 30 sec in 65% acetone, 3% formaldehyde, 20 mM HEPES pH 7.0 and washed twice for 5 min each in 150 mM NaCl, 20 mM HEPES pH 7.5. The plates were then floated on a 70°C water bath for 30 min to inactivate cellular phosphatases. After rinsing with 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂, the cells were stained for 1 h in the same buffer containing 0.17 mg/ml bromochloroindolyl phosphate and 0.33 mg/ml nitro blue tetrazolium.

Metabolic Labeling and Immunoprecipitation

For analysis of ³⁵S-labeled proteins, COS cells 62 h after transfection were rinsed twice in methionine-free DMEM then incubated with 200 μCi/ml of ³⁵S-methionine (NEG-072, New England Nuclear, Boston, MA) in 1 ml of methionine-free DMEM (Gibco-BRL) containing 10% dialyzed calf serum (Sigma). After 8 h the supernatants were taken and concentrated to 200 μl on a Centricon-10 microconcentrator (Amicon, Beverly, MA). The cells were washed twice in HBHA buffer and lysed in 200 μl of Triton-Tris buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF); the nuclei were spun out for 10 min in a microfuge. Both the concentrated supernatants and the cell lysates were then incubated with an equal volume of concentrated 152.7 supernatant containing ~10 μg/ml of AP-KIT fusion protein. After 60 min at room temperature, the AP-KIT protein was immunoprecipitated by incubating on a rotator at room temperature for 60 min with CNBr-Sepharose beads coupled to excess monoclonal antibody against human placental alkaline phosphatase (MIA 1801, Medix Biotech, Foster City, CA). The beads were then washed five times in modified RIPA buffer (0.5% NP40, 0.5% NaDOC, 0.025% sodium dodecyl sulfate [SDS], 0.1% NaN₃, 144 mM NaCl, 50 mM Tris-HCl pH 8.0) and heated for 3 min at 100°C in SDS sample buffer. ³⁵S-labeled proteins were analyzed on a 12 or 15% SDS-polyacrylamide gel. The gel was fixed in 20% methanol, 10% acetic acid, treated

with Enlightning (Dupont, Wilmington, DE), dried, and exposed to X-ray film.

For deglycosylation analysis, metabolic labeling and immunoprecipitation were done as described above. Before gel electrophoresis, the beads were further washed twice in deglycosylation buffer (10 mM sodium phosphate pH 7.5, 0.1% NP-40, 1 mM PMSF) and incubated in 200 μl deglycosylation buffer containing 0.5 U N-glycanase (Genzyme, Boston, MA), 1 mU O-glycanase (Genzyme), and 0.01 U neuraminidase (Calbiochem, La Jolla, CA) at 37°C for 20 h.

For phorbol 12-myristate 13-acetate (PMA) stimulation experiments, transfected COS cells were rinsed twice and preincubated in serum-free and methionine-free DMEM for 30 min at 37°C. Cells were then incubated with 200 μCi/ml ³⁵S-methionine in 1 ml serum-free and methionine-free DMEM for 3 h and chased with serum-free medium containing 0.5 μM PMA (Sigma) for 2 h. The supernatants and cell lysates were then processed for immunoprecipitation as described above.

RESULTS

Cell Surface Expression and Cleavage of KL Do Not Require the Cytoplasmic Domain

A C-terminal valine residue was previously reported to be required for cleavage of TGF-α and also for cleavage of a fusion protein consisting of the extracellular TGF-α domain joined to the transmembrane and cytoplasmic domains of KL (Bosenberg *et al.*, 1992). We therefore tested the possibility that a C-terminal valine might also be an essential requirement for proteolysis of KL. As an initial test, mutants of KL were produced where the C-terminal valine was either deleted (KL-ΔV248) or substituted by glycine (KL-V248G). The structures of these mutants are illustrated in Figure 1. The equivalent C-terminal modifications were previously found to block cleavage of TGF-α transfected into Chinese hamster ovary (CHO) cells or NIH-3T3 cells (Bosenberg *et al.*, 1992).

Expression of the KL mutant molecules was analyzed using an AP-KIT soluble receptor fusion protein as an affinity reagent as described previously (Flanagan and Leder, 1990; Flanagan *et al.*, 1991). Cell surface expression of KL was assessed by two methods. Quantitation of bound AP-KIT, measured as bound AP activity, indicated that the KL-ΔV248 and KL-V248G mutants were expressed on the cell surface at levels comparable to native KL-M1 (Table 1). In situ staining of AP activity, to determine the distribution of the bound AP-KIT, indicated expression over the surface of the whole cell and did not reveal any obvious differences in expression pattern between KL-M1, KL-ΔV248, and KL-V248G (Figure 2).

To assess the proteolytic cleavage of KL, transfected cells were metabolically labeled with ³⁵S-methionine, and radiolabeled KL polypeptides were then coimmunoprecipitated with AP-KIT and analyzed by SDS gel electrophoresis. Kinetic pulse-chase experiments in the presence of 10% serum indicated that KL-M1 is cleaved with a half-time of ~3 h, consistent with previously published results (Flanagan *et al.*, 1991; Huang *et al.*,

1992). Figure 3 shows a comparison of the mutant and normal KL polypeptides following continuous labeling for 8 h in the presence of 10% serum. The lysates of cells transfected with the KL-M1, KL- Δ V248, and KL-V248G cDNAs gave polypeptide bands of the expected apparent molecular weight of ~ 38 kDa for the encoded transmembrane KL molecules (Figure 3). Each of these transmembrane molecules was cleaved to a soluble form, as the supernatants also showed a KL band, with the same migration distance in each case, corresponding to an apparent molecular weight of ~ 27 kDa (Figure 3). Although these experiments do not rule out the possibility of minor effects on the rate of proteolysis, it is clear that the C-terminal valine residue is not an essential requirement for the cleavage of KL and that removal or alteration of this residue does not cause a major reduction in the rate of cleavage, nor does it change the site of cleavage.

To study the effects of the C-terminal mutations of KL further, we tested their effects on KL cleavage stimulated by phorbol ester. As others reported previously (Huang *et al.*, 1992), we found that in pulse-chase labeling experiments in the absence of serum, phorbol ester treatment resulted in an increased rate of KL-M1

Table 1. Cell surface expression of KL in transfected cells

Transfected construct	AP-KIT binding (AP activity, OD/h)
KL-M1	24.94
KL-V248G	16.46
KL- Δ V248	17.25
KL- Δ Cyto	5.00
KL-SL ^{17H}	1.91
KL-M2	28.29
KL-A164N	41.59
KL-(exon6) ₂	6.69
KL-SI ^d	0.020
KL-S	0.017
Vector	0.012

Supernatant containing ATP-KIT fusion protein was incubated with transfected COS cells in six-well plates, and then the cells were washed and analyzed for bound fusion protein, and assayed as bound AP activity.

cleavage. In the presence of PMA about half the KL appeared in the supernatant after a 1-h chase, and most of the KL appeared in the supernatant after a 2-h chase,

Figure 1. Schematic representation of polypeptides encoded by wild-type and mutant KL cDNAs. The transmembrane domain is indicated by ■ and the alternatively spliced exon 6 by ▨. The locations of cysteine residues are indicated by the letter C and the potential N-linked glycosylation sites by •. (A) The sequence of the cytoplasmic domain. (B) The part of the extracellular sequence located between the last cysteine residue and the transmembrane domain. The amino acid sequences and numbering are for the wild-type mouse KL-M1 cDNA. Variant sequences are indicated below this with identical amino acids indicated by -, and changed amino acids are indicated in the single letter code with an asterisk for stop codons. An internal deletion in KL-M2 is indicated by a bracketed space. Construct KL-(exon6)₂ contains a duplication, as indicated, and also contains an additional valine residue between the duplicated copies of exon 6.

Figure 2. Cell surface expression of KL detected by in situ staining. COS cells were transfected with the indicated KL cDNAs. Cell surface KL was detected by treating with supernatant containing AP-KIT fusion protein. After washing, the cells were fixed and stained for AP activity.

whereas there was little cleavage ($\leq 10\%$) after the same period in the absence of PMA and serum. Figure 4 shows a comparison of mutant and normal KL polypeptides after pulse labeling and a 2-h chase in the presence of PMA. Neither of the mutants KL- $\Delta V248$ or KL-V248G blocked the stimulation of cleavage by

Figure 3. Polypeptides encoded by KL cDNAs that differ in the sequence of the cytoplasmic region. COS cells were transiently transfected with KL cDNAs or with vector as a control. Sixty-two hours after transfection, the cells were labeled with ^{35}S -methionine for 8 h in the presence of 10% serum. KL polypeptides were then isolated from the supernatant (S) or cell lysate (C) by treating with the AP-KIT fusion protein followed by immunoprecipitation with Sepharose beads coupled to monoclonal antibody against alkaline phosphatase. Proteins were separated on 15% polyacrylamide gels and autoradiographed. The left and right panels are from different gels.

phorbol ester with almost all of the KL appearing in the supernatant after the 2-h chase period (Figure 4). This contrasts sharply with similar experiments on TGF- α , where mutants lacking a C-terminal valine residue were barely cleaved at all even after exposures to PMA lasting up to 4 h (Bosenberg *et al.*, 1992).

As no effect of altering the C-terminal valine residue was detected, we investigated further whether other sequences in the cytoplasmic domain might have a role. This was tested with the KL- ΔCyto mutant (Figure 1)

Figure 4. Effect of PMA on cleavage of KL variants. COS cells were transfected with KL cDNAs or vector as a control. After 62 h, the cells were metabolically labeled with ^{35}S -methionine in the absence of serum then chased for 2 h with serum-free medium containing 0.5 μM PMA. KL polypeptides were isolated from the supernatant (S) or cell lysate (C) by coimmunoprecipitation with AP-KIT fusion protein. Proteins were then separated on a 15% polyacrylamide gel and autoradiographed.

in which the entire cytoplasmic domain is deleted, leaving only three lysine residues after the transmembrane domain as a stop-transfer sequence to ensure correct insertion in the membrane. The KL- Δ Cyto mutant was expressed at the cell surface (Table 1 and Figure 2). Coimmunoprecipitation gave a cell-associated band with the expected apparent molecular weight of ~ 33 kDa for the encoded transmembrane molecule and was cleaved to give a band in the supernatant that comigrated with the normal KL-M1 cleavage product (Figure 3). As with the valine-248 mutants, there was no indication of a significant effect of the KL- Δ Cyto mutant on cleavage efficiency (Figure 3).

In addition to testing our KL constructs in the COS cell line, a derivative of the monkey fibroblast line CV-1, we also tested for cleavage in the MOP-8 cell line (Muller *et al.*, 1984). The MOP-8 cell line, which supports good expression from the pcDNA1 vector, is a derivative of the mouse NIH-3T3 embryonic fibroblast line and expresses low amounts of endogenous mouse KL. For each construct, the same results were obtained with the MOP-8 cells as with the COS cells.

Expression and Cleavage of the Sl^{17H} Mutant Allele of KL

The mutation in the Sl^{17H} allele of KL results in a splicing defect that causes replacement of the normal cytoplasmic domain with a different sequence (Figure 1) (Brannan *et al.*, 1992). A possible explanation of the defect in Sl^{17H} mice would be a failure of normal expression or processing of KL. This was assessed by constructing an expression plasmid encoding the Sl^{17H} variant of KL. Like the valine-248 mutants and the KL- Δ Cyto mutant, the KL- Sl^{17H} variant was expressed at the cell surface, though the amounts of AP-KIT binding to cells transfected with the KL- Sl^{17H} cDNA were consistently lower than those seen with other constructs (Table 1). Coimmunoprecipitation showed a cell-associated form migrating at an apparent molecular weight of ~ 36 kDa, consistent with the alterations in the cytoplasmic domain of the Sl^{17H} variant (Figure 3). A corresponding soluble form comigrating with the KL-M1 cleavage product was also seen (Figure 3). Neither proteolytic cleavage (Figure 3) nor the stimulation of cleavage by phorbol ester (Figure 4) were blocked by the Sl^{17H} mutation.

Proteolytic Cleavage Does Not Occur in KL Peptides Lacking a Transmembrane Domain

The lack of any absolute requirement of a cytoplasmic domain for KL cleavage raises the question of whether the transmembrane domain might also be dispensable for this process. The requirements for a transmembrane domain were addressed with two constructs that lack a transmembrane domain: KL-S and KL- Sl^d . KL-S is an

artificially constructed cDNA that encodes the first 164 amino acids of KL, corresponding to the soluble form of KL generated by cleavage of KL-M1 (Figure 1). The KL- Sl^d cDNA encodes a form of KL that is truncated by the Sl^d mutation (Flanagan *et al.*, 1991) and includes the first 180 amino acids of the KL-M1 molecule, ending nine amino acids before the transmembrane domain (Figure 1).

Both KL-S and KL- Sl^d are soluble molecules that lack a transmembrane domain and are not expressed detectably at the cell surface (Flanagan *et al.*, 1991) (Table 1 and Figure 2); however, both molecules contain potential proteolytic cleavage sites. KL-S contains the normal KL-M2 cleavage site (see below for mapping of the KL-M2 site), whereas KL- Sl^d contains both the normal KL-M1 and KL-M2 sites. Coimmunoprecipitation of KL peptides from cells transfected with KL-S or KL- Sl^d cDNAs showed that neither of these soluble polypeptides is susceptible to cleavage at the sites normally recognized in the corresponding transmembrane forms (Figure 5). No trace of the normal cleavage product could be detected, even when the labeled peptides were incubated in the presence of cells for several hours, and even when the autoradiograms were heavily overexposed (Figure 5, far right panel). As with all the immunoprecipitation experiments, some faint minor bands are detectable in this lane, but none of them comigrate with the normal cleavage product, indicating that they probably represent some other form of processing heterogeneity rather than cleavage at the usual site (Figure 5, far right panel). Figure 4 shows that cleavage of the Sl^d polypeptide was also not detected after PMA treat-

Figure 5. Polypeptides encoded by KL cDNAs that differ in the sequence of the extracellular region. COS cells were transiently transfected with KL cDNAs or with vector as a control. Sixty-two hours after transfection, the cells were labeled with 35 S-methionine for 8 h in the presence of 10% serum. KL polypeptides were then isolated from the supernatant (S) or cell lysate (C) by coimmunoprecipitation with AP-KIT fusion protein. Proteins were separated on 15% polyacrylamide gels and autoradiographed. The left and middle panels are from different gels. The KL- Sl^d supernatant lane in the far right panel is the same as that in the left panel but exposed six times longer to detect any faint bands. The arrowhead to the right is to indicate the absence of a detectable band with the mobility of the soluble KL generated by proteolysis at the KL-M1 cleavage site.

ment. A low amount of SL^d polypeptide is seen in this experiment because of the exchange of supernatant during the pulse-chase protocol, but all the detectable material appears at the size expected for the uncleaved polypeptide (Figure 4). These results imply that the presence of a transmembrane domain is strictly required for cleavage within the extracellular domain.

Extracellular Determinants of Site Selection: Cleavage Does Not Depend on a Specific Substrate Sequence but Does Correlate with Spacing from the Membrane

Particularly as KL proteolysis did not seem to require cytoplasmic sequences, we were interested to know what the extracellular determinants of cleavage might be. Structural analysis of naturally occurring soluble rat KL indicated that it ends after alanine-164 or alanine-165 (Zsebo *et al.*, 1990b) presumably representing the cleavage site for the KL-M1 form. In the KL-M2 form this site is removed by alternative splicing, and KL-M2 is cleaved at a slower but still detectable rate (Flanagan *et al.*, 1991; Huang *et al.*, 1992).

To map the approximate cleavage site of the KL-M2 form, soluble KL was immunoprecipitated from the supernatant of cells transfected with KL cDNAs and was then sized by SDS-polyacrylamide gel electrophoresis after deglycosylation (Figure 6). In each lane of the gel shown in Figure 6 several bands can be seen, with the lowest band apparently corresponding to the fully deglycosylated product. The size of the deglycosylated 164 residue KL-S product was estimated at 18.3 kDa from this experiment, consistent with the deglycosylated molecular weight of 18 301 Da predicted from the cDNA sequence. The soluble cleavage product of KL-M1 migrated slightly more slowly, consistent with a predominant cleavage site after alanine-165. This result with mouse KL is consistent with a recent report identifying alanine-165 as the predominant cleavage site of the KL-M1 sequence from humans (Lu *et al.*, 1992). In repeated experiments where soluble KL derived from KL-M2 was run in adjacent lanes with the soluble KL derived from KL-M1, the apparent size difference was estimated at 2400 Da (Figure 6), indicating that the KL-M2 cleavage site is at or near serine-142. The sequence at this region (VLSSTLG) contains a stretch of amino acids that have small side chains with low polarity, a feature also noted at the KL-M1 cleavage site, as well as the cleavage sites of TGF- α and CSF-1 (Massague and Pandiella, 1993).

To determine whether KL cleavage could be blocked by substituting a polar region at the KL-M1 cleavage site, we made construct KL-A164N, where an alanine residue at the cleavage site is converted to an asparagine (Figure 1). In addition to substituting a more polar amino acid residue, this modification creates an N-linked gly-

Figure 6. Size of deglycosylated polypeptides encoded by KL cDNAs. COS cells transfected with KL mutants were metabolically labeled, and KL polypeptides were isolated from the supernatants by coimmunoprecipitation with AP-KIT fusion protein. Before processing on a 15% gel the precipitates were treated with N-glycanase, O-glycanase, and neuraminidase. The polypeptides that appear to correspond to the fully deglycosylated forms are indicated by an asterisk to the right of the band. For clarity, lanes 2, 3, and 4 were more heavily exposed than the other lanes.

cosylation sequence (asparagine-x-serine) and would be expected to cause asparagine 164 to be glycosylated. Cells transfected with KL-A164N display KL on the cell surface (Table 1), and gel analysis indicated a transmembrane KL polypeptide appearing as a broad band centered at ~40 kDa, consistent with glycosylation of the new asparagine-164 residue (Figure 5). After enzymatic deglycosylation, this band drops to approximately the same apparent molecular weight as transmembrane KL-M1, as expected (Figure 6). The A164N mutation causes a moderate reduction in cleavage of transmembrane KL to a soluble form (Figure 5). However, it is perhaps surprising that addition of both a highly polar amino acid residue and a bulky multimeric carbohydrate side chain at the proteolysis site of KL-M1 does not have a greater effect on cleavage.

Although the exact amino acid sequence at the KL cleavage site does not seem to be critical, as judged by the KL-A164N mutant as well as the comparison of KL-M1 with KL-M2, it is of interest that the KL-M1 cleavage site is located ~24 amino acids from the transmembrane domain, whereas our experiments place the KL-M2 cleavage site at a similar spacing of ~19 amino acids from the transmembrane domain. This suggested the possibility that distance from the membrane might be a determinant of cleavage. To test this possibility further, we made construct KL-(exon6)₂ (Figure 1). This construct contains an insertion of 30 amino acids that gives it two successive copies of the sequence of the alternatively spliced exon 6, and therefore two copies of the normal KL-M1 cleavage sequence, as well as one copy of the normal KL-M2 cleavage sequence. The KL-(exon6)₂ cDNA is expressed at the cell surface (Table 1 and Figure 2). On immunoprecipitation the KL-(exon6)₂ polypeptide has an apparent molecular weight of ~43 kDa, as expected, though this peptide is somewhat obscured by background bands (Figure 5). The soluble form of KL produced from KL-(exon6)₂ migrates with an apparent molecular weight of 32 kDa (Figure 5). After deglycosylation the soluble product of KL-(exon6)₂ migrates

with an apparent molecular weight difference of 1300 Da relative to KL-Sl^d run in an adjacent lane, indicating cleavage at or near alanine-195 of KL-(exon6)₂ (Figure 6). Thus, in accordance with the idea that distance from the membrane determines the cleavage site, the KL-(exon6)₂ peptide is evidently cut at the proteolytic site located at the usual spacing (~24 amino acids) from the transmembrane domain, rather than at either of the two potential cleavage sequences located at different spacings from the transmembrane sequence.

DISCUSSION

Although growth factors are classically viewed as soluble molecules, several are now known to be produced as active transmembrane molecules that can give rise to soluble forms by proteolytic cleavage. Important aspects of understanding the biological significance of this finding will be to understand the exact functions as well as the mechanism and control of the proteolytic cleavage that converts the transmembrane forms to the soluble forms.

For TGF- α , one signal required for proteolytic cleavage is apparently the presence of a valine residue at the C-terminus of the cytoplasmic domain, as it was recently reported that deletion or modification of this residue, or deletion of the whole cytoplasmic domain, blocks TGF- α cleavage in transfected CHO cells or mouse fibroblasts (Bosenberg *et al.*, 1992). The possibility of a general mechanism was suggested by the presence of a valine residue at the C-terminus of two additional growth factors, KL and CSF-1 (Bosenberg *et al.*, 1992), though other transmembrane growth factors do not have a valine in this position (Lyman *et al.*, 1993; Mas-sague and Pandiella, 1993).

We have found here that deletion or modification of the C-terminal valine residue of KL did not block the cleavage of KL transfected into cultured cells (see Figure 7, top). The same results were seen after transfection either into COS cells or into mouse fibroblasts that express endogenous KL. Indeed, even deletion of the entire cytoplasmic domain past a three amino acid stop transfer sequence did not discernibly interfere with cleavage. One possible explanation for the different requirement for a C-terminal signal in KL and TGF- α would be the involvement of different proteases. Possibly arguing against this, a recent survey of proteolysis inhibitors concluded that, although some difference was seen with KL-M2, the inhibition profiles of KL-M1 and TGF- α were not distinguishable (Pandiella *et al.*, 1992). Other explanations for the difference in requirement for a C-terminal valine would be different recognition mechanisms involving the same protease or the involvement of different accessory proteins. Alternatively, removal of the C-terminal valine might have different effects on the conformation or oligomerization of the transmembrane growth factor itself.

Figure 7. Diagrammatic summary and model for the cleavage of KL polypeptides. KL variants illustrated at top differ in the cytoplasmic domain, whereas those at bottom have sequence differences in the extracellular domain. Long horizontal lines represent the cell membrane. ■, the portion of each molecule containing the four extracellular cysteine residues, which form intrachain disulfide bridges. Potential proteolysis sequences that are cleaved in one or more KL variants are indicated by short horizontal lines: the number 1 denotes the cleavage site characteristic of the KL-M1 polypeptide, the number 2 denotes the site characteristic of the alternatively spliced KL-M2 form, and 1' indicates the site altered by a substitution mutation that introduces an asparagine residue with N-linked glycosylation. The triangular flags indicate actual sites of proteolytic cleavage determined for each of these KL molecules. The data are consistent with a model where the triangular flag would represent a membrane bound protease that does not require a highly specific substrate sequence in the extracellular domain, or signals in the cytoplasmic domain, but its activity may be limited to a narrow range of distances from the cell membrane.

Although the KL cytoplasmic domain is apparently not an essential requirement for proteolysis, some important function of this domain is implied by its strong evolutionary conservation. In sequence alignments of several species, this region is at least as well conserved as other parts of the molecule with an amino acid identity of $\geq 89\%$ in pairwise comparisons of human, mouse, pig, and dog (Anderson *et al.*, 1990; Huang *et al.*, 1990; Martin *et al.*, 1990; Shull *et al.*, 1992). A role for the cytoplasmic tail is further supported by the nature of the Sl^{17H} allele, a splicing mutation that results in an altered amino acid sequence in this part of the KL molecule. Like the Sl^d molecule, the Sl^{17H} KL molecule can stimulate receptor-bearing cells in a coculture assay (Flanagan *et al.*, 1991; Brannan *et al.*, 1992). However, unlike Sl^d, we find that the Sl^{17H} KL is displayed at the cell surface and does not show any obvious abnormality with regard to cleavage or the protein kinase C-mediated stimulation of cleavage. On the other hand, the

amounts of KL polypeptide expressed in transfected tissue culture cells did appear to be reduced by the SI^{17H} mutation, suggesting that the SI^{17H} phenotype could be because of defects such as impaired transport to the cell surface or instability of the KL polypeptide. The SI^{17H} phenotype is also compatible with the possibility that transmembrane KL could itself be a receptor with a cytoplasmic tail capable of transducing a signal into the cell carrying it. This possibility could be consistent with some of the phenotype characteristics of *SI* and *W* mice, such as alterations in the composition of the extracellular matrix (Morrison-Graham and Weston, 1989).

Regarding the extracellular sequence requirements for the cleavage of KL, there does not appear to be a precise requirement for a specific substrate sequence. The site of KL-M1 cleavage and the site of KL-M2 cleavage mapped here do not have any obvious recognition sequence in common. Although the presence of a cluster of amino acids with small side chains of low polarity is found at both sites and is also a feature of the cleavage sites of CSF-1 and TGF- α , other transmembrane growth factors do not share this feature (Massague and Pandiella, 1993). Moreover, when alanine-164 at the KL cleavage site was converted to a polar asparagine residue, also resulting in addition of a large polar N-linked carbohydrate moiety, we found that cleavage was only somewhat impaired and was not blocked. Previous studies on TGF- α and TNF- α have similarly found that cleavage could only be blocked by major alterations of the sequences at the cleavage sites (Brachmann *et al.*, 1989; Wong *et al.*, 1989; Perez *et al.*, 1990), and a study of the β -amyloid precursor concluded that proteolysis is largely independent of primary structure though it may require an α -helical conformation (Sisodia, 1992). The finding that cleavage does not require a highly specific substrate sequence could be explained by two extreme models: either there are a large number of proteases at the cell surface that can collectively recognize many different substrate sequences, or, perhaps more likely, there is one protease (or a small number of them) that is not tightly restricted as to the primary amino acid sequence it will accept.

Although there is not a tight requirement for primary amino acid sequence, there must be some way to restrict proteolytic cleavage, because only some proteins must be cleaved and only at specific sites. Our results support the idea that certain aspects of the spatial positioning of the cleavage site may be important in generating this specificity. One essential element for the cleavage of KL appears to be the presence of a linked transmembrane domain. We were unable to detect even a minor amount of cleavage of KL variants lacking a transmembrane domain, even when those soluble molecules contain one or more sequences that would be cut in a comparable transmembrane molecule (Figure 7, bottom, constructs KL-S and KL-SI^d). It thus appears that KL cannot be cut

at the appropriate sites by a proteolytic activity in the medium nor can it be cleaved effectively by adjacent cells after secretion. Instead it seems likely that the protease is itself a membrane bound molecule that is active on the surface of the same cell that displays transmembrane KL (Figure 7). The existence of a cell surface protease for TGF- α is also likely (Massague and Pandiella, 1993). However, although membrane anchored proteases are known, no good candidate for a protease responsible for the cleavage of these transmembrane growth factors has yet been identified.

Not only membrane anchorage, but also distance from the membrane, may be important for cleavage site selection. When different combinations of potential sites in KL molecules were tested, the site selected for proteolysis was always located at approximately the same spacing within the primary sequence relative to the transmembrane domain: ~ 24 residues (KL-M1, KL-A164N, and KL-(exon6)₂) or ~ 19 residues (KL-M2) (Figure 7, bottom). Sequences that were cleaved when located at this spacing from the membrane were not recognized when placed at more distant locations (Figure 7). These observations provide further support for the idea of cleavage by a membrane-anchored protease. In addition they imply that this protease may only be able to recognize sites within a limited range of distances from the membrane.

Cleavage by a protease that recognizes sites within a specific range of distances from the membrane may be a widespread phenomenon, because the known cleavage sites in KL, as well as other transmembrane growth factors, are almost invariably located between eight and 24 residues from the transmembrane domain (see references in Massague and Pandiella, 1993). This model for cleavage is compatible with our results and also the results of other studies involving mutations of the region outside the membrane, including a study of the β -amyloid precursor showing that the site of proteolysis remained at the same spacing from the membrane despite the introduction of a small insertion just outside the transmembrane domain (Sisodia, 1992), and a recent study of KL showing that deletion of four amino acids around the KL-M1 cleavage site resulted in usage of a secondary site that appeared to be located approximately nine to 12 amino acids from the membrane (Majumdar *et al.*, 1994). In these experiments cleavage was always correlated with spacing within the primary sequence, though presumably the conformation of some other proteins might allow sequences located more distantly in the primary sequence to come near the membrane in the tertiary structure.

Selection of cleavage sites based on distance from the membrane could be an important factor in preventing indiscriminate proteolysis of proteins near the cell surface and in ensuring that only the correct sites within cell surface molecules are selected. This mech-

anism of site selection also has implications for the potential design of molecules that might affect the cleavage process, providing a way to modulate the action of membrane anchored growth factors as well as other classes of protein that undergo proteolytic release from the cell surface.

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