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THE KIT-LIGAND (STEEL FACTOR) AND ITS RECEPTOR C-*KIT*: PLEIOTROPIC ROLES IN CELL GROWTH, CELL SURVIVAL AND CELL MIGRATION - INSIGHTS FROM GERMLINE MUTATIONS.

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INTRODUCTION

Receptor tyrosine kinases and their cognate ligands function in the transduction of extracellular signals and control processes such as cell proliferation, cell survival, motility and differentiation. Therefore, receptor tyrosine kinases play key roles in the formation of patterns in embryonic development, in organogenesis and in the adult life of invertebrate and vertebrate animals. Receptor tyrosine kinases and their cognate ligands which determine developmental processes are known in Drosophila, the nematode C. elegans and in mammals. In mice, germ line mutations with developmental consequences in several receptor tyrosine kinases have been described in the recent past. The c-*kit* receptor tyrosine kinase and the *kit*-ligand, KL, were shown to be allelic with the *white spotting (W)* and the *steel (Sl)* loci respectively; the platelet derived growth factor receptor A chain (PDGFRA) with the *patch (Ph)* locus and the macrophage colony stimulating factor (CSF-1) with the *osteopetrosis (op)* locus [1,2]. In this paper insights into the c-*kit* receptor system that arise from the molecular characterization of c-*kit* and its ligand and from the analysis of various alleles at the *W* and *Sl* loci will be discussed.

Mutations at the murine *white spotting* and the *steel* loci generate deficiencies in three cell systems: the pigmentary system, germ cells and hematopoiesis during embryogenesis and in the adult animal [for reviews see: 3,4]. During normal development melanoblasts arise from the neural crest, migrate to the periphery, where they enter the epidermal ectoderm and colonize hair follicles. Postnatally amelanotic melanoblasts differentiate to become melanocytes. *W* and *Sl* mutations affect several aspects of melanogenesis causing varying degrees of white spotting. Primordial germ cells migrate from the base of the allantois through the hindgut endoderm and the mesentery to the genital ridges, and spermatogenesis and oogenesis then proceed following well known developmental programs. *W* and *Sl* mutations affect the survival, migration and proliferation of primordial germ cells and aspects of spermatogenesis and oogenesis causing impaired fertility. In hematopoiesis *W* and *Sl* mutations affect cells within the stem cell hierarchy, distinctive cell

populations in the erythroid cell lineage and mast cells, during early development as well as in the adult animal. Therefore, mutant animals suffer from macrocytic anemia and they lack tissue mast cells. While *W* mutations are cell autonomous, *Sl* mutations affect the microenvironment of the targets of the mutations [5-7]. These findings were a strong indication that the *white spotting* and *steel* gene products function in the same biochemical pathway possibly as receptor and ligand [8,9].

THE C-*KIT* RECEPTOR AND ITS LIGAND KL / *STEEL* FACTOR

The proto-oncogene *c-kit* is the cellular homolog of v-*kit* the oncogene of an acute transforming feline retrovirus, the Hardy-Zuckermann 4 - feline sarcoma virus, which was isolated from a feline leukemia virus-associated feline fibrosarcoma [10]. v-*kit* encodes the catalytic domain of a tyrosine protein kinase, which is most closely related to the tyrosine kinases of the PDGF receptor subfamily and is expressed as an $80kD^{gag\text{-}kit}$ fusion protein in HZ4-FeSV infected cells. *c-kit* encodes a receptor tyrosine kinase with an extracellular domain that contains 5 imunoglobulin domains and a cytosolic kinase which is divided into two subdomains by the so-called kinase insert segment characteristic of the platelet derived growth factor receptor subfamily [11,12]. A normal variant of c-*kit*, formed as the result of alternate usage of a 3' splice site, contains a four amino acid insert in the extracellular domain between amino acids 512 and 513 of the known murine c-*kit* sequence [13,14].

The identity of the c-*kit* proto-oncogene with the *white spotting* locus was established by linkage analysis [9], the demonstration of rearrangements in the c-*kit* gene in some *W* alleles [15] and the finding of missense mutations which inactivate the c-*kit* kinase [16,17].

The soluble form of the ligand of the c-*kit* receptor kinase was identified by three independent approaches: 1) on the basis of the known function of c-*kit*/*W* in bone marrow derived mast cells [18], 2) as a factor which promotes the formation of colonies from early hematopoietic progenitors [19.20], or as a mast cell growth factor [21]. Subsequently, two alternatively spliced KL RNA transcripts have been shown to encode two cell associated KL protein products, KL-1 and KL-2, which differ in their sequences N-terminal of the transmembrane segment and are expressed in a tissue specific manner [22-25]. The KL-2 protein product lacks sequences which include the major proteolytic cleavage site for the generation of the soluble KL protein product of KL-1. The KL-1 protein is efficiently processed by proteolytic cleavage to produce soluble KL; by contrast KL-2 is also processed to form soluble KL but not as effectively and therefore KL-2 represents a differentially more stable cell associated form of KL. Interestingly, the protein kinase C inducer PMA accelerates the proteolytic cleavage of both KL-1 and KL-2 suggesting that this process is subject to regulation [25]. Consequently, differential expression of variant cell membrane associated KL molecules and their proteolytic cleavage to generate soluble forms of KL provide different means to control and modulate c-kit function in various

cell types during development and in the adult animal. The defects in *W* and *Sl* mutant mice are consistent with a role of the c-*kit* receptor system in facilitating cell proliferation, cell survival of precursor cells as well as promoting cell migration and functions in differentiated cells. It then is plausible that the cell membrane and the soluble forms of KL serve different aspects of c-kit function.

C-*KIT* AND KL EXPRESSION IN ORGANOGENESIS AND ADULT LIFE

The synchronous expression of the c-*kit* receptor and its ligand in close cellular environments are a good predictor for c-*kit* function *in vivo*. Therefore, the examination of c-*kit* and KL expression during embryogenic development and in the adult animal by using RNA blot analysis, *in situ* hybridization and histochemistry has provided important insights into our understanding of c-*kit* function. In agreement with the cell autonomous nature of *W* mutations, c-*kit* is expressed in targets of *W* and *Sl* mutations during embryogenesis and in the adult animal in melanogenesis, gametogenesis (primordial germ cells, oogenesis and spermatogenesis) and in cells of the hematopoietic system [16,26-31]. In addition, expression of KL was shown to be associated with migratory pathways of melanoblasts and germ cells and homing sites of germ cells and hematopoietic progenitors during embryonic development, i.e. the genital ridges and the fetal liver [32].

Interestingly, c-*kit* and KL are also expressed in cell types that are not targets of *W* and *Sl* mutations. During embryonic development c-*kit* expression is seen in the neural tube, dorsal root ganglia, portions of the developing central nervous system, the olfactory epithelium, the digestive tract, the lung, and other tissues, whereas KL expression is seen in the floor plate of the neural tube, the thalamus and in the olfactory epithelium [33,34 and K Manova, unpublished data]. In the developing nervous system c-*kit* expression is typically seen in cells which have ceased to divide and began their differentiation. In the adult animal c-*kit* and KL expression are prominent in the lung and in the brain, including the cerebellum where c-*kit* expression is evident in basket, stellate and Golgi neurons and KL expression in Purkinje neurons [16,34,35]. Although, many mutations are known at the *W* and *Sl* loci, no neurologic phenotypes and no histopathological changes have been noted in these mice. It may be, that as a result of redundancies, other signaling mechanisms compensate for the lack of c-*kit* function in neuronal cells. The elucidation of a role for the c-*kit* receptor system in the developing and the mature central nervous system is an important task of the future.

MOLECULAR BASES OF *W* AND *SL* MUTATIONS

An easily recognizable phenotype, namely spotting, has made possible the isolation of many distinct mutations at the *W* and the *Sl* loci [3,4]. These mutations provided the opportunity to characterize both the molecular basis of these mutations as well as the consequences of these mutation on different cell lineages and tissues thus furthering our understandin of c-*kit* function. Many of

the *W* and *Sl* mutations vary in the degree of severity in the homozygous and the heterozygous state and the effect on the different cell lineages. In the homozygous state the original *W* and *Sl* alleles and several others are perinatal lethal while others are viable and possibly fertile. A number of different *W* and *Sl* alleles have been characterized at the molecular level and they were shown to be loss of function mutations.

***W* mutations:** The W^{19H} and the original *W* allele are essentially recessive mutations with a severe homozygous phenotype. The W^{19H} allele results from of a chromosomal deletion that includes the entire c-*kit* gene and the *W* allele from a splice site mutation that generates a nonfunctional c-*kit* protein product which is not expressed on the cell surface [9,13,15,36 and Tan and Besmer, unpublished]. Several semidominant mutations exist at the *W* locus ($W^{42,37,V,55,41}$). Even though the various alleles vary in their degree of severity in the homzygous state, each allele affects all three cell lineages fo comparable degrees. These alleles result from missense mutations which affect the c-*kit* activity to differing degrees [17,36,37]. Work on the mechanism of the activation of several tyrosine kinase receptors, including the PDGF receptor family including the c-*kit* receptor, implies receptor dimers or oligomers as intermediates [46]. The dominant phenotypes of these mutations indicates that the mutant c-*kit* proteins in receptor heterodimers interfere with KL induced signal transmission effectively reducing the number of normal receptor molecules on the cell surface. Consequently these mutations give rise to more severe heterozygous mutant phenotypes and, thus, have the hallmarks of dominant negative mutations.

W-sash is an interesting allele at the *W* locus [38]. The W^{sh} mutation differs from most *W* mutations in that it affects primarily mast cells and melanogenesis but not other cellular targets of *W* and *Sl* mutations. Thus, W^{sh}/W^{sh} mice are fertile and not anemic, but they lack mast cells in their skin and intestine and are devoid of coat pigment. Heterozygotes are black with a broad white sash/belt in the lumbar region. Non-parallel display of mutant characteristics might be the consequence of a defect that affects c-*kit* expression in one and not in an other cell type. The unique W^{sh} phenotype in the heterozygous state furthermore indicates a dominant property of this mutation. To determine the basis for the phenotypes of *W-sash* mice, we investigated c-*kit* RNA and protein expression patterns in adult W^{sh}/W^{sh} mice and during embryonic development. c-*kit* expression was absent in bone marrow derived W^{sh}/W^{sh} mast cells, the fetal and the adult lung, and the digestive tract at embryonic day 13½ (E13½), tissues which normally express c-*kit*. In addition, at E13½ W^{sh}/W^{sh} embryos lack c-*kit* positive cells in the skin. All other tissues which normally express c-*kit* also express c-*kit* in W^{sh}/W^{sh} embryos and adults. Unexpectedly, in E10½ W^{sh}/W^{sh} embryos ectopic c-*kit* expression was observed in the dermatome of the somites, the mesenchyme around the otic vesicle and the floorplate of the neural tube, structures known to express the c-*kit* ligand in wild-type embryos. The ectopic c-*kit* expression in W^{sh} homozygous embryos does not affect KL expression. This suggests that c-*kit* expression in different tissues is regulated

by different cis-acting elements and that positive regulatory elements specific for mast cells and mesenchymal cells in the lung and the digestive tract are affected by the W^{sh} mutation. Furthermore, the observation of c-*kit* expression in tissues which normally express KL implies that the regulatory region of the c-*kit* gene includes negative control elements which are also affected by this mutation. The inappropriate c-*kit* expression in the dermatome of E10½ and E11½ mutant embryos is an important feature of the W^{sh} mutation which could provide an explanation for the dominant pigmentation defect in adult mutant mice. We propose that inappropriate expression of c-*kit* receptor in the dermatome of W^{sh}/W^{sh} mice may sequester (bind and neutralize) the kit-ligand. As a result the amount available for c-*kit* expressing melanoblasts migrating over the dermatome is reduced, thus affecting their survival and/or proliferation. It is also conceivable that the co-expression of c-*kit* and its ligand results in the activation of dermatomal cells by an autocrine or paracrine mechanism and this in turn may lead to changes in the extracellular matrix. In either model, the W^{sh}/W^{sh} melanocyte precursors would die betweeen day E11½ and day E13½. In support of our model is the fact that in the trunk region migration of melanocyte precursors over the somites occurs at E10½-11½ and thus coincides with the time of inappropriate c-*kit* expression in the dermatome. Also in agreement with this paradigm, we found c-*kit* expressing melanocyte precursors in E10½-11½ W^{sh}/W^{sh} embryos apparently on a dorsolateral pathway, whereas in 13½ day embryos no c-*kit* positive cells were detected in the skin. Although melanocyte precursors appear to express c-*kit* normally in day 10½ W^{sh}/W^{sh} embryos, these results do not preclude the possibility, that c-*kit* expression in more mature melanoblasts/melanocytes is affected by the W^{sh} mutation. Taken together these results may suggest that c-*kit* is required between E10½-13½.

***Sl* mutations:** Several severe *Sl* alleles ($Sl, Sl^{J,gb,8H,10H,12H,18H}$), based on Southern blot analysis, have been shown to contain deletions which include the KL gene, and therefore are KL loss of function mutations [20,22,39]. The *Steel-Dickie* allele (Sl^d), although viable and less severe, when homozygous displays all of the pleiotropic effects associated with *steel* mutations, suggesting some residual functional activity of KL. Molecular analysis indicates that the Sl^d allele arose as a result of an intragenic deletion which includes the transmembrane domain and the C-terminus generating a secreted, biologically active KL protein product [24,25,40]. The biological characteristics of homozygous Sl^d/Sl^d mice and of Sl/Sl^d mice suggests, that although the Sl^d KL protein product is able to sustain some function, it is in major ways defective in facilitating cell proliferation and cell survival. This indicates that the cell membrane form of KL plays a critical role in c-*kit* function.

Steel-Panda is a week *Sl* allele. Homozygous $\boldsymbol{Sl^{pan}/Sl^{pan}}$ mice are black eyed whites with pigmented ears and scrotum and they have a mild macrocytic anemia. Interestingly, females are sterile, whereas males are fertile [41]. Molecular analysis indicated that the KL coding sequences are normal in the Sl^{pan} allele, but that the levels of the KL transcripts are consistently reduced in most tissues analyzed; and therefore, the Sl^{pan} mutation appears to affect KL

gene expression [42].

Work by Bennett and by Mintz and Russell had indicated that *Sl* and *W* null mutations affect primordial germ cells during embryonic development [8,43]. The observation of differential sterility in males and females in some *steel* mutations and Sl^{pan}, Sl^{t} and Sl^{con} are examples, indicated that the c-*kit* receptor is essential also after sexual differentiation. In normal mice the c-*kit* receptor is expressed highly in all stages of postnatal oogenesis [27], by contrast KL is expressed in follicle cells increasing during follicle development to high levels in the three layered cuboidal stages [44]. Taken together these results might suggest a role for c-*kit* in oocyte maturation or oocyte growth.

Histological analysis of ovaries from homozygous Sl^{pan} mice indicates that the number of primordial oocytes in neonatal animals was reduced to 20% of that in heterozygotes, and that in juvenile and adult mice ovarian follicle development was arrested at the one-layered cuboidal stage, with a few exceptions. Therefore, a reduced level of KL in Sl^{pan}/Sl^{pan} ovary in follicle cells appears to arrest ovarian follicle development in agreement with an essential role for c-*kit* in oocyte growth/maturation. Whereas KL is limiting in oogenesis, a reduced level of KL does not affect spermatogenesis. In addition the Sl^{pan} mutation appears to have a partial effect on primordial germ cells which is reflected in the reduced number of oocytesand spermatogonia in the Sl^{pan} ovaries and testis, respectively.

GENERATION OF *W* PHENOTYPES

Formal proof for the molecular basis of *W* mutations may be obtained either by correcting the germ line defects in mutant mice or by generating *W* mutant phenotypes in normal mice. We thought to generate a germ line modification which would generate *W* phenotypes by exploiting the dominant negative characteristics of the W^{42} mutation. Transgenic mice were derived in which the c-kit^{W42} gene products were expressed ectopically under the instruction of the human actin promotor to generate *W* phenotypes [45]. The predictions of the outcome of this experiment were: (1) expression of the c-kit^{W42} transgene in cells which express the endogenous c-*kit* gene could result in effects similar to those seen in $W^{42}/+$ mice; (2) expression of the transgene in cells which espress KL could effect the neutralization of KL and thus affect normal c-*kit* function; (3) Expression of the c-kit^{W42} transgene in other cells would be of no consequence since the transgene is functionally inactive. Transgenic mice expressing the c-kit^{W42} transgene showed an effect on pigmentation and the number of tissue mast cells. A patchy coat color pattern was observed in some mice presumably as the result of variable expression of the transgene in melanoblast progenitors. However, germ cell development and erythropoiesis in these mice were not affected by the transgene. Therefore, mice expressing the c-kit^{W42} transgene recapitulate some of the phenotypes of mice with *W* mutations in agreement with the molecular basis of the W^{42} mutation and the dominant negative characteristics of the c-kit^{W42} protein product. By using cell lineage specific promoters it should now be possible to express the c-

kit^{W42} gene products and inhibit c-*kit* function in specific cell types. A function in cell differentiation and development is not known for many mammalian kinase receptors due to the scarcity of germ line mutations. Null mutations in these receptor genes, presumably like *W* mutations, may be recessive lethals. Cell-type restricted or ectopic expression in transgenic or chimeric mice of newly manufactured dominant negative mutations, like c-*kit*^{W42}, may help define the function of tyrosine kinase receptors in development and differentiation.

CONCLUSIONS

The discovery of the identity of c-*kit*, a member of the PDGF receptor family, with the W locus brought to light the interesting pleiotropic roles of this gene in developmental processes, particularly melanogenesis, gametogenesis and hematopoiesis. Knowledge of c-*kit* function facilitated the identification and characterization of the ligand of the c-*kit* receptor and the demonstration of allelism between the *kit*-ligand and *Steel* provided a molecular notion to the relationship between the *W* and *Sl* mutations which had been anticipated by mouse geneticists years ago. A role for c-*kit* is now known in many cell types and cell lineages because of the numerous mutations that are available in this gene system. Interestingly, in hematopoiesis, gametogenesis and melanogenesis W mutations affect immature and mature cell populations suggesting that c-*kit* is critically involved in the formation and maintenance of these cell systems.

A corollary of the recent studies of the c-*kit* receptor system is the realization that c-*kit* may function in cell types that are not targets of *W* and *Sl* mutations. Redundant signaling mechanisms may compensate for the lack of c-*kit* function in these cell systems. On one hand redudant signaling systems in higher vertebrates may provide safeguards in case of deficiencies, and on the other hand similar and redundant systems may facilitate fine tuning of complex mechanisms of regulation of homeostasis. Undoubtedly, investigation of the functions, if any, of the c-*kit* receptor system in cell types that are not targets of *W* and *Sl* mutations such as the central nervous system will be a challenging task of the future.

The detailed knowledge of the functional significance of the c-*kit* receptor system *in vivo*, provided through mutant phenotypes, should facilitate the elucidation of the mechanisms underlying cell proliferation, cell adhesion/migration, cell survival and other post mitotic functions in various cell systems during development and in adult life. In addition, the c-*kit* receptor system should also provide an excellent model to study the mechanisms underlying synergism between different signaling systems.

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Discussion - THE KIT-LIGAND (STEEL FACTOR) AND ITS RECEPTOR C-*KIT*: PLEIOTROPIC ROLES IN CELL GROWTH, CELL SURVIVAL AND CELL MIGRATION - INSIGHTS FROM GERMLINE MUTATIONS

H.F. Lodish

As you know, in the Drosophila bride of sevenless system the cells that express the receptor tyrosine kinase bind the ligand presented to it by the opposite cell and actually internalise pieces of the plasma membrane on that cell. Does anything like that happen here?

P. Besmer

This is an interesting point. We actually have done experiments to address this issue. In the cerebellum receptor expressing basket and stellate cells take up KL presumably by receptor mediated endocytosis. By using antibodies specific to the extracellular domain (ligand portion) and to the cytoplasmic domain we found that the material contained within these cells comprised predominantly soluble KL and not the entire KL molecule as with bride of sevenless.

During oogenesis KL taken up by oocytes is found in granules and appears to consist of soluble KL as well as of a smaller fraction of KL containing the cytoplasmic domain. The uptake of KL containing the cytoplasmic domain in oocytes may result by phagocytosis of small fragments of follicle cell membranes as suggested by Zamboni or alternatively fragments of KL-containing membrane may be taken up by a receptor mediated process as in the sevenless system.

H.F. Lodish

I was implying that, in fact, there was receptor mediated internalisation of the bound factor presented by the adjacent cell.

J. Baguñá

In the hemopoietic lineage which are the cells expressing the Steel factor (kit ligand)? Are they stroma cells? Finally, can you make a brief comment on the state

of the art of finding the grand-grandmother of the stem cell, that is the true stem cell?

P. Besmer

To answer your first question: Very little information is available today about KL expressing cells in the bone marrow or other sites of active hematopoiesis. Let me rephrase your second question: Which progenitors in the hematopoietic system express the c-kit receptor? The c-kit receptor is expressed on cell populations at very early stages of hematopoiesis, i.e. c-kit expression has been reported on the primitive "stem cell" populations isolated by Visser, Weissman and Dexter and their colleagues. In addition, c-kit is expressed on most of the committed progenitors of the hematopoietic system including lymphoid, myeloid and erythroid progenitors.

J. Baguñá

I would also make a brief comment to an earlier lecture where Lodish mentioned that he knew of no invertebrates having system like hematopoietic lineages. I am not going to say that invertebrates have such an elaborate system as blood cells of vertebrates, but I would like to mention that lower invertebrates (e.g. hydra and flatworms) have renewal systems involving very different cell lineages and pluripotent stem cells that work very well like the hemopoietic system in mammals. So I think that before hematopoietic lineages were just set up genetic systems had all the wiring to make such a beautiful lineage of cells.

J. Massagué

If the transmembrane cytoplasmic domains of kit-ligand were to serve the function of merely providing an anchor, one might expect that there would be no pressure to conserve them. Are these regions conserved, suggesting that they might be playing some further additional function?

P. Besmer

Between mouse and human the cytoplasmic domains of KL are highly homologous suggesting functional significance of these sequences. However, at this

time we do not have a good appreciation of the roles these sequences may play.

J. Massagué

It should be noted that in the case of boss (bride of sevenless), the transmembrane anchor spans the membrane seven times and has a cytoplasmic domain, so it looks like a receptor.

P. Besmer

We are in the process of investigating the functional significance of the cytoplasmic domain sequences of the kit-ligand and made serial deletions which remove cytoplasmic domain sequences of the kit-ligand. Most of these deletion mutations do not affect processing of kit-ligand to the cell surface and proteolytic cleavage in significant ways. However, deletion of the entire cytoplasmic domain severely affects processing to the cell surface. Interestingly, with the cytoplasmic domain minus mutation proteolysis and release of soluble kit-ligand into the medium still occurs and actually can be accelerated by PMA.