W mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the c-kit receptor

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Mutations at the mouse W/c-kit locus lead to intrinsic defects in stem cells of the melanocytic, hematopoietic, and germ cell lineages. W alleles vary in the overall severity of phenotype that they confer, and some alleles exhibit an independence of pleiotropic effects. To elucidate the molecular basis for these biological differences, we analyzed the c-kit locus and the c-kit-associated autophosphorylation activities in five different W mutants representative of a range of W phenotypes. Mast cell cultures derived from mice or embryos homozygous for each W allele were deficient in c-kit autophosphorylation activity, the extent of which paralleled the severity of phenotype conferred by a given W allele both in vivo and in an in vitro mast cell coculture assay. The mildly dominant, homozygous viable alleles W 44 and W 57 were found to express reduced levels of an apparently normal c-kit protein. In contrast, c-kit kinase defects conferred by the moderately dominant, homozygous viable alleles W 44 or W 55 or the homozygous lethal allele, W 37, were attributed to single-point mutations within the kinase domain of the c-kit polypeptide, which result in point substitutions of amino acid residues highly conserved in the family of protein tyrosine kinases. The nature and location of these amino acid substitutions account for the relative severity of phenotypes conferred by these W alleles and demonstrate that the pleiotropic developmental defects associated with the W/c-kit locus arise as the result of dominant loss-of-function mutations in a transmembrane receptor tyrosine kinase.

[Key Words: c-kit tyrosine kinase; W locus; dominant-negative mutations]

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The mechanisms that underlie the determination of cell fate and cell lineage during mammalian development remain to be determined. In Drosophila, progress toward an understanding of the strategies utilized by multicellular organisms to ensure orderly development has derived largely from the molecular analysis of the genes responsible for various developmental mutations. This has revealed a limited number of protein families that are involved in developmental decisions. Included among these are proteins with tyrosine kinase activity. Tyrosine kinases were identified initially as the products of various oncogenes. They can be divided into those that are associated with the cytoplasmic aspect of the cell membrane and transmembrane receptors that have a single membrane-spanning domain (for review, see Hanks et al. 1988; Yarden and Ullrich 1988). This latter group of proteins are thought to play important roles in transduction of extracellular signals to the cytoplasm by means of ligand–receptor interactions at the cell surface, activating the intracellular tyrosine kinase domain. The potential of these molecules to mediate intercellular signaling, along with the high degree of evolutionary conservation of this gene family, suggests that receptor tyrosine kinases play important roles in cell–cell communication during embryonic development of multicellular organisms.

Consistent with this hypothesis, distinct developmental abnormalities have been found to result from mutation of receptor tyrosine kinases. For example, sevenless is a cell autonomous homeotic mutation that results in the failure of undifferentiated cells to detect and respond to positional cues to differentiate to the R7 photoreceptor within each ommatidium of the compound eye (Tomlinson and Ready 1987). The sevenless gene has been isolated and shown to be the Drosophila homolog of the proto-oncogene c-ros, a member of the receptor tyrosine kinase gene family (Hafen et al. 1987). Similarly, mutations in another transmembrane protein tyrosine kinase, the Drosophila epidermal growth factor (EGF) receptor homolog [DER] gene, have been found to
result in abnormal egg development (Price et al. 1989; Schejter and Shilo 1989) and to affect differentiation of ommatidia in compound eye development (Baker and Rubin 1989). Torso, mutation of which affects pattern formation in the early Drosophila embryo, has also been shown to encode a receptor tyrosine kinase (Sprenger et al. 1989).

In mammalian systems, genetic analysis of the roles played by tyrosine kinases in development has been hampered by the paucity of known germ line mutations in these genes. We have shown recently that the proto-oncogene c-kit, which encodes a transmembrane receptor tyrosine kinase, maps to the mouse W locus (Chabot et al. 1988). Moreover, genomic rearrangements within the c-kit locus have been reported for two W alleles (Geissler et al. 1988), and the kinase activity associated with the c-kit receptor is impaired in mast cell cultures derived from W/W embryos (Nocka et al. 1989; Bernstein et al. 1990). These data, along with the demonstration that c-kit is expressed in those cell lineages affected by mutations at the W locus (André et al. 1989; Nocka et al. 1989; Bernstein et al. 1990) have provided strong evidence that W and c-kit are allelic.

Mutations at the murine dominant white spotting (W) locus (Little 1915) have pleiotropic effects on the development of hematopoietic, melanocyte, and germ cell lineages (for reviews, see Russell 1979; Silvers 1979). A large number of W alleles have been independently isolated that not only vary in the overall severity of the mutant phenotype but also exhibit an independence of pleiotropic effects (Table 1). By definition, melanogenesis is affected in animals heterozygous for any allele at the W locus, although the extent of white spotting can vary. Some W alleles are lethal in the homozygous state, with embryos being severely anemic and dying at or near parturition despite having normal hematocrits in the mid-gestation period (Little and Cloudman 1937). W v (Little and Cloudman 1937), W a4, W 4z (Geissler et al. 1981) are spontaneous mutants, with homozygotes having a severe anemia both as embryos being severely anemic and dying at or near parturition despite having normal hematocrits in the mid-gestation period. W az mostly white normal normal postimplantation lethal. W mostly white normal normal postimplantation lethal. W sz white spot normal normal white patches mild anemia normal. W white spot normal normal mostly white normal reduced. W 44 white spot normal normal mild anemia normal. W 57 white spot normal normal mild anemia normal. W 41 white spot mild anemia normal normal. W 55 white spot mild anemia normal normal. W 4 white spot mild anemia normal normal. W s7 white spot normal normal white patches mild anemia normal. W white spot normal normal mostly white normal reduced. W v white spot mild anemia normal normal. W v white spot mild anemia normal normal. W white spot normal normal mostly white severe anemia normal. W s7 white spot normal normal white patches mild anemia normal. W s7 white spot normal normal white patches mild anemia normal. W s7 white spot normal normal white patches mild anemia normal. W s7 white spot normal normal white patches mild anemia normal. W s7 white spot normal normal white patches mild anemia normal. W s7 white spot normal normal white patches mild anemia normal. W s7 white spot normal normal white patches mild anemia normal.

Table 1. Pleiotropic effects of various W mutations

| Heterozygote [W+/+ | Homozygote [W+/W+] |
|-----------------|-----------------|
| melanogenesis | hematopoiesis | fertility | melanogenesis | hematopoiesis | fertility |
| mostly white | normal | normal | postimplantation lethal | postimplantation lethal |
| W | white spot | normal | normal | all white | severe anemia | sterile |
| W v | white spot | mild anemia | normal | all white | severe anemia | sterile |
| W 55 | white spot | mild anemia | normal | mostly white | severe anemia | normal |
| W 41 | white spot | mild anemia | normal | mostly white | normal | reduced |
| W 44 | white spot | normal | normal | white patches | mild anemia | normal |
| W 57 | white spot | normal | normal | white patches | mild anemia | normal |

The phenotypic effects of various W alleles on melanogenesis, hematopoiesis, and gametogenesis are shown. The alleles are listed in order of decreasing severity. W (Little 1915) and W 57 (Geissler et al. 1981) are spontaneous mutants, with homozygotes having a severe macrocytic anemia detectable at mid-gestation and dying at or near parturition. W v (Little and Cloudman 1937), W 41 (Geissler et al. 1981), W 55, and W 57 [J. Barker, pers. comm.; data not shown] are all spontaneous homozygous viable mutants.

c-kit point mutations in W mice

The overall severity of different W alleles can be reproduced in vitro by a mast cell coculture assay

Homogenous populations of mast cells can be generated in vitro by suspension culture in the presence of an exogenous source of interleukin-3 [IL-3] (for review, see Schrader 1986). An alternate mode of mast cell growth has been described (Fujita et al. 1988b) that has no requirement for exogenous growth factors but is dependent on contact with embryonic fibroblasts. W/W mast cell cultures show normal proliferation in suspension culture but are deficient in fibroblast-dependent growth, a result of a defect in transition from G1 to S phase of the cell cycle (Fujita et al. 1988a).
Mast cell cultures derived from bone marrow or fetal liver of mouse strains WB/Re W/W and C57BL/6 +/+ W/W', W37/W37, W44/W44, W55/W55 or W57/W57 were found to proliferate at similar rates in suspension culture in the presence of exogenous IL-3 (data not shown). However, as seen in Figure 1, markedly different growth properties were observed in the fibroblast coculture assay. After 8 days in coculture, a decrease in mast cell viability was apparent in cultures derived from the homozygous viable mutants W44, W41, W55, W57, and the viable compound heterozygote W/W v relative to mast cells from normal mice. In contrast, mast cell cultures derived from the fetal liver of embryos homozygous for the severe alleles W and W37 were unable to survive in coculture with normal fibroblasts. Thus, the mast cell coculture assay reflected the relative severity of phenotypes conferred by different W alleles on the whole organism.

Both severe and mild W mutations confer deficiencies in c-kit in vitro autophosphorylation activity

Mast cells from normal mice contain two glycosylated forms of c-kit with molecular weights of 160 and 124 kD, both of which have in vitro kinase activity [Nocka et al. 1989; Bernstein et al. 1990]. In contrast, mast cells derived from adult bone marrow of W/W' animals, despite expressing normal levels of both glycosylated forms, are deficient in c-kit kinase activity [Nocka et al. 1989; Bernstein et al. 1990]. Antiserum raised against a TrpE-kit fusion protein containing part of the intracellular domain of murine c-kit was used to assess any quantitative or qualitative changes in c-kit protein expression in mast cell cultures homozygous for the lethal allele W37 or the viable W alleles, W44, W55, and W57.

Three patterns of expression were observed. First, approximately equivalent amounts of c-kit protein were observed in mast cells derived from the bone marrows of +/+ mice and mice homozygous for the mild alleles W41 and W55 (Fig. 2A). In contrast, mast cells homozygous for the W44 and W57 mutations contained much lower amounts of c-kit protein, suggesting that these W alleles confer quantitative changes in c-kit expression. A third pattern of c-kit expression was found with W37/W37 mast cells in which an alteration in the relative abundance of the two glycosylated forms of c-kit was observed, although the total amount of immunoprecipitable c-kit protein was similar to that found in +/+ mast cells. This difference cannot be attributed to the fetal origin of these cultures, as mast cell populations isolated
from the fetal livers of mice not carrying the \( W^{37} \) allele expressed both forms of \( c\)-kit in a ratio similar to that observed in mast cells derived from adult bone marrow (data not shown).

To determine whether these different \( W \) mutations affected \( c\)-kit-associated kinase activity, in vitro autophosphorylation assays were performed on \( c\)-kit protein immunoprecipitated from mast cell cultures. Three patterns of \( c\)-kit activity were observed: (1) The decreased levels of \( c\)-kit protein in \( W^{44}/W^{44} \) and \( W^{57}/W^{57} \) mast cells were reflected by a proportionate decrease in \( c\)-kit autophosphorylation activity (Fig. 2B); (2) the levels of \( c\)-kit-associated kinase activity were markedly reduced in mast cells derived from the homozygous viable mutants \( W^{41} \) and \( W^{55} \) (Fig. 2B), and (3) no kinase activity associated with either the 160- or 124-kD \( c\)-kit proteins was detectable in \( W^{37}/W^{57} \) mast cell cultures (Fig. 2B).

\( W^{37} \), \( W^{55} \), and \( W^{41} \) alleles contain point mutations in the \( c\)-kit tyrosine kinase domain

Southern blot analyses failed to detect genomic rearrangements of \( c\)-kit in \( W^{37} \), \( W^{55} \), or \( W^{41} \) alleles (data not shown), suggesting that the molecular basis for the deficiencies in these \( W \) mutants lay in point mutations or small deletions/insertions in the \( c\)-kit coding sequence. To identify such changes, \( c\)-kit cDNA was cloned from cell types homozygous for each of these \( W \) mutations by means of polymerase chain reaction (PCR) using a series of \( c\)-kit-specific oligonucleotide pairs (Fig. 3A). When compared with the published murine \( c\)-kit cDNA sequence (Qui et al. 1988), nucleotide sequence analyses of cDNA clones revealed single GC→AT transitions in the \( c\)-kit coding sequence that result in distinct amino acid substitutions in the \( c\)-kit polypeptide in each of these three \( W \) alleles (Fig. 3B–D). Glu\(^{382}\) is mutated to lysine in \( W^{37} \), whereas methionine replaces threonine at residue 660 in \( W^{55} \). Both of these point mutations lie within or near the ATP-binding pocket of the \( c\)-kit kinase domain. Val\(^{831}\) within the phosphotransferase domain of the \( c\)-kit protein is mutated to methionine in \( W^{41} \) (Fig. 3D). All of the mutations described above were found consistently in a total of six \( W^{37} \), four \( W^{55} \), and seven \( W^{41} \) clones analyzed from two independent PCR reactions for each given \( W \) allele and were never found in \( c\)-kit cDNA clones generated from other \( W \) alleles.

Two other substitutions were detected in our \( c\)-kit nucleotide sequences compared with the sequence published previously for BALB/c brain cDNA (Qui et al. 1988). A GC→AT transition at nucleotide 1618 was found consistently in our sequence. This substitution was not \( W \) allele specific, did not alter Val\(^{530}\) encoded at this position, and was assumed to be a strain-specific polymorphism. An AT→GC substitution at position 648 was also always found in our sequences resulting in Glu\(^{207}\) being replaced by Ala\(^{207}\) in the \( c\)-kit polypeptide. This substitution was also observed in a \( c\)-kit brain cDNA clone isolated from BALB/c mice (D. Fowlkes and O. Smithies, pers. comm.).

Discussion

\( c\)-kit kinase activity in different \( W \) mutants

We have shown that the kinase activity associated with the \( c\)-kit transmembrane receptor is impaired in physiologic target cell populations derived from mice bearing each of five spontaneous \( W \) mutations. As summarized in Table 1, the alleles analyzed confer a wide range of \( W \) mutant phenotypes from severe (homozygous lethal) to mild (homozygous viable and fertile). The in vitro coculture assay described here reflected the overall severity of different \( W \) phenotypes. Mast cells homozygous for viable alleles [\( W^{41} \), \( W^{44} \), \( W^{55} \), \( W^{57} \)] were found to have a greater capacity to survive on fibroblast feeders than those homozygous for the lethal \( W^{37} \) allele. Similarly, the amount of residual \( c\)-kit kinase activity in these mast cell cultures correlated with the overall severity of \( W \) phenotype. These results support and extend previous observations suggesting that \( c\)-kit and \( W \) are allelic (Chabot et al. 1988; Geissler et al. 1988; Nocka et al. 1989; Bernstein et al. 1990) and provide direct evidence that the tyrosine kinase activity associated with the \( c\)-kit protein is a biochemical parameter relevant to \( c\)-kit function in those lineages affected by the \( W \) locus.

To obtain a more complete understanding of the range of phenotypes conferred by different \( W \) alleles, we also analyzed the molecular basis of the deficiencies in \( c\)-kit kinase activity. Two broad categories of mutations were revealed. The decreased \( c\)-kit kinase activity associated with the mild alleles \( W^{44} \) and \( W^{57} \) appears to be a consequence of an equivalent decrease in the level of \( c\)-kit protein. Rearrangement of the \( c\)-kit locus, associated with decreased levels of \( c\)-kit mRNA in adult brain, has been reported for the \( W^{44} \) allele (Geissler et al. 1988). The data presented here demonstrate that the \( W^{57} \) mutation also affects \( c\)-kit protein expression in a physiologic target of \( W \) mutations. In contrast, no gross rearrangement of the \( c\)-kit locus in \( W^{37} \) mice was detectable at the level of resolution afforded by Southern blot analysis using cDNA probes (data not shown). Nevertheless, the \( W^{57} \) mutation also appears to lower the level of \( c\)-kit mRNA in mast cells (data not shown), by affecting either \( c\)-kit transcription or mRNA stability.

The other mutant alleles analyzed here were characterized by the expression of normal levels of \( c\)-kit proteins with reduced or undetectable in vitro autophosphorylation activity. These results raised the possibility that this class of \( W \) alleles arose as a consequence of mutations that directly affect the \( c\)-kit tyrosine kinase domain. Consistent with this prediction, DNA sequence analysis revealed the presence of single-point mutations within the kinase domain of \( c\)-kit isolated from mice bearing each of these mutant alleles. Formal proof that these single amino acid substitutions confer the phenotypes of \( W^{37} \), \( W^{44} \), and \( W^{55} \) animals awaits the generation of transgenic mice expressing recombinant mutant \( c\)-kit proteins. However, the nature and location of these point substitutions, along with the inability to detect additional allele-specific changes in the \( c\)-kit-coding sequence, suggest that they are responsible for the defi-
Figure 3. Nucleotide sequence analysis of c-kit cDNA from W37, W55, and W41 animals. (A) Cloning strategy. The entire coding sequence of c-kit was cloned from total cellular RNA by PCR using the c-kit-specific oligonucleotide pairs K6–K3, K5–K2, and K4–K1 [see Methods]. Line indicates noncoding regions of c-kit mRNA; box indicates c-kit-coding sequence; solid area indicates transmembrane domain; shaded area indicates c-kit variable region within the tyrosine kinase domain. Nucleotide sequence determination utilized the series of c-kit-specific oligonucleotides K1 through K16 [see Methods]. (B) GC→AT mutation in c-kit cDNA isolated from W37/W37 mast cells was detected in K14 oligonucleotide-primed sequence reactions resulting in substitution of lysine for Glu82. (C) GC→AT point mutation in c-kit from W55/W55 mast cells was detected in K4 oligonucleotide primed sequence reactions resulting in substitution of methionine for Thr660. (D) c-kit cDNA prepared from W41/W41 placenta was found to contain a GC→AT point mutation in K7 oligonucleotide-primed sequence reactions. This results in Val831 being mutated to methionine. (B and D) Lower strand sequence of the relevant regions of representative mutant c-kit cDNA clones; (C) upper strand. (*) Mutated nucleotides. (A) alanine; (C) cysteine, (E) glutamate; (F) phenylalanine; (G) glycine; (K) lysine; (L) leucine; (M) methionine; (P) proline; (T) threonine; (V) valine; (W) tryptophan.

iciencies in hematopoietic, melanocyte, and germ cell lineages of these three W mutants.

All three allele-specific mutations involve GC→AT transitions [Fig. 3]. Spontaneous hydrolytic deamination of 5-methylcytosine to thymine is known to occur at an appreciable frequency in mammalian cells [Wang et al. 1982; Riggs and Jones 1983]. Although a specific mismatch repair system has been identified that restores GT mismatches to GC pairs, a small proportion of such mismatches are repaired to mutant AT pairs in mammalian cells [Brown and Jiricny 1987; Wiebauer and Jiricny 1989]. Such a mechanism could account for all three mutations detected in the spontaneous W mutants, W37, W55, and W41, as well as the strain-specific polymorphism observed in comparisons of c-kit cDNAs from C57BL/6 and BALB/c mice. The allele-specific mutations result in point substitutions of amino acids within the kinase domain of c-kit that are highly conserved among the family of protein tyrosine kinases [Fig. 4]. Moreover, the nature of these amino acid substitutions is consistent with the observed phenotypes induced by each W allele; the moderately dominant, homozygous viable alleles W55 and W41, contain more conservative substitutions than that detected in the homozygous lethal allele, W37.

Lethal allele W37

The amino acid residue Glu82, replaced by lysine in c-kit from W37 mice, lies amino terminal to the ATP-binding consensus sequence G-X-G-X-X-G found in all
protein kinases, a subdomain known to be critical for kinase activity [Kamps et al. 1984; Weinmaster et al. 1986, Chou et al. 1987; Odawara et al. 1989; for review, see Yarden and Ullrich 1988]. Glu\(^{882}\) forms part of the consensus sequence W-E-X-X-R, found not only in all known members of the platelet-derived growth factor [PDGF] subfamily but also in proteins of the insulin receptor subfamily of transmembrane tyrosine kinases and some, but not all, members of c-src and c-abl subfamilies [Fig. 4A]. Strikingly, this consensus sequence is not

**Figure 4.** c-kit residues mutated in W\(^{87}\), W\(^{55}\), and W\(^{42}\) animals are highly conserved in protein tyrosine kinases. Amino acid residues 578–602 (A), 650–670 (B), and 818–839 (C) of murine c-kit (Qui et al. 1988) are compared with homologous regions of other protein tyrosine kinases representing PDGF receptor, insulin receptor and EGF receptor subfamilies of transmembrane receptor tyrosine kinases, src and abl subfamilies of cytoplasmic tyrosine kinases, and c-kit isolated from animals bearing mutant alleles W\(^{87}\), W\(^{55}\), and W\(^{42}\), respectively. Conserved residues mutated in each W allele are shown in open boxes. Shaded boxes in A indicate the invariant glycine residues of the ATP-binding domain within conserved subdomain I; in B, conserved residues within subdomain IV; and in C, invariant residues within conserved subdomain VIII of protein tyrosine kinases (Hanks et al. 1988), as well as the major autophosphorylation site (Tyr\(^{821}\)) in c-kit of many tyrosine kinases. [c-fms] Human c-fms (Coussens et al. 1986), [PDGF] human platelet-derived growth factor receptor [Yarden et al. 1986], [RET] human ret proto-oncogene [Takahashi and Cooper 1987], [INS.R] human insulin receptor [Ullrich et al. 1985]; [IGF1.R] human insulin-like growth factor I receptor [Ullrich et al. 1986], [DILR] Drosophila insulin-like receptor [Nishida et al. 1986], [EGF] human epidermal growth factor receptor [Ullrich et al. 1984], [NEU] human c-neu [Yamamoto et al. 1986], [DER] Drosophila EGF receptor [Livneh et al. 1985]; [c-src] human c-src [Anderson et al. 1985]; [Dsrc28] Drosophila src [Gregory et al. 1987]; [c-abl] human c-abl [Shibelman et al. 1986]; and [Nabl] Caenorhabditis elegans abl [Goddard et al. 1986]. (A) alanine; (C) cysteine; (D) aspartate; (E) glutamate; (F) phenylalanine; (G) glycine; (H) histidine; (I) isoleucine; (K) lysine; (L) leucine; (M) methionine; (N) asparagine; (P) proline; (Q) glutamine; (R) arginine; (S) serine; (T) threonine; (V) valine; (W) tryptophan; (Y) tyrosine. Dashes indicate residues identical to those in c-kit. Gaps were introduced into conserved subdomains IV and VIII of EGFR subfamily for optimal alignment. Sequences were aligned by eye.

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found in members of the EGF receptor [EGFR] subfamily of transmembrane receptor tyrosine kinases but is replaced by another conserved sequence R-I-L/V-K-E/D [Fig. 4A]. Deletion analysis of v-fps has established that, although this region is not essential for catalytic function, its inclusion greatly stimulates kinase activity [Sa
dowski and Pawson 1987]. The definition of a point mutation in this region that results in markedly decreased in vitro autophosphorylation activity is consistent with a positive function for this domain and identifies Glu382 as a residue critical for this activity.

A change in the relative abundance of the 160- and 124-kD forms of c-kit was also associated with the W37 mutation. Point mutations within the extracellular domains of c-fms and the insulin receptor have been shown to affect processing of receptor tyrosine kinases [Roussel et al. 1988; Woolford et al. 1988; Accili et al. 1989]. However, no allele-specific amino acid substitutions were detected in the extracellular domain of c-kit cloned from cells homozygous for the W37 mutation [data not shown]. It is possible that the glutamic acid to lysine mutation detected in W37 c-kit alters the conformation of the protein in such a way as to impair processing or destabilize the 160-kD form of c-kit. However, because neither form was found to have autophosphorylation activity, it is likely that the primary phenotypic consequences of this mutation arise from the loss of kinase activity.

**Moderate alleles W55 and W41**

Thr460, mutated to methionine in c-kit from animals bearing the W55 allele, lies within conserved subdomain IV of protein tyrosine kinases, within the ATP-binding domain [Fig. 4B; Hanks et al. 1988]. This residue is conserved as either threonine or serine not only in other members of the PDGF receptor and insulin receptor subfamilies of transmembrane protein tyrosine kinases but also in some proteins of the src and abl subfamilies of cytoplasmic tyrosine kinases [Fig. 4B]. Like the W77 allele, the mutated residue in W55 is not conserved within the EGF receptor subfamily. To date, a putative role for this subdomain in tyrosine kinase catalytic activity has been deduced solely from sequence comparisons [Hanks et al. 1988]. The data presented here indicate that substitution of Thr460 by methionine directly affects c-kit kinase activity. Conservation of residues with aliphatic hydroxyl side chains at this position may reflect constraints necessary to retain suitable secondary structure of the ATP-binding domain.

C-kit residue Val831, mutated to methionine in W47 animals, lies within conserved subdomain VIII of protein kinases [Fig. 4C; Hanks et al. 1988]. The strong evolutionary conservation of this subdomain and its proximity to the major autophosphorylation site of many tyrosine kinases suggests that this region is critical for catalytic activity, a hypothesis confirmed by mutational analyses [for review, see Yarden and Ulrich 1988]. However, within this subdomain, Val831 is part of a sequence conserved across tyrosine kinases but not serine/threonine kinases, indicating that this residue may be important for recognition of the correct hydroxyamino acid.

An independence of pleiotropic effects of mutations at the W locus is seen in W55 heterozygotes which, unlike W55/W55 animals, retain normal fertility [Table 1]. There are several possible explanations for this phenotype. The c-kit signal transduction pathway may involve substrates in germ cells that are different from those in hematopoietic stem cells or melanoblasts. Tyrosine phosphorylation of such substrates may be differentially affected by the Val831 to Met831 mutation. Alternatively, the residual level of c-kit kinase activity present in W55 animals may be sufficient for normal germ cell development but inadequate for normal melanogenesis or hematopoiesis, or the germ cell migration pathway may provide larger amounts of c-kit ligand than those for melanocyte and hematopoietic stem cell migrations.

**Dominant-negative W phenotypes**

W alleles have all been identified initially by their ability to confer a dominant phenotype on the melanocyte lineage. In some, but not all, W mutants, dominant phenotypes are also apparent in the hematopoietic and germ cell lineages [Table 1]. Both the data presented here and that reported previously [Chabot et al. 1988; Geissler et al. 1988; Nocka et al. 1989; Bernstein et al. 1990] indicate that dominant W phenotypes arise as a consequence of a loss of c-kit kinase function. Our data demonstrate that such dominant phenotypes can arise by one of two classes of mutation: those that affect c-kit expression levels and those that result in expression of a defective protein as a consequence of point substitutions within highly conserved residues in the c-kit polypeptide. Mutations that confer reduced levels of an apparently normal protein give mild heterozygous phenotypes [W444/+ or W571/+; Table 1], whereas point substitutions in c-kit give more strongly dominant heterozygous phenotypes [W77/+ , W41/+ , or W55/+ ; Table 1], suggesting that coexpression of normal and defective c-kit proteins in the same cell inhibits proper signal transduction by the wild-type protein. There are precedents for mutations in transmembrane receptor tyrosine kinases inducing such dominant-negative phenotypes. Truncated forms of EGFR have been shown to act in a dominant-negative manner in in vitro cell lines [Basu et al. 1989]. Deletion of the kinase domain of the insulin receptor results in autosomal dominant inheritance of insulin resistance in humans [Kadowaki et al. 1988; Taika et al. 1989], and a point mutation in the ligand-binding domain of the human insulin receptor confers decreased insulin binding in cell culture and insulin resistance in heterozygote individuals [Klinkhamer et al. 1989].

The occurrence of such dominant-negative phenotypes is presumably a consequence of the mechanism by which this class of proteins transduces extracellular signals. The regulation and activation of transmembrane...
receptor tyrosine kinases appears to be controlled by a dynamic equilibrium between monomeric and aggregated forms of receptors (for review, see Carpenter 1987). Ligand-dependent activation of nonaggregated forms of EGFR has been reported [Biswas et al. 1985; Basu et al. 1986; Koland and Cervione 1988; Northwood and Davis 1988], but the activity of aggregated forms of transmembrane protein tyrosine kinases is unclear. There is some data to suggest that binding of ligand to EGFR pushes the dynamic equilibrium in favor of aggregates, thus activating kinase activity [Bonischnetzler and Pilch 1987; Yarden and Schlessinger 1987], and it has been shown that the oncogenic form of neu exists preferentially on the cell surface in the aggregated form [Drebin et al. 1985, Weiner et al. 1989]. In such a model, the point mutations described here for W37, W55, and W41 could act in a dominant-negative manner by producing defective heterodimers with impaired kinase activity or nonfunctional monomers that bind ligand nonproductively in situations where ligand is limiting. An alternative model suggests that aggregation of receptors results in negative regulation, with ligand binding acting as a positive regulator by pushing the dynamic equilibrium in favor of active monomers possibly stabilized by nucleotide binding [Basu et al. 1986; Koland and Cervione 1988, Northwood and Davis 1988]. In this case, the point mutations may confer an inherent instability in monomers even in the presence of ligand, thus pushing the equilibrium in favor of inactive dimers. The mutations could also produce defective heterodimers insensitive to ligand-mediated activation.

Transmembrane receptor tyrosine kinases and mammalian development

The functional definition of amino acid residues and domains critical for protein tyrosine kinase function has been largely restricted to analyses of mutations that activate the kinase domain, as determined by their ability to transform cells in vitro or cause tumors in transgenic mice. These mutations all constitute gain-of-function alterations and thus represent only a subset of possible alterations in this developmentally important class of molecules. The data presented here provide the first examples of the consequences of single amino acid substitutions in a transmembrane tyrosine kinase gene on mammalian development. A large number of spontaneous and mutagen-induced alleles at the Wc-kit locus are available [Green 1981]. The molecular characterization of additional W mutations will greatly facilitate the definition of the structural elements necessary for signal transduction by transmembrane protein tyrosine kinases in vivo and also provide further insight into the roles played by c-kit in mammalian embryogenesis and cellular differentiation. The mutations defined here all mapped to amino acids highly conserved among the family of protein tyrosine kinases. It will be of considerable interest to determine whether the introduction of analogous germ line mutations in other members of the receptor tyrosine kinase gene family also results in dominant-negative developmental defects in the mouse.

Methods

Animals

Normal +/+ mice and the W mutants W/+ , W07/+ , W07/+ , W07/+ , W07/+, W07/+, and W07/+, all on a C57BL/6 background, and WB/Re W/+ mice were purchased from Jackson Laboratory [Maine]. New Zealand rabbits were obtained from the Animal Division, Mt. Sinai Hospital Research Institute. Mouse embryos were obtained by natural matings (normal light cycle), fertilization being assumed to occur at midnight prior to detecting vaginal plugs. The developmental age of dissected embryos was assessed in accordance with Theiler [1972]. Fetuses homozygous for lethal W alleles were distinguished from heterozygous litter mates by their anemic appearance and verified by their inability to form CFU-S in irradiated syngeneic hosts and their deficiency in CFU-E.

Cell culture

Single cell suspensions of adult bone marrow or 15.5-day postcoitum fetal liver were prepared in Iscove’s modified Dulbecco’s medium [IMDM], seeded at 5 × 10⁶ cells/ml in IMDM containing 5% fetal calf serum [FCS] supplemented with 10% WEHI-3-conditioned medium, and grown at 37°C in an incubator gassed with 5% CO₂ at a relative humidity 100%. After 6 days, cells were pelleted and resuspended in 1 ml of the original culture medium supplemented with 4 ml of mast cell mix [IMDM, 0.05% BSA, 1% FCS, 2 µg/ml concanavalin A, 5 µg/ml transferrin, 5 µg/ml insulin, 0.5 U/ml murine IL-3 from the myeloma cell line X63Ag8-653 carrying a recombinant IL-3 cDNA expression vector [Karasuyama and Melchers 1988]]. Every 3 days, 80% of the medium was changed until cells achieved a concentration >1.5 × 10⁹ cells/ml, at which point cells were maintained at 4 × 10⁹ to 5 × 10⁹ cells/ml in mast cell mix. Mature mast cell populations were generated after 5–6 weeks of culture.

For coculture experiments, 5 × 10⁵ mast cells were seeded onto confluent monolayers of normal mouse embryo fibroblasts [Fujita et al. 1989] in IMDM containing 5% FCS. Medium was changed every 4 days. Mast cells were identified by staining a cytocentrifuge preparation of trypsinized cultures with Alcian Blue.

For metabolic labeling, 5 × 10⁶ mast cells were washed twice in prewarmed Tris–saline and incubated for 4 hr at 37°C in 2 ml of DMEM lacking methionine [Flow Laboratories], supplemented with 5% FCS [Flow], 10% WEHI-conditioned medium and 200 µCi [35S]methionine [Amersham].

Nucleic acids

pTrpE–kit contains a 928-bp PvuII–PvuII restriction fragment of a 3.7-kb murine c-kit cDNA clone ligated in frame into the TrpE fusion vector pATH2 [Crivellone et al. 1988]. Total cellular RNA was prepared by the guanidinium isothiocyanate method [Chirgwin et al. 1979]. Mouse c-kit-specific oligonucleotides were purchased from the Banting Research Institute [Toronto] and correspond to the following nucleotides in the published murine c-kit cDNA sequence [Qui et al. 1988]: K1: 2967–2988, K2: 1959–1980, K3: 1066–1087, K4: 1981–2002, K5: 1088–1109, K6: 7–15, K7: 2611–2632, K8: 1734–1755, K9: 413–429, K10: 601–617, K11: 811–827, K12: 1401–1417, K13: 1607–1623, K14: 1804–1820, K15: 2415–2431, K16: 2801–2817.
c-kit-specific antisera

Twenty milliliters of M9 medium, supplemented with 0.5% [wt/vol] casamino acids, 10 μg/ml thiamine, 20 μg/ml tryptophan, and 50 μg/ml of ampicillin, was inoculated with a single ampicillin-resistant *Escherichia coli* colony bearing the plasmid pTrpE-kit. Following overnight incubation at 37°C, the culture was added to 200 ml prewarmed M9 medium supplemented as described above but with the omission of tryptophan. After a 3-hr incubation at 37°C, culture medium was supplemented with indole acetic acid (Sigma) to a final concentration of 5 μg/ml and incubated at 37°C with vigorous aeration for an additional 2 hr. Cells were harvested by centrifugation, resuspended in 5 ml of cracking buffer [0.01 M sodium phosphate (pH 7.2), 1% 2-mercaptoethanol, 1% SDS, 6 M urea] and incubated at 37°C for 1 hr prior to preparative SDS-PAGE. The TrpE-kit fusion protein (74-kD) was excised from the preparative gel, electroeluted, and concentrated with polyethylene glycol to a final concentration of 1.5 mg/ml.

For immunization, rabbits were inoculated with 150 μg of TrpE-kit fusion protein in 500 μl of Freund’s complete adjuvant [Difco] and boosted with 50 μg of fusion protein in Freund’s incomplete adjuvant. Antibera reactivity was tested by Western blot of TrpE-kit-induced protein.

**Immunoprecipitation analyses**

Cells were washed twice with ice-cold Tris–saline and lysed in 1 ml of RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% [vol/vol] Triton X-100, 1% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS, 100 μM sodium vanadate, 100 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride]. Lysates were pre-cleared by centrifugation for 45 min at 4°C and incubation of 500 μl of supernatant for 2 hr at 4°C with 50 μl rabbit preimmune serum plus 50 μl of 10% protein-A/Sepharose (Phar-macia) for 2 hr at 4°C. Immunoprecipitates were washed three times in 500 μl ice-cold RIPA buffer, two times in ice-cold 50 mM Tris-HCl (pH 7.5), resuspended in 50 μl 1 × SDS sample buffer [10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 2.3% [wt/vol] SDS, 0.0625 M Tris-HCl at pH 6.8, 0.002% [wt/vol] bromphenol blue] and subjected to 7.5% SDS-PAGE. Gels were fixed with 10% acetic acid and 30% methanol and treated with Enhance [DuPont] prior to autoradiography.

**In vitro kinase assays**

Following immunoprecipitation, immune complexes were washed twice in ice-cold RIPA buffer, twice in ice-cold 50 mM Tris-HCl (pH 7.5), and 1% [vol/vol] Triton X-100 and resuspended in 10 μl kinase reaction buffer [10 mM MnCl₂, 1% [vol/vol] Triton X-100, 10 μCi [32P]ATP/4000 Ci/m mole, Amer-sham]. Following incubation for 10 min at 30°C, an equal volume of 2 × SDS sample buffer was added, and samples were subjected to 7.5% SDS-PAGE. Following electrophoresis, gels were fixed with 10% acetic acid and 30% methanol and treated with 1 M potassium hydroxide [Cooper et al. 1983] prior to autoradiography.

cDNA cloning

Total cellular RNA (20 μg) was mixed with 50 ng of each K1, K2, and K3 c-kit-specific oligonucleotides in 0.2 mM sodium chloride, 20 mM PIPES [pH 6.5], and 0.5 mM EDTA in a final volume of 20 μl, denatured at 65°C for 2 min, and annealed by incubation at 42°C for 3 hr. First-strand cDNA was then generated by incubation of the above mix in 50 mM Tris-HCl [pH 8.3], 10 mM MgCl₂, 20 mM Na₂PO₄, 40 mM KCl, 2 mM DTT, 0.4 mM each of dCTP, dGTP, dATP, and dTTP, 40 units RNAsin (Promega Biotech), 20 units AMV reverse transcriptase (Boehringer-Mannheim), and 10 units M-MuLV reverse transcriptase [Pharmacia] in a final volume of 50 μl and incubated at 42°C for 2 hr.

Ten percent of the above reaction mix was then denatured in 0.4 μl NaOH for 10 min at 50°C, neutralized by the addition of hydrochloric acid, and PCR was carried out by incubation in 1 x TaqI buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% gelatin], 0.2 μM dCTP, dGTP, dATP, and dTTP, 0.2 mg/ml gelatin, 500 ng of each of c-kit-specific oligonucleotide pairs K1–K4, K2–K5, or K3–K6, as appropriate, and 2.5 units TaqI polymerase [Cetus] in a final volume of 100 μl. The reaction mix was subjected to incubation at 93°C for 90 sec, 55°C for 2 min, and 72°C for 3 min for a total of 25 cycles in an Ercorp twin block variable temperature cycler. PCR products were electrophoresed in 0.8% agarose gel. cDNA fragments of the appropriate size were excised, eluted, blunted with T4 DNA polymerase [Boehringer-Mannheim], and ligated with HincII-linearized pKS plasmid vector [Stratagene] at 4°C for 12–16 hr. The ligation mix was transformed into *E. coli* DH5αF’ and recombinant clones were detected by blue/white color selection on ampicillin plates supplemented with 0.5 mM IPTG and 100 μg/ml X-gal.

**Nucleotide sequence analysis**

Double-stranded plasmid DNA was prepared for DNA sequence determination as described previously [Hattori and Sakaki 1986]. DNA sequence reactions were performed using the modified T7 DNA polymerase Sequenase [U.S. Biochemicals] essentially in accordance with the conditions recommended by the manufacturer.

**Autoradiography**

Detection of 35S-radiolabeled proteins and nucleic acids was carried out using Kodak XAR-5 or Fuji X-ray film, respectively, at room temperature. Detection of 32P-radiolabeled proteins was performed using Kodak XAR-5 film with intensifying screens at ~70°C.

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