Characterization of Ws Mutant Allele of Rats: A 12-Base Deletion in Tyrosine Kinase Domain of c-kit Gene

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Homozygous mutant rats at the newly found white spotting (Ws) locus were anemic and deficient in mast cells and melanocytes. Because the phenotype of Ws/Ws rats resembled the phenotype of mice possessing a double-gene dose of mutant alleles at the W locus and because the c-kit gene was mapped at the W locus of mice, we characterized the c-kit gene of Ws/Ws rats. The authentic sequence of the rat c-kit cDNA was determined by using a cDNA library prepared from the hippocampus of Sprague-Dawley rats. The c-kit cDNA of Ws/Ws and normal (+/+ ) control rats was obtained by reverse transcriptase modification of the polymerase chain reaction. When compared with the authentic sequence, a deletion of 12 bases was found in the c-kit cDNA of Ws/Ws rats. This change was shown to be a result of the deletion of the genomic DNA. Four amino acids encoded by the deleted 12 bases (ie, Val-Lys-Gly-Asn) were located at two amino acids downstream from the tyrosine autophosphorylation sites in the c-kit kinase and were conserved not only in mouse and human c-kit kinases but also in mouse and human c-fms kinases (ie, receptors of colony-stimulating factor-1). Taken together, the Ws/Ws rat is the first characterized mutant of the c-kit gene in an animal species other than the mouse.

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C-KIT gene mutation of rats

Fig 1. Schematic representation of the cDNA for the rat c-kit gene. Rat c-kit cDNA clones, which were shown by solid lines, were isolated from the cDNA library by using the 1.6 kb of mouse c-kit cDNA as a probe (shaded box). The solid box shows the coding region. Arrows numbered 1 to 7 indicate the position and orientation of oligonucleotide primers. An open vertical arrow points the deletion site that was found in the mutant Ws allele.

RESULTS

A 1.6-kb fragment of the mouse c-kit cDNA was used as a probe, and four positive clones were isolated from 6 × 10⁶ phages of the Agt10 cDNA library, which was prepared from the hippocampus of Sprague-Dawley rats (Fig 1). The clone 1-6 contained an insert of 2.3 kb, clone 2-3 an insert of 2.6 kb, clone 9-2 an insert of 2.7 kb, and clone 6-3 an insert of 1.2 kb. Because clones 1-6 and 2-3 appeared to cover the principal portion of the c-kit cDNA (Fig 1), we first sequenced these two clones. The clones 9-2 and 6-3 were also sequenced for confirmation. The complete sequence of 3,816 bp consisted of the entire coding region of the c-kit gene (2,934 bp with 5' (44 bp) and 3' (838 bp) untranslated sequences (Fig 2). The coding region of the c-kit gene showed 91% homology between rats and mice and 83% homology between rats and humans. The acquired rat sequence was used as the authentic sequence. The predicted amino acid sequence was also shown in Fig 2. The overall amino acid homology was 92% between rats and mice and 84% between rats and humans.

Three sets of primers were used to cover the whole coding region of the c-kit cDNA (Fig 1). Fragments of cDNA obtained by reverse transcriptase modification of PCR amplification from Ws/Ws and BN-+/+ rats were sequenced. The sequences were compared with the authentic sequence determined by using the cDNA library from the hippocampus of Sprague-Dawley rats. In the cDNA fragments that intervened between primers 1 and 2, three independent substitutions of a base were found in both Ws/Ws and BN-+/+ rats: a GC → CG transversion at nucleotide 197, a CG → TA transition at nucleotide 434, and an AT → GC transition at nucleotide 659. However, all three substitutions do not result in alteration of amino acids and appear to be polymorphic changes between the Sprague-Dawley and BN strains. In the fragment that intervened between primers 3 and 4, a deletion of 12 bases (nucleotides 2520 through 2531) was detected in cDNA derived from Ws/Ws rats but not in cDNA derived from BN-+/+ rats (Fig 2). The sequence of the deleted 12 bases predicted four amino acids (Val-Lys-Gly-Asn), which were residues 826 through 829 of the c-kit protein (Fig 2). Two independent clones obtained from a Ws/Ws rat and three indepen-dent clones from another Ws/Ws rat showed the same deletion. In contrast, no changes from the authentic sequence were detectable in a clone from a BN-+/+ rat and two independent clones from another BN-+/+ rat. In the cDNA fragment that intervened between primers 5 and 6, the deletion of 12 bases was confirmed in cDNA derived from two Ws/Ws rats, but no other changes from the authentic sequence were detectable in cDNA derived from both Ws/Ws and BN-+/+ rats.

In the next experiment, we examined if the deletion of 12 bases existed in the c-kit genomic DNA of Ws/Ws rats. DNA isolated from the liver of Ws/Ws, Ws/+ , and BN-+/+ rats was used as a template, and the DNA fragment between nucleotides 2485 and 2569 was amplified by PCR with primers 5 and 7 (Fig 1). When the sizes of PCR products were compared, the fragment derived from the Ws/Ws rats was apparently smaller than that of the BN-+/+ rats (Fig 3). Moreover, two distinct bands were detected in PCR products derived from Ws/+ rats.

DISCUSSION

First we characterized the rat c-kit cDNA, which was isolated from the cDNA library prepared from the hippocampus of Sprague-Dawley rats. An open reading frame of 2,934 bases predicted a protein composed of 978 amino acids. Proteins predicted from the mouse⁰ and human¹ c-kit cDNA consist of 975 and 976 amino acids, respectively. Sequences of both nucleotides and amino acids are highly conserved among rats, mice, and humans.

Sequencing of the c-kit cDNA from Ws/Ws rats showed the deletion of nucleotides 2520 through 2531. This change was a result of the deletion of the genomic DNA. Four amino acids predicted from the deleted 12 bases are residues 826 through 829, which are located at two amino acids downstream from the tyrosine autophosphorylation site (residue 824) (Figs 2 and 4). These four amino acid residues are conserved not only in murine and human c-kit proteins, but also in murine and human c-fms proteins (i.e., colony-stimulating factor-1 receptors) and appear to have an important physiologic role (Fig 4). Therefore, the Ws/Ws rat is the first mutant of the c-kit gene in the animal species other than the mouse.

Heterozygous Ws/+ rats showed a coat color dilution, an amenia, and a mast-cell deficiency of considerable degree. The phenotype of Ws/+ heterozygous rats is similar to that of W¹/+ , W²/+ (= W³/+ ), W⁴/+ , and W⁵/+ heterozygous mice, but not to that of W/+ and W⁶/+ mice.¹²,¹⁷-²⁰ Nocka et al¹⁹ and Reith et al²⁰ pointed out an interesting aspect of W (c-kit) mutations of mice, in that they display the differing degrees of dominance in the heterozygous state. W/+ mice have a ventral spot but no coat color dilution and normal numbers of erythrocytes and mast cells.¹⁵,¹⁶ In their weak mutant phenotype W/+ heterozygous mice resemble heterozygotes of the W¹⁰ mutation, which results from a large deletion including the W (c-kit) gene. In agreement with the null phenotype of the W mutation, the W allele has been shown to encode a nonfunctional c-kit protein that is not expressed on the cell surface.¹⁹,²⁰ By contrast, the W¹⁰, W⁴, and W⁶ alleles
Tyrosine autophosphorylation site that located at residue 824 is encircled. A box indicates the deletion of four amino acids, which was observed in sequence is displayed over the DNA sequence and numbered from the initiation codon. The transmembrane domain is underlined. The potential and give rise to more severe heterozygous phenotype than the and W~Miss~ alleles. The mutations belonging to the group showing the severe phenotypes are dominant negative mutations and result from missense mutations that affect the tyrosine kinase function of the c-kit protein. The molecular nature of the W~Miss~ mutant allele, i.e., a small deletion at the tyrosine kinase domain of the c-kit gene, is consistent with its dominant negative expression.

Fig 2. Nucleotide sequence of rat c-kit cDNA. The DNA sequence is numbered from the first base of the cloned cDNA. The predicted amino acid sequence is displayed over the DNA sequence and numbered from the initiation codon. The transmembrane domain is underlined. The potential tyrosine autophosphorylation site that located at residue 824 is encircled. A box indicates the deletion of four amino acids, which was observed in sequence is displayed over the DNA sequence and numbered from the initiation codon. The transmembrane domain is underlined. The potential and give rise to more severe heterozygous phenotype than the W and W~Miss~ alleles. The mutations belonging to the group showing the severe phenotypes are dominant negative mutations and result from missense mutations that affect the tyrosine kinase function of the c-kit protein. The molecular nature of the W~Miss~ mutant allele, i.e., a small deletion at the tyrosine kinase domain of the c-kit gene, is consistent with its dominant negative expression.
Segregation of the mutant allele with a 12-base deletion at the Ws locus. Oligonucleotide primers 5 and 7 (the sequences and the position were shown in the text and Fig 1) were used to amplify the intervening genomic DNA sequences. The annealing temperature of PCR was 58°C. Amplified products were analyzed on 4% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME) with Haelll-digested pBR322 plasmid DNA as a size marker (M). The PCR-amplified products individually obtained from two Ws/Ws rats were shown in lanes 2 and 3, those of two BN-/+ rats in lanes 3 and 4, and those of two Ws/+ rats in lanes 1 and 6.

Tan et al., Nocka et al., and Reith et al. have proposed that signal transduction induced by the ligand of the c-kit protein involves the formation of receptor oligomers. Therefore, the mutant c-kit protein in receptor heterodimers could interfere with c-kit ligand-induced normal signal transduction, and this would result in a reduction of the number of functional c-kit receptors on the cell surface and explain the phenotype of heterozygous mutant animals. The cell surface expression of mutant proteins is an important aspect of their ability to function as dominant negative mutations, and this is best seen by the fact that the truncated product of the W mutation is not detectable on the cell surface. In fact, the dominant negative W (c-kit) mutations W37, W4, and W41 primarily affect the tyrosine kinase and not other aspects of c-kit receptor function. This finding might suggest that receptor oligomers cannot contain nonfunctional kinases for normal ligand-induced receptor activation and receptor function. Although the Ws allele has a small deletion at the tyrosine kinase domain, its extracellular domain appears to be normal. The dominant negative nature of the Ws allele may be explained by the mechanism proposed by Tan et al., Nocka et al., and Reith et al.

We have not examined whether the deletion of four amino acids impairs the tyrosine kinase activity of the c-kit protein encoded by the mutant Ws allele. Moreover, we have not confirmed the normal expression of the c-kit protein on the surface of cultured mast cells derived from the bone marrow of Ws/Ws rats, as reported in the case of W+/W+, W37/W37, and W41/W41 mice. We are now attempting to perform these experiments. Without them, we cannot exclude the possibility that the phenotype of Ws/Ws rats is a result of mutations at genes other than the c-kit. However, Mendelian segregation of the Ws mutant allele with the 12-base deletion among Ws/Ws, Ws/+ and +/+ rats strongly suggests that the 12-base deletion of the c-kit genomic DNA is responsible for the mutant phenotype.

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