Brief Definitive Report

The Reduced Expression of 6Ckine in the plt Mouse Results from the Deletion of One of Two 6Ckine Genes

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Summary

6Ckine is an unusual chemokine capable of attracting naive T lymphocytes in vitro. It has been recently reported that lack of 6Ckine expression in lymphoid organs is a prominent characteristic of mice homozygous for the paucity of lymph node T cell (plt) mutation. These mice show reduced numbers of T cells in lymph nodes, Peyer’s patches, and the white pulp of the spleen. The genetic reason for the lack of 6Ckine expression in the plt mouse, however, has remained unknown. Here we demonstrate that mouse 6Ckine is encoded by two genes, one of which is expressed in lymphoid organs and is deleted in plt mice. A second 6Ckine gene is intact and expressed in the plt mouse.

Key words: chemokines • cell trafficking • leukocyte chemotaxis • T lymphocytes • mutation

Also reported as secondary lymphoid organ chemokine (1), Exodus-2 (2), and thymus-derived chemotactic agent 4 (3), 6Ckine is a unique CC chemokine containing six cysteines (4). Four of these cysteines occur in the expected conserved positions, and two additional cysteines are arrayed in a highly charged COOH-terminal domain unique to 6Ckine (4). In humans as well as mice, expression of 6Ckine mRNA is highest in lymphoid tissues, particularly LN and spleen, but 6Ckine is also expressed at lower levels in some nonlymphoid tissues (1–9). Expression of 6Ckine in LN’s and Peyer’s patches (PPs) has been more precisely localized by in situ hybridization to high endothelial venules (6). Expression of 6Ckine also occurs in the T cell areas of spleen, LN, and PP (5–7), as well as in thymic stromal cells (3). Expression of 6Ckine in other tissues, such as liver and small intestine, has been localized to the lymphatics (6).

In vitro, 6Ckine is reported to attract T cells, B cells, thymocytes, dendritic cells, and mesangial cells but not neutrophils or monocytes (1–9). It has been reported that 6Ckine preferentially attracts naive T cells over memory T cells (6), although this preference has not been universally observed (2, 7). Several investigators have also reported that activated T cells migrate more vigorously toward 6Ckine (4, 5, 7). In addition to its chemotactic activities, 6Ckine promotes adhesion of lymphocytes under shear flow conditions (6, 10, 11). These reports also suggest that the adhesion-promoting effects of 6Ckine are more pronounced for naive versus memory T cells. Taken together, these observations suggest that 6Ckine is involved in homing of lymphocytes or dendritic cells to secondary lymphoid organs. One prediction of this hypothesis is that 6Ckine null mice would show defects in homing of these cells to LNs, leading to a reduction in LN cellularity. Interestingly, a mutant mouse displaying just such a phenotype had been previously described by Nakano et al. (12–14) as the DDD/1 mouse.

The DDD/1 mouse carries an autosomal recessive mutation designated paucity of lymph node T cells (plt) and shows dramatically reduced numbers of T cells in the peripheral LN, PP, and spleen, whereas memory T cell numbers are unchanged (14). Interestingly, B cell numbers in the lymphoid organs of these mice are also relatively normal. Accumulation of neutrophils and macrophages in response to intraperitoneal injection of thioglycolate also appears normal in plt mice. Recent characterization of the plt phenotype by Gunn et al. has shown that these mice lack 6Ckine expression in lymphoid organs (15). This report also found that dendritic cell numbers were reduced in LNs. However, no significant alterations in the 6Ckine gene were found by these investigators, and thus the precise nature of the genetic defect in the plt mouse has remained unknown.

Our interest in the normal function of 6Ckine led us to begin work on a targeted disruption of the 6Ckine gene. In the process, we discovered that 6Ckine is encoded by two genes in the mouse genome. The predicted products of these two genes are nearly identical, differing by only a single amino acid. Furthermore, we demonstrate that one of the two 6Ckine genes is deleted in the plt mouse, leading

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to a loss of 6Ckine expression in lymphoid organs, whereas expression in various nonlymphoid organs, presumably arising from the remaining gene, is maintained.

Materials and Methods

Isolation and characterization of the genomic C copies of the 6C kine gene. Two bacterial artificial chromosome (BAC) clones containing genomic copies of the mouse 6C kine gene were identified in a mouse 129/sv embryonic stem cell genomic library (Genome Systems, Inc.) using PCR primers corresponding to the mouse 6Ckine cDNA: GV100 (5'-CTG CAA GAG AAC TGA ACA GAC-3') and GV105 (5'-CTG ACT CTC TAG GTC TAC-3'). Several overlapping fragments containing the 6C kine gene were identified by Southern blot analysis of BAC plasmid DNA using a 275-bp PCR-generated probe (GV100/GV105) labeled with [32P]dCTP (Amersham Corp.; 3,000 Ci/mmol) by random priming (Megaprime DNA Labeling System; Amersham Corp.). They were subcloned into pBluescript (Stratagene Inc.) and mapped by restriction digest. 6C kine-containing SacI fragments (7.5 kb) from each of the two BAC clones were sequenced using an Applied Biosystems 377 sequencer (Applied Biosystems, Inc.).

PCR Analysis of the 6C kine Locus. A pair of PCR primers GV104 (5'-GTA GAC CTA GAG AGT CAG AAG-3') and GV125 (5'-CGA TCC TGG GAG GAA CCA CAG T-3'), shown in Fig. 2, were used to amplify 1.35- and 1.2-kb fragments that included part of the 3' untranslated region (UTR) and ~1 kb downstream of the gene. PCR conditions were: 94°C for 2 min; 25 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min; and 72°C for 5 min. Both fragments were subcloned into pCR2.1 TA cloning vector (Invitrogen Corp.) and sequenced. Another pair of PCR primers GV95 (5'-AGT CTC GAG ATG ATG ACT CT-3') and GV105 (5'-CTT CAG CTC TAG GTC TAC-3') (see Fig. 2) were used to amplify the 5' coding region and part of the 3' UTR of the 6C kine gene (899 bp) from wild-type and BALB/c-plt (10th backcrossed generation) tail DNA. These fragments were also subcloned into pCR2.1 TA cloning vector (Invitrogen Corp.) and sequenced.

Southern Blot Analysis of 6Ckine Locus. Tail DNA was isolated from 129/sv (The Jackson Laboratory) and BALB/c-plt mice. EcoRI and HindIII (New England Biolabs Inc.) digested mouse tail DNA and BAC DNA were denatured and blotted onto Duralon membrane (Stratagene Inc.) and hybridized with probes A and B (see Fig. 3).

RNA Analysis. Total RNA was extracted from different tissues of plt and BALB/c-plt mice using Ultraspec RNA Reagent (Biotec Labs, Inc.), and 20 μg/lane was electrophoresed in a 1% agarose gel. The RNA was blotted onto Duralon nylon membrane (Stratagene Inc.). A 6C kine probe was labeled with [32P]dCTP (Amersham Corp.; 3,000 Ci/mmol) by random priming (Megaprime DNA Labeling System; Amersham Corp.). Hybridization was carried out in QuikHyb hybridization solution (Stratagene Inc.).

Results and Discussion

Mouse 6CK1 is encoded by two separate genes. As part of an effort to generate 6C kine null mice, a genomic library generated from 129/sv embryonic stem cells was screened by PCR (see Materials and Methods). Two BAC clones were identified, which we designated 6CKBAC1 and 6CKBAC2. Southern blot analysis of EcoRI-digested DNA from each BAC clone revealed two distinct banding patterns (Fig. 1 A). 6CKBAC1 contained bands of 6.0 and 1.35 kb, whereas 6CKBAC2 contained two bands of 3.0 and 1.2 kb. Southern blot analysis of 129/sv mouse genomic DNA showed that all four bands were present. These results immediately suggested the possibility of two distinct 6C kine genes in the mouse genome. Interestingly, hybridization of plt mouse genomic DNA showed only bands corresponding to those found in 6CKBAC1 (Fig. 1 A), suggesting the possibility that one of the two 6C kine genes in the plt mouse is altered.

To further investigate this possibility and to determine if this was a strain-specific observation, two primers (GV104 and GV125; Fig. 2) amplifying a segment of the 3' UTR of the 6C kine gene were used to amplify genomic DNA from various mouse strains as well as the two BAC clones. DNA from three separate mouse strains, BALB/c, 129/sv, and C57BL/6, all demonstrated two bands of 1.35 and 1.2 kb (Fig. 1 B). 6CKBAC1 showed only a 1.35-kb band and 6CKBAC2 only a 1.2-kb band (Fig. 1 B). These data suggest that wild-type mice have two 6C kine genes, one of which is represented on 6CKBAC1 and the other on 6CKBAC2.

Interestingly, PCR amplification of plt mouse genomic DNA showed only a single 1.35-kb band identical to the size of the band amplified from 6CKBAC1 (Fig. 1 B). This result again suggested that the plt mouse has an alteration in one of the two 6C kine genes.

To conclusively demonstrate that the 6C kine gene on 6CKBAC1 was indeed different from that residing on 6CKBAC2, SacI fragments (7.5 kb) were isolated from each BAC, subcloned, and completely sequenced. Analysis of the sequence data clearly showed that each BAC contained DNA from each BAC clone revealed two distinct banding patterns (Fig. 1 A). 6CKBAC1 contained bands of 6.0 and 1.35 kb, whereas 6CKBAC2 contained two bands of 3.0 and 1.2 kb. Southern blot analysis of 129/sv mouse genomic DNA showed that all four bands were present. These results immediately suggested the possibility of two distinct 6C kine genes in the mouse genome. Interestingly, hybridization of plt mouse genomic DNA showed only bands corresponding to those found in 6CKBAC1 (Fig. 1 A), suggesting the possibility that one of the two 6C kine genes in the plt mouse is altered.

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a distinct 6Ckine gene (Fig. 2). Furthermore, although the overall genomic organization of the two 6Ckine genes was similar, consisting of four exons and three introns (Fig. 2), there were a number of sequence differences. These differences included numerous single base changes as well as various small (<200 bp) deletions (Fig. 2). Within the coding regions of the two genes, only two single nucleotide differences were found, one of which resulted in an amino acid difference at position 65. 6CKBAC1 encodes a leucine at this position, whereas 6CKBAC2 encodes a serine. A series of deletions/insertions in a region of the gene downstream from the polyadenylation signal results in a net difference of 136 bp between the two genes that accounts for the observed difference in the size of the PCR products amplified by GV104/GV125 primers (Fig. 2B). Sequencing of these two PCR fragments amplified from wild-type genomic DNA shows that the sequence of each exactly matches that of the appropriate BAC, further proving that 6Ckine is encoded by two separate genes in the wild-type mouse genome and suggesting that the plt mouse is likely to contain mutations in the serine form of 6Ckine. Furthermore, the fact that two separate primer pairs failed to amplify the 6Ckine-ser gene suggested that the plt mouse has a deletion in this gene.

To demonstrate that the plt mouse has indeed deleted one 6Ckine gene, two DNA probes corresponding to the 5' end of the gene (probe A, Fig. 3C) and the 3' end of the gene (probe B, Fig. 3C) were hybridized to DNA from 129/sv and plt mice as well as 6CKBAC1 and 6CKBAC2. Hybridization with probe A revealed two bands of the expected sizes in EcoRI-digested DNA from 129/sv mice (Fig. 3A). Only one band, corresponding to the leucine form

![Figure 2](image_url)

(A) The 6Ckine genes found on the two BACs are distinct. A schematic of the sequence of SacI fragments (7.5 kb) from 6CKBAC1 (leu) and 6CKBAC2 (ser) is shown. Black and white boxes represent 6Ckine exons. Open triangles indicate larger deletions. Single base differences outside of the exons are not shown. The four nucleotide differences identified in coding regions (black boxes) and noncoding regions (white box) of the two 6Ckine genes are shown. Vertical arrows identify the amino acid difference at position 65. Horizontal arrows identify the amino acid difference at position 65. Horizontal arrows identify the nucleotide sequence differences.

(B) Sequences of the intron-exon boundaries are identical between the two copies with the exception of the intron2-exon3 junction. The 6Ckine-ser sequence is added to the intron2-exon3 boundary sequence to illustrate the single nucleotide difference (arrow). These sequence data are available from EMBL/GenBank/DDBJ under accession numbers AF171085 and AF171086 for 6Ckine-leu and 6Ckine-ser, respectively.
Mice Lack One of Two Ckine Genes

of Ckine, was detected in plt mice. A band of the same size was also detected in 6CKBAC1 (encoding Ckine-leu) but not in 6CKBAC2 (encoding Ckine-ser). Similarly, hybridization with probe B showed a pattern of bands in HindIII-digested 129/sv DNA consistent with the presence of both genes, whereas plt mice showed only bands of the same size as those present in 6CKBAC1. The failure of two independent probes, encompassing at least 7 kb of the Ckine gene, to hybridize to plt mouse DNA corresponding to Ckine-ser gene shows that the plt mouse has a deletion in the Ckine-ser gene and that this deletion likely includes all of the Ckine-ser coding region.

The plt Mouse Expresses Ckine in Nonlymphoid Organs. Our data demonstrates that the plt mouse has deleted the Ckine-ser gene but that it retains an intact Ckine-leu gene. A previous report has shown that the plt mouse lacks Ckine expression in lymphoid tissue, but expression in other tissue types was not reported (15). As the Ckine-leu gene is present in the plt mouse, we thought it possible that these mice might express Ckine in nonlymphoid tissue. To examine this possibility, we conducted Northern blot analysis on a variety of both wild-type (BALB/c) and plt mouse organs. Consistent with our previously reported findings (4), normal mice showed expression of Ckine in both lymphoid and nonlymphoid organs (Fig. 4 A). As expected, plt mice were found to lack detectable expression of Ckine mRNA in lymphoid organs (Fig. 4 B). Expression of Ckine was observed, however, in a number of nonlymphoid organs of the plt mouse (Fig. 4 B). As the Ckine-ser gene is deleted in these mice, the observed Ckine message likely derives from expression of the Ckine-leu gene.

Ckine/secondary lymphoid organ chemokine has been demonstrated in vitro to be important for lymphocyte adhesion and migration. Recent findings have also suggested a role for this chemokine in dendritic cell migration (8, 9). The lack of Ckine expression in the lymphoid organs of the plt mouse, along with the reported phenotype of these mice, suggests that at least some of these observations are also true in vivo. The conclusions drawn from the plt mouse regarding Ckine are confounded by the fact that the precise nature of the mutation in these mice remains unknown. We have presented data here that demonstrates that murine Ckine is, in fact, encoded by two separate genes and that one of these is deleted in the plt mouse. The two Ckine genes are nearly identical in the sequences of their open reading frames and show only one amino acid difference at position 65.

Analysis of the public expressed sequence tag (EST) database shows that cDNAs for both forms of mouse Ckine

Figure 3. Southern blot analysis of Ckine genomic loci suggests that the Ckine-ser gene of the plt mouse contains significant deletions. (A) EcoRI-digested genomic DNA from 6CKBAC1 and 6CKBAC2 and genomic DNA from 129/sv and plt mice were probed with a 0.8-kb SacI/NheI fragment (probe A, indicated). An 8-kb fragment was recognized in 6CKBAC1, and a 6.5-kb fragment was recognized in 6CKBAC2. The plt mouse genomic DNA contained only an 8-kb fragment DNA, whereas both 8- and 6.5-kb fragments hybridized in 129/sv DNA. (B) HindIII-digested DNA from 6CKBAC1 and 6CKBAC2 and genomic DNA from 129/sv and plt mice was probed with a 271-bp NheI/BstXI fragment (probe B, indicated). A 9-kb fragment hybridized in 6CKBAC1 and an 11-kb fragment hybridized in 6CKBAC2 DNA. Only a 9-kb fragment was recognized in plt genomic DNA, whereas both 9- and 11-kb fragments were present in 129/sv DNA. (C) Schematic of the Ckine genomic locus, with positions of initiation and stop codons indicated by ○ and ●, respectively. The positions of the hybridization probes are indicated by shaded boxes, and the sizes of the fragments recognized by these probes relative to each BAC are indicated.
are present and, in fact, both forms have been reported and characterized in the literature but not recognized as arising from independent genes. Hedrick and Zlotnik (4) reported the mouse 6C kine-leu form, and Tanabe et al. (3) reported the mouse 6C kine-ser form; although a direct comparison of the two mouse 6C kine proteins had not been made, these two reports showed similar findings regarding their chemotactic activity. Furthermore, computer modeling of the differences between the 6C kine-ser and 6C kine-leu proteins based on known chemokine crystal structures does not predict any radical differences in the structures of the two proteins (Murgolo, N., and E. Coates, personal communication). An analysis of the human 6C kine gene shows that it encodes a leucine at position 65 (1, 2, 6), and we were unable to find any ESTs corresponding to a human serine form in any of over 300 ESTs examined (Hedrick, J., and L. Wang, unpublished observation). We cannot, however, formally exclude the possibility of a second human gene. Chemokine gene duplication is relatively common, and indeed most members of this cytokine superfamily are thought to have arisen through a series of duplications of "primordial" chemokines. The duplication of mouse 6C kine is likely to have been a relatively recent evolutionary event, as there have been few changes within the exon/intron regions of the genes. It is also possible, however, that some selective pressure has maintained the two genes in a relatively unchanged state, and thus it will be important to determine the relative contributions of each form of 6C kine to the process of lymphocyte and dendritic cell trafficking in vivo.

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