Demethylated CD8 Gene in CD4$^+$ T Cells Suggests That CD4$^+$ Cells Develop from CD8$^+$ Precursors

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Mature T cells and medullary thymocytes bear either the CD4 or CD8 differentiation antigen. Precursor cells in the thymus express neither CD4 nor CD8 (CD4$^-$8$^-$), but most cortical thymocytes are CD4$^+$8$^+$. Whether CD4$^+$ and CD8$^+$ mature T cells arise directly from CD4$^-$8$^-$ precursors or from a CD4$^+$8$^+$ intermediate remains unresolved. In this study, methylation of the CD8 gene in murine T cells and thymocytes was examined. There was progressive demethylation of the CD8 gene in the thymus during the transition from CD4$^-$8$^-$ to CD4$^+$8$^+$. A similar pattern of demethylation of the CD8 gene was seen in CD4$^+$ mature T cells, suggesting previous expression of CD8 in the CD4$^+$ lineage.

THE CD4 AND CD8 MOLECULES play a crucial role in T cell responses to antigen. T cells use the αβ receptor to recognize antigen bound to either class I or class II products of the major histocompatibility complex (MHC). In general, T cells with αβ receptors that recognize antigen in association with class II MHC molecules express CD4, whereas those that recognize antigens bound to class I MHC express CD8 (*1*). Evidence suggests that CD4 and CD8 interactions with MHC are important in T cell activation (*2*). To understand the coordinated expression of either CD4 or CD8 with a T cell receptor of a particular specificity, we must know the sequence of expression of these molecules during T cell differentiation, when the repertoire of αβ receptors is expressed and selected in the thymus.

The precursor of all thymocytes and mature T cells lies in a minor cortical thymocyte subpopulation that is CD4$^-$8$^-$ (*3*). During ontogeny or during repopulation of an irradiated thymus, CD4$^+$8$^+$ cortical cells appear before CD4$^+$ or CD8$^+$ mature cells (*4, 5*); this order of events suggests that thymocytes first express both of these antigens and then shut off one or the other depending, for instance, on the specificity of their αβ receptors. Although there is some direct evidence for this pathway (*6*), attempts to induce isolated CD4$^+$8$^+$ cells to develop into CD4$^+$ and CD8$^+$ T cells have failed. This led to the hypothesis that CD4$^+$ and CD8$^+$ cells may arise directly from CD4$^-$8$^-$ precursors; the CD4$^+$8$^+$ phenotype may mark thymocytes that have not differentiated properly and are destined to die in the thymus (*5*).

Our study of CD8 expression in murine T cell hybridomas made by fusion of CD8$^+$ T cells to the T cell thymoma BW5147 (*7*) showed that, despite the continued expression of many T cell functions in these hybrids, CD8 expression was rapidly lost. We examined the methylation state of the CD8 gene in normal cells and in these CD8$^-$ hybrids. The 5′ end of the CD8 gene is heavily methylated in non-T cells, but a number of sites become unmethylated in CD8$^+$ T cells and tumors. In the hybrids, loss of CD8 expression is accompanied by remethylation of some, but not all, of these sites. We postulated that, if this partial resistance to remethylation occurs in normal T cells, the CD8 gene in normal CD4$^+$ cells may contain residual demethylated sites associated with CD8 expression at a previous CD4$^+$8$^+$ stage.

Therefore, DNA from fetal thymocytes, CD4$^-$8$^-$ thymocytes, CD4$^+$8$^+$ thymocytes, and CD8$^+$ and CD4$^+$ mature T cells was isolated, and methylation of the CD8 gene was analyzed with Msp I and Hpa II, an isoschizomer pair of restriction enzymes, and the methylation-sensitive enzyme Hha I. Msp I cleaves the recognition sequence CCGG whether or not the DNA is methylated, whereas Hpa II cuts unmethylated DNA only. Hha I cleaves GCGC but not Gm^{5}CGC. DNA was first digested with Bam HI and then cleaved with Msp I, Hpa II, or Hha I. Bam HI digestion resulted in an 8-kb fragment that encompasses the first three exons of the CD8 gene (*8*) and approximately 6 kb of 5′ flanking sequences (*7*) (Fig. 1). We mapped at least two Msp I/Hpa II (M/H) sites and one Hha I site 5′ of the CD8 gene (Fig. 1, sites 1, 2, and 7) (*7*). There is also a cluster of four M/H sites located in exon 2, intron 2, and exon 3 of the CD8 gene (Fig. 1, sites 3 to 6) (*8*).

We analyzed DNA from fetal thymuses at day 15 of gestation, at which time virtually all of the cells are CD4$^-$8$^-$, to day 19, when 98% of thymocytes express both CD4 and CD8 (Fig. 2A). In all stages of development, digestion with Bam HI yielded the expected 8-kb fragment (Fig. 2A, lanes B). Digestion with Bam HI and then with Msp I generated a 2.8-kb fragment as a result of cleavage at site 2 at the 5′ end and site 3 at the 3′ end (Fig. 2A, lanes M; Fig. 1). Digestion of day 15 fetal thymocyte DNA with Bam HI and then with Hpa II resulted in two fragments, 8 kb and 5 kb. The 8-kb band represents DNA that is still completely methylated at sites 1 to 6. The 5-kb band represents demethylation at site 1 but not at sites 2 to 6. In day 16 fetal thymus DNA the 8-kb band was very faint, the 5-kb band was much stronger, and faint bands of 4.3, 3.4, and 2.8 kb were present. These fragments represent extensive demethylation of the CD8 gene at site 1 and less complete demethylation at sites 2 to 6. In day 17 to day 19 fetal thymus DNA, as demethylation at sites 2 to 6 progressed, the intensity of the 4.3-, 3.4-, and 2.8-kb bands increased. The kinetics of demethylation and the kinetics of thymocyte expression of cell surface CD8 were similar, which suggested a relation between demethylation and CD8 gene expression.

We next examined DNA from isolated subpopulations of adult thymocytes and mature peripheral T cells (Fig. 2B). Digestion

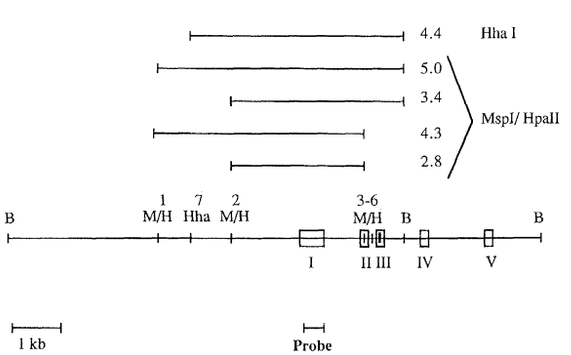

Fig. 1. Restriction map of the murine CD8 gene and 5′ flanking region. I to V, exons encoding the CD8 gene (*8*); B, Bam HI; M/H, isoschizomers Msp I and Hpa II; Hha, Hha I.

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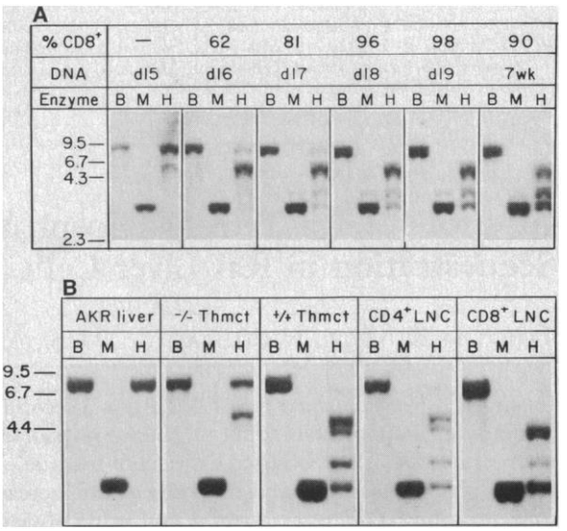

Fig. 2. Methylation patterns of the CD8 gene detected with Msp I and Hpa II. B, Bam HI alone; M, Bam HI followed by Msp I; H, Bam HI followed by Hpa II. (**A**) DNA from day 15, 16, 17, 18, and 19 fetal thymocytes, and adult thymocytes from B6 mice. (**B**) DNA from AKR liver, $CD4^-8^-$ thymocytes from B6 mice (−/− Thmct), $CD4^+8^+$ thymocytes from B6 mice (+/+ Thmct), $CD4^+$ lymphocytes from B6 lymph node ($CD4^+$ LNC), and $CD8^+$ lymphocytes from SJL/J lymph node ($CD8^+$ LNC). The isolation of T cell subpopulations was as described (*12*). Southern analyses of DNA from the above cell preparations were carried out by standard techniques (*17*). A complementary DNA composed of the 5′ end of the murine CD8 gene [PstI–EcoRI fragment of pcLy2-7b, provided by J. Parnes (*18*)] was used for hybridization.

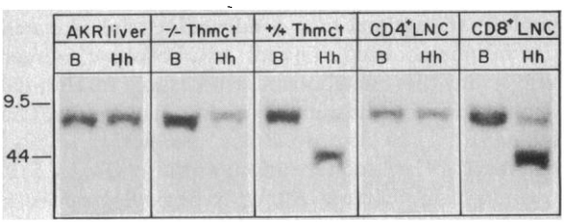

Fig. 3. Methylation patterns of the CD8 gene detected with Hha I. B, Bam HI alone; Hh, Bam HI followed by Hha I. Cells prepared as in Fig. 2.

of these DNAs with Bam HI again yielded the expected 8-kb fragment, and cleavage with Bam HI and then with Msp I resulted in the 2.8-kb fragment (Fig. 2B, lanes B and M). Liver DNA from AKR mice was methylated at M/H sites 1 to 6 since the size of the 8-kb Bam HI fragment was not further reduced upon treatment with Hpa II. Digestion of $CD4^-8^-$ precursor thymocyte DNA with Bam HI and then with Hpa II resulted in two fragments, 8 kb and 5 kb, representing some demethylation at site 1 but not at sites 2 to 6. This pattern of demethylation was almost identical to that of day 15 fetal thymocytes, which were also of the $CD4^-8^-$ phenotype. The $CD4^+8^+$ thymocytes had more extensive demethylation. Cleavage of DNA from $CD4^+8^+$ thymocytes with Bam HI and then with Hpa II yielded fragments of 5, 4.3, 3.4, and 2.8 kb. These fragments represent variable demethylation both within the CD8 gene and at sites 5′ of the gene, as seen in day 17 to day 19 fetal thymus DNA (Fig. 1, sites 1 to 6). The methylation pattern of $CD4^+$ lymph node cells was identical to that of $CD4^+8^+$ thymocytes at these sites. Digestion of DNA from $CD8^+$ lymph node cells with Bam HI and then with Hpa II yielded two bands of approximately 4.3 and 2.8 kb, as well as a less prominent band of 3.4 kb. The 4.3- and 2.8-kb fragments were from demethylation of the CD8 gene at site 3 and at either site 1, site 2, or both (Fig. 1). In a small percentage of CD8 alleles, sites 3 to 6 were still methylated, yielding the 3.4-kb fragment.

These methylation patterns reflect a continuum of CD8 gene demethylation corresponding to CD8 surface expression. In early $CD4^-8^-$ precursor thymocytes the gene region is either completely methylated or unmethylated only at the most upstream M/H site. In $CD4^+8^+$ thymocytes demethylation has progressed further; all of the identified M/H sites are demethylated to some degree. In mature cells committed to CD8 expression, there is further demethylation, with loss of the 5-kb and the 3.4-kb bands. The similar demethylation of the CD8 gene in $CD4^+$ cells and in $CD4^+8^+$ thymocytes is consistent with the idea that mature $CD4^+$ cells arise from a $CD4^+8^+$ precursor, but stop further demethylation of the CD8 gene when CD8 expression is halted.

We used the restriction enzyme Hha I to examine an additional methylation site located approximately 2.6 kb 5′ of the CD8 gene (Fig. 1, site 7). This site is demethylated in $CD8^+$ T cells, but is always remethylated in T cell hybridomas that have lost CD8 expression (*7*). We postulated that this site might be demethylated in $CD4^+8^+$ thymocytes, but, unlike the sites detected with Msp I/Hpa II, it might be remethylated in $CD4^+$ T cells. This was indeed the case (Fig. 3). This Hha I site is methylated in liver DNA and in $CD4^-8^-$ thymocytes, as the Bam HI fragment was not further cleaved by Hha I (Fig. 3). This site is largely unmethylated in $CD4^+8^+$ thymocytes and in $CD8^+$ lymph node cells, as a 4.4-kb Bam HI–Hha I fragment was generated. However, this Hha I site is methylated in mature $CD4^+$ lymph node cells. It is likely that this particular methylation-sensitive Hha I site is one that is remethylated in association with conversion from the $CD4^+8^+$ phenotype to the mature $CD4^+$ phenotype, similar to its remethylation in T cell hybridomas that have lost expression of the CD8 molecule (*7*). It is tempting to speculate that this particular site is important in the regulation of CD8 expression; remethylation of this site might affect chromatin structure or binding of regulatory proteins.

Our results favor, but do not prove, that $CD4^+$ T cells pass through $CD4^+8^+$ intermediate during differentiation. After differentiation most of the sites that are demethylated at the 5′ end of the CD8 gene become resistant to remethylation, even after the loss of CD8 expression. We must, however, interpret our results with caution. Demethylation of a gene often accompanies expression, but the two processes are not absolutely linked. Therefore, we cannot formally rule out the alternative possibility that, in arising directly from a $CD4^-8^-$ precursor, $CD4^+$ T cells permanently demethylate the CD8 gene in a manner identical to $CD4^+8^+$ thymocytes, but nevertheless fail to express CD8. In an experiment relevant to this question, we examined the immunoglobulin κ gene, which is tightly linked to the murine CD8 gene on chromosome 6. Despite the fact that this gene is unmethylated in B cells (*9*), we found no demethylation of the κ gene in any T cell or thymocyte population (*10*), arguing against nonspecific demethylation of genes in this chromosomal region or of genes of the lymphocytic lineage in general, and strengthening the conclusion that demethylation of the CD8 gene is a specific event accompanying expression.

Despite the difficulty of directly demonstrating the transition of $CD4^+8^+$ cells to either $CD4^+$ or $CD8^+$ T cells, our results add to recent evidence in support of this view. The development of $CD4^+$ T cells is impaired by in vivo administration of antibodies to CD8 (*6*). Also, in transgenic mice, in which expression of a self-reactive αβ receptor is forced on all thymocytes, extensive deletion of both $CD4^+8^+$ and mature thymocytes is observed, suggesting the participation of $CD4^+8^+$ cells in the thymic processes that select the T cell repertoire (*11*).

Although gene methylation has not been

proven to regulate gene expression, demethylation of newly expressed genes is often observed during differentiation. Our results suggest that genes transiently expressed during differentiation of a cell lineage may bear a stable residual demethylation pattern at later times. We have compared our results to other gene systems in which methylation has been studied, but found none that parallel ours in identifying a gene transiently expressed during differentiation (including the globin genes in which expression of fetal and adult forms of α and β globins does not necessarily occur in the same cell). However, analysis of methylation patterns in other systems may provide a general tool for sorting out complex precursor-product relationships in hemopoetic and other cell lineages.

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12. $CD4^-8^-$ thymocytes from B6 mice were prepared by removal of $CD8^+$ T cells on plastic petri dishes coated with AD4(15) [mouse immunoglobulin M (IgM) specific for murine CD8.2] (*13*). Nonadherent cells were coated with KJ16 (rat IgG specific for murine T cell receptor Vβ8.1 and 8.2 regions) (*14*), GK1.5 (rat IgG specific for murine CD4) (*15*), and 2.43 (rat IgG specific for CD8.2) (*16*) and incubated on petri dishes coated with goat antibody to rat IgG (Jackson ImmunoResearch Laboratories Inc.). Nonadherent cells were collected and passed two more times over goat antibody to rat IgG–coated plates. Purified $CD4^-8^-$ cells were analyzed by flow cytometry (EPICS C, Coulter Electronics), with 2.43 and GK1.5 as primary staining reagents and fluroscein isothiocyanate (FITC)–labeled RG7/9.1 (mouse antibody to rat κ chain, gift of T. A. Springer) as the secondary reagent. By this analysis 90% of the purified cells were $CD4^-8^-$. The remaining 10% expressed only low density surface CD4, CD8, or both. High density J11d (gift of J. Sprent) was observed on 97% of purified double negative cells, and low density T3 (145-2C11, gift of J. Bluestone) was observed on 6% of these cells. The $CD4^+8^+$ thymocytes were isolated from newborn B6 mice; 97% of these cells stained with both GK1.5 and 2.43. The $CD8^+$ T cells (94% pure) were isolated as described (7). The $CD4^+$ T cells from B6 lymph node were isolated by passage over nylon wool columns and nonadherence to AD4 (*15*) coated plates; 97% of these T cells expressed surface CD4.
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Inositol 1,3,4,5-Tetrakisphosphate Induces Ca^{2+} Sequestration in Rat Liver Cells

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Inositol 1,4,5-trisphosphate [$I(1,4,5)P_3$] is a second messenger generated along with diacylglycerol upon the binding of various physiological agents with their cell surface receptors. $I(1,4,5)P_3$ mobilizes Ca^{2+} from intracellular storage sites through a receptor-coupled mechanism, and the subsequent increased intracellular free calcium ion concentration ($[Ca^{2+}]_i$) activates a multitude of cellular responses. Electropermeabilized neoplastic rat liver epithelial (261B) cells were used to study Ca^{2+} sequestration, a process that reverses the elevated $[Ca^{2+}]_i$ to resting levels and replenishes intracellular Ca^{2+} pools. Although $I(1,4,5)P_3$-mobilized Ca^{2+} is readily sequestered into storage pools by the action of Ca^{2+}–adenosine triphosphatases, Ca^{2+} mobilized by addition of the nonmetabolized inositol trisphosphate isomer $I(2,4,5)P_3$ is not sequestered, suggesting that metabolism is necessary to eliminate the stimulus for Ca^{2+} release. Several inositol phosphate compounds were examined for their ability to lower the buffer $[Ca^{2+}]$ to determine if a specific $I(1,4,5)P_3$ metabolite might be involved in stimulating Ca^{2+} sequestration; of these, $I(1,3,4,5)P_4$ alone was found to induce Ca^{2+} sequestration, demonstrating a physiological role for this inositol trisphosphate metabolite.

THE SECOND MESSENGER ROLE OF $I(1,4,5)P_3$ as a Ca^{2+} mobilizing agent is well characterized (*1*). Transient and prolonged alterations in $[Ca^{2+}]_i$ in response to the liberation of $I(1,4,5)P_3$ are known to stimulate such diverse physiological functions as secretion, egg fertilization, and cell proliferation (*2*). Many enzymes are activated by a rise in $[Ca^{2+}]_i$ either directly by free Ca^{2+} or through association with the calcium-binding protein calmodulin (*3*). Moreover, a number of calcium-binding proteins exist that might participate in important regulatory mechanisms when complexed with Ca^{2+}, for which a functional role has yet to be assigned (*4*). Although the release of intracellularly stored Ca^{2+} induced by $I(1,4,5)P_3$ appears to be receptor mediated (*5*) and in tight association with calmodulin (*6*), the mechanism for initiating a reversal of high $[Ca^{2+}]_i$ after the Ca^{2+} mobilization response, and thereby removing the stimulatory signal generated by Ca^{2+}, has not been identified. To study Ca^{2+} sequestration we used an electropermeabilized neoplastic rat liver epithelial (261B) cell system, in which changes in buffer $[Ca^{2+}]$ were monitored with the fluorescent indicator fura-2, and we found that inositol 1,3,4,5-tetrakisphosphate [$I(1,3,4,5)P_4$] induces Ca^{2+} sequestration.

When 2 μ*M* $I(1,4,5)P_3$ (a maximal dose) was added to permeable 261B cells, there was a rapid release of Ca^{2+} into the buffer, followed by a sequestration phase that reduced the buffer $[Ca^{2+}]$ to starting levels (Fig. 1A). In contrast, Ca^{2+} mobilized by 2 μ*M* $I(2,4,5)P_3$ was not sequestered by intracellular membranes (Fig. 1B). This finding could be the result of receptor de-

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Table 1. Effect of inositol phosphate compounds on Ca^{2+} sequestration. Inositol phosphate (IP) compounds were added 30 s before $I(2,4,5)P_3$, and the amount of Ca^{2+} released was determined. The amount of Ca^{2+} sequestered after a 15-min incubation period was determined on the same permeabilized cell samples. Experiments were repeated ten times with similar results.

Inositol phosphate (2 μ*M*)	Ca^{2+} released by 2 μ*M* $I(2,4,5)P_3$ (n*M*)	Ca^{2+} sequestered in response to IP (n*M*)
$I(1)P_1$	289	0
$I(2)P_1$	322	0
I cyclic$(1,2)P_1$	278	0
$I(1,4)P_2$	312	0
$I(1,3,4)P_3$	354	0
$I(1,3,4,5)P_4$	368	368
$I(1,4,5,6)P_4$	397	0
$I(w,x,y,z)P_4$	264	0
$I(1,3,4,5,6)P_5$	292	0
$I(1,2,3,4,5,6)P_6$	388	0