B-cell-specific demethylation of BTK, the defective gene in X-linked agammaglobulinemia

Abstract BTK, the gene that is defective in X-linked agammaglobulinemia, encodes a cytoplasmic tyrosine kinase that is critical for B-cell proliferation, or survival. To identify regulatory elements that control the expression of BTK we evaluated the methylation pattern of this gene in cell lines and in freshly isolated cells. An Hpa II site that was specifically demethylated in mature B cells but not in pre-B cells, T cells, neutrophils, or nonhematopoietic cells was identified in the tenth intron of BTK. In a 40 kilobase (kb) segment of DNA spanning the entire coding region of BTK plus 3 kb upstream of the first exon there were no other sites that demonstrated lineage-specific demethylation. The B-cell-specific demethylation site in intron 10, which falls within the SH2 domain, 26 kb distal to the first exon, occurs in a region rich in regulatory elements including two E2 boxes, two AP-2 sites, and a cAMP response element. It is likely that this site plays a role in maintaining BTK transcription in mature B cells.

Introduction

In early 1993, three groups independently reported the identification of a novel cytoplasmic tyrosine kinase expressed throughout B-cell and myeloid differentiation (Tskakada et al. 1993; Vetrie et al. 1993; N. Yamada et al. 1993). The gene coding for this protein, which has been called Btk (Bruton’s tyrosine kinase), shows 40%-50% base pair (bp) identity with members of an src subfamily which includes: Dsrc28C, tecI, itk, and BMX (Tsukada et al. 1993; Tamagnone et al. 1994). Members of this subfamily contain SH3, SH2, and carboxy-terminal kinase domains, like other src family members; however, they differ from typical src family members in that they have an unusually long 5’ basic region that contains a pleckstrin homology domain (Musacchio et al. 1993). In addition, the members of this subfamily do not have the typical amino-terminal myristylation signal seen in src family members, and they lack the carboxy-terminal down regulatory phosphorylation site. Although the exact function of Btk is not yet known, cytoplasmic tyrosine kinases are thought to play a role in the cellular signaling pathways that regulate cell proliferation and/or differentiation (Bolen 1993). In support of this, recent studies suggest that Btk is activated by cross-linking of the antigen receptor on B cells and the high-affinity IgE receptor on mast cells (Aoki et al. 1994; de Weers et al. 1994a; Kawakami et al. 1994). Whether Btk is involved in signaling through other cell surface molecules is not yet clear.

We and others have shown that mutations in BTK result in the B-cell-specific defect, X-linked agammaglobulinemia (XLA) (Tsukada et al. 1993; Vetrie et al. 1993; Vorechovsky et al. 1993; Bradley et al. 1994; Conley et al. 1994; de Weers et al. 1994b; Duriez et al. 1994; Hagemann et al. 1994; Ohta et al. 1994; Saffran et al. 1994; Zhu et al. 1994a; Zhu et al. 1994b; Vobechevsky et al. 1995). Originally described in 1952 by Bruton (1952), XLA is characterized by profound hypogammaglobulinemia and markedly reduced numbers of circulating B cells (Conley 1992). In the normal child or adult, 5%-15% of the peripheral blood lymphocytes are B cells, whereas in patients with XLA less than 1% of lymphocytes can be stained with antibody to surface immunoglobulin (Ig) or CD19 (Conley 1985). Other hematopoietic cells lines do not seem to be affected by the gene defect. T-cell numbers
EBV-transformed cell line from the male contains an unmethylated site approximately 0.5 kb from the 5' site of the gene. The two Eco RI fragments are 10.7 kb and 5.8 kb. The digestion with Hpa II shows that the 5.8 kb Eco RI fragment from the EBV-transformed cell line from the male contains an unmethylated Hpa II site approximately 0.5 kb from the Eco RI site. In the EBV-transformed cell line from the female, approximately half of the DNA is digested with Hpa II, as would be expected if the active but not the inactive X chromosome was demethylated at this site.

Analysis of genomic DNA

DNA was prepared from fresh cells or cultured cell lines using the Applied Biosystems Nucleic Acid Extractor, model 240A (Foster City, CA). For double digestions, DNA (30 μg) was digested to completion with Eco RI (E), Eco RI plus Hpa II (E+H), or Eco RI plus Msp I (E+M) and analyzed by Southern blot using a probe derived from the 5' region of BTK (bp 93 to 900). The two Eco RI fragments are 10.7 kb and 5.8 kb. The digestion with Hpa II shows that the 5.8 kb Eco RI fragment from the EBV-transformed cell line from the male contains an unmethylated Hpa II site approximately 0.5 kb from the Eco RI site. In the EBV-transformed cell line from the female, approximately half of the DNA in the 5.8 kb fragment is digested with Hpa II, as would be expected if the active but not the inactive X chromosome was demethylated at this site.

Materials and methods

Cell separations and cell lines

Cell separations were performed as previously described (Wengler et al. 1993). Briefly, venous blood from healthy donors was centrifuged through a Ficoll-Hyphaque gradient; neutrophils were retrieved from red blood cell pellet and lymphocytes, which were isolated from the Ficoll interface, were separated into CD19 + and CD19- cells using anti-CD19 coated immunomagnetic beads. Over 95% of the CD19 + cells were cytoplasmic g-negative and cytoplasmic g-positive; both groups express Btk. DNA was extracted from an EBV-transformed B-cell line produced in our laboratory, which was derived from the bone marrow of a patient with severe combined immunodeficiency who had no B cells or T cells in the peripheral circulation. It is CD19+ and has Ig genes in germline configuration. The REH and the Nalm-1 are human CD19 + and CD10 + B-cell precursor cell lines that are respectively cytoplasmic Ig-negative and cytoplasmic Ig-positive; both groups express Btk.

Methylation status of BTK in activated T cells and EBV-transformed cell lines

To explore lineage specific demethylation of BTK, we examined genomic DNA from B cells, a lineage known to express Btk, and T cells, a lineage that does not express Btk. DNA was extracted from an EBV-transformed B-cell line and from activated T cells from healthy males and females digested with Eco RI (E). Aliquots were further digested either with Hpa II (E+H), which cleaves the sequence CCGG only when both cytosine residues are unmethylated, or with the isoschizomer MspI (E+M), which cleaves the same sequence independently of the methylation status of the internal cytosine. The digestion with Msp I reveals potential Hpa II sites and results of the digestion with Hpa II show the methylation status of these sites. As shown in...