expression. We reconstituted the functional chemiluminescent protein by incubating the intact cells with 2.5 μM coelenterazine for 2 to 3 hours before an experiment (8); cells were also incubated with fura-2 for the last 30 min before an experiment (11). After reconstitution, the monolayer of HeLa cells, grown on a small glass cover slip (13 mm in diameter), was washed with fresh medium and incubated into the perfusion chamber; light emission was measured with a luminometer [P. H. Cobbold and J. A. Lee in Cellular Calcium, A Practical Approach, J. G. McCormack and P. H. Cobbold, Eds. (IRL Press, Oxford, 1991), pp. 55–81]. The perfusion chamber was maintained at 37°C by a water jacket. To obtain a rapid and synchronous equilibrium of medium in the chamber, a flow of 1 ml/min was used and the flow rate was increased from 2.5 to 6 ml/min. The time necessary for the new medium to reach the perfusion chamber was 12 ± 1 s (n = 5), and 50% equilibration was obtained in 2 s. The light emission from aequorin was transformed into [Ca^{2+}]i by means of a program provided by R. Cuthbertson, assuming an intramitochondrial Mg^{2+} concentration of 1 mM [G. A. Rutter, N. J. Os- baldiston, J. G. McCormack, R. M. Denton, Biochim. J. 271, 627 (1990)]. In each experiment we obtained the final discharge of uncharged aequorin, necessary for calibration, by exposing the cells to a solution of 10 mM CaCl2.


11. Fura-2 loading was done as described [A. Malgo- rral, G. Milani, M. D. Bootman, T. Pozzan, J. Cell Biol. 105, 2145 (1988)]. Two types of cover slips were used. One, 20 mm long and 8 mm wide, was held in place in the cuvette of a multimode fluorimeter maintained at 37°C (Carm Research Ltd., Sittingbourne, Kent, UK, excitation at 350 and 380 nm, emission at 500 nm), as described [F. Di Virgilio, B. C. Meyer, S. Greenberg, S. C. Silverstein, J. Cell Biol. 106, 657 (1989)]. In this type of experiment the signal represents the average of a few thousand cells illuminated by the incident beam. At the end of each experiment, iodination (2 μM) and then Mn^{2+} (1 mM) were added to obtain the background signal to be subtracted before calculating [Ca^{2+}]. The second type of cover slip, diameter 24 mm, was placed in a chamber held at 37°C in a water bath and illuminated with a multimode fluorimeter (Medical System Corporation, Greenville, NY) on the stage of an inverted fluorescent microscope (Zeiss Axiovert 100TV) equipped with the imaging apparatus (AxioCam, Imaging Concepts, Atlanta, GA) for fura-2 measurement. The [Ca^{2+}]i images (1 image per second) were calculated off-line after subtraction of the background (obtained as above). The absolute values of [Ca^{2+}]i were calculated as described [G. Grynkiewicz, M. Poenie, R. Y. Tsien, J. Biol. Chem. 260, 3440 (1985); A. Malgorral, G. Milani, M. D. Bootman, T. Pozzan, J. Cell Biol. 105, 2145 (1988)].


13. Permeabilization with digitonin, a detergent prefer- entially directed toward membranes containing cholesterol, has been used to study the function of mitochondria in situ. Digitonin treatment of HeLa cells for results in complete release of the cytosolic marker enzyme lactate dehydrogenase, whereas the mitochondrial marker enzymes citrate synthase or glutamate dehydro- genase remain associated with the chromatohorm. The present work indicates that the cytosolic marker remains associated with sedimented cells.

14. Similar values of [Ca^{2+}]i were measured with fura-2 and with injected aequorin in hepatocytes [N. M. Wood, Biochem. J. 170, 1 (1978)]. These results confirm the extensive application of aequorin to study intracellular calcium, the role of which is still poorly understood.

15. The concentration of Ca^{2+} in the buffers was calculated as described [A. Fabiato, in Cellular Calcium, A Practical Approach, J. G. McCormack and P. H. Cobbold, Eds. (IRL Press, Oxford, 1991), pp. 159–176]. The calculated concentrations of free Ca^{2+} in the medium were always compared with the values measured directly with fura-2 in an aliquot of the same buffers. If a discrepancy was noticed, the values measured with fura-2 were considered correct.

16. A discrepancy between the increases in [Ca^{2+}]i in intact cells measured with fura-2 and the calculated extramitochondrial [Ca^{2+}]i is observed also in the case of the other indicators. The difference (approximately a factor of 2) may be ascribed to the loss of a cytosolic factor, perhaps spermine [J. G. McCormack, H. M. Brown, N. J. Dawes, with fura-2 were considered correct.

17. R. Rizzuto et al., unpublished data.


19. If, after permeabilization, ATP was omitted, the response to IP3 was reduced and eventually disappeared. Marginal increases of [Ca^{2+}]i were observed if the uncoupler of mitochondrial respiration (trifluoro-methoxyphenylhydrazine (FCCP) was included in the buffer before the addition of IP3. Substitution of EGTA with an equivalent concentration of the faster Ca^{2+} chelator BAPTA did not change the effect of IP3 on [Ca^{2+}]i. Conversely, when high concentrations of each chelator were used (for example, 500 μM), the IP3-induced [Ca^{2+}]i transient was virtually abolished.


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Alterations of a Zinc Finger-Encoding Gene, BCL-6, in Diffuse Large-Cell Lymphoma

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The molecular pathogenesis of diffuse large-cell lymphoma (DLC), the most frequent and clinically relevant type of lymphoma, is unknown. A gene was cloned from chromosomal translocations affecting band 3q27, which are common in DLC. This gene, BCL-6, codes for a 79-kilodalton protein that is homologous with zinc finger–transcription factors. In 33 percent (13 of 39) of DLC samples, but not in other types of lymphoid malignancies, the BCL-6 gene is truncated within its 5’ noncoding sequences, suggesting that its expression is deregulated. Thus, BCL-6 may be a proto-oncogene specifically involved in the pathogenesis of DLC.

The molecular analysis of specific chromosomal translocations has improved our understanding of the pathogenesis of non-Hodgkin lymphoma (NHL), a heterogeneous group of B cell and (less frequently) T cell malignancies (1, 2). The (14;18) chromosomal translocation, which causes the deregulated expression of the anti-apoptosis gene BCL-2, plays a critical role in the development of follicular lymphoma (FL) (3), which accounts for 20 to 30% of all NHL diagnoses (4). Burkitt’s lymphoma (BL) and mantle-cell lymphoma, two relatively rare NHL types, are characterized by chromosomal translocations that cause the deregulated expression of the cell-cycle progression genes MYC and BCL-1/cyclin D1, respectively (5, 6).

Relatively little is known about the molecular pathogenesis of DLC, the most frequent and most lethal human lymphoma (4). It accounts for ~40% of initial NHL diagnoses and is often the final stage of progression of FL (4). A small percentage of DLCs display MYC rearrangements (7) and 20 to 30% display alterations of BCL-2, reflecting the tumor’s derivation from FL (8). However, no consistent molecular alteration has been identified that is specific for DLC.
Chromosomal translocations involving reciprocal recombinations between band 3q27 and several other chromosomal sites are found in 8 to 12% of NHL cases, particularly in DLCL (9). From NHL samples displaying recombinations between 3q27 and the immunoglobulin heavy chain (IgH) locus on 14q32, we and others have cloned the chromosomal junctions of several (3;14)(q27;q32) translocations and identified a cluster of breakpoints at a 3q27 locus named BCL-6 (10). Genomic sequences flanking the cluster region are transcriptionally active, suggesting that they contain a gene potentially involved in DLCL pathogenesis (10). In this study, we identify the BCL-6 gene and its predicted protein product. We also demonstrate that structural lesions of this gene are common in DLCL.

To isolate normal BCL-6 complementary DNA (cDNA), we screened a cDNA library constructed from the NHL cell line Bjab (11) with a probe (10) derived from the chromosomal region flanking the breakpoints of two t(3;14)(q27;q32) cases. Sequence analysis (Fig. 1) revealed that the longest clone (3549 bp), about the same size as BCL-6 RNA, codes for a protein of 706 amino acids with a predicted molecular mass of 79 kD. The putative ATG initiation codon at position 328 is surrounded by a Kozak consensus sequence (12) and is preceded by three upstream in-frame stop codons. The 1101-bp 3' untranslated region contains a polyadenylation signal followed by a track of polyadenylate. These features are consistent with the idea that BCL-6 is a functional gene (see Fig. 1A for a schematic representation of the cDNA clone).

The NH2- and COOH-termini of the BCL-6 protein (Fig. 1) (13) have homologies with zinc finger–transcription factors (14). A gene sequence encoding a nearly identical protein was recently deposited in GenBank. This gene, termed LAZ-3, was cloned on the basis of its involvement in 3q27 translocations, and it is therefore likely that BCL-6 and LAZ-3 are the same gene (15). Because the COOH-terminal region of BCL-6 contains six C3H2 zinc finger motifs (Fig. 1) and a conserved stretch of six amino acids (the His-Cys link) connecting the successive zinc finger repeats (16), BCL-6 can be assigned to the Krüppel-like subfamily of zinc finger proteins. The NH2-terminal region of BCL-6 is devoid of the FAX (17) and KRAB (18) domains sometimes seen in Krüppel-related zinc finger proteins, but it does have homologies (Fig. 1) with other zinc finger–transcription factors including the human ZPFS protein, a putative human transcription factor that regulates the major histocompatibility complex II promoter (19), the Tramtrack (ttk) and Broad-complex (Br-c) proteins in Drosophila that regulate developmental transcription (20), the human KUP protein (21), and the human PLZF protein, which is occasionally involved in chromosomal translocations in human promyelocytic leukemia (22). The regions of NH2-terminal homology among ZPFSJ, ttk, Br-c, PLZF, and BCL-6 also share homology with viral proteins (for example, VA55R) of the poxvirus family (23) as well as with the Drosophila kelch protein involved in nurse cell–oocyte interaction (24). These structural homologies suggest that BCL-6 may function as a DNA binding transcription factor that regulates organ development and tissue differentiation.

The cDNA clone was used as a probe to investigate BCL-6 RNA expression in a variety of human cell lines by Northern (RNA) blot analysis. A single 3.8-kb RNA species was readily detected (Fig. 2) in cell lines derived from mature B cells, but not from pro–B cells or plasma cells, T cells, or other hematopoietic cell lineages (25). The BCL-6 RNA was not detectable in other normal tissues, except for skeletal muscle, which expressed low amounts (25). Thus, the expression of BCL-6 was detected in B cells at a differentiation stage corresponding to that of DLCL cells. This selective expression in a “window” of B cell differentiation suggests that BCL-6 may play a role in the control of normal B cell differentiation and lymphoid organ development.

To characterize the BCL-6 genomic locus, we used the same cDNA probe to screen a genomic library from human placenta (26). By restriction mapping, hybridization with various cDNA probes, and limited nucleotide sequencing, the BCL-6 gene was found to contain at least eight exons spanning ~26 kb of DNA (Fig. 3). Sequence analysis of the first and second exons indicated that they are noncoding and that the translation initiation codon is within the third exon.

Various cDNA and genomic probes were used in Southern (DNA) blot hybridizations to determine the relation between 3q27 breakpoints and BCL-6 sequences in a panel of 17 DLCL cases carrying chromosomal translocations involving 3q27 (Table 1). Monosomic rearrangements of BCL-6 were detected in 12 of 17 tumors with combinations of restriction enzymes (Bam HI and Xba I) and probes that explore ~16 kb of the BCL-6 locus. These 12 positive cases carry recombinations between 3q27 and several different chromosomes (Table 1), indicating that heterogeneous 3q27 breakpoints cluster in a restricted genomic locus irrespective of the partner chromosome involved in the translocation. Some DLCL samples (5 of 17) do not display BCL-6 rearrangements despite cytogenetic alterations in band 3q27, suggesting that another gene is involved or, more likely, that there are other breakpoint clusters 5' or 3' to BCL-6. If the latter is true, the observed frequency of BCL-6 involvement in DLCL (33%) may be an underestimate.

We also analyzed a panel of tumors not previously selected on the basis of 3q27 breakpoints but representative of the major subtypes of NHL as well as of other lymphomas.
phoproliferative diseases. Rearrangements of BCL-6 were detected in 13 of 39 DLCLs, but not in other tumors including other NHL subtypes (28 FL, 20 BL, and 8 small lymphocytic NHL, acute lymphoblastic leukemia (ALL; 21 cases), and chronic lymphocytic leukemia (CLL; 31 cases). These findings indicate that BCL-6 rearrangements are specific for and frequent in DLCL. In addition, the frequency of rearrangements in DLCL (33%) significantly exceeds that (8 to 12%) reported on the basis of cytogenetic studies (9), suggesting that some of the observed rearrangements may involve submicroscopic chromosomal alterations.

All the breakpoints in BCL-6 mapped to the putative 5' flanking region, the first exon, or the first intron (Fig. 3). For two patients that carry (3;14)(q27;q32) translocations, the chromosomal breakpoints have been cloned and precisely mapped to the first intron (SM1444) or to 5' flanking sequences (KC1445) of BCL-6 on 3q27 and to the switch region of IgH on 14q32 (10). In all rearrangements, the coding region of BCL-6 was left intact, whereas the 5' regulatory region, presumably containing the promoter sequences, was either completely removed or truncated. The resultant fusion of BCL-6 coding sequences to heterologous (from other chromosomes) or alternative (within the BCL-6 locus) regulatory sequences may disrupt the gene's normal expression pattern (27).

Zinc-finger-encoding genes are plausible candidate oncogenes as they have been shown to participate in the control of cell proliferation, differentiation, and organ pattern formation (14). In fact, alterations of zinc finger genes have been detected in a variety of tumor types. These genes include PLZF (22) and PML in acute promyelocytic leukemia, ETV-1 in multiple myeloma and human myeloid leukemia, TTG-1 in T cell ALL, HTRX in acute mixed-lineage leukemia, and WT-1 in Wilms tumor (28). Terminal differentiation of hematopoietic cells is associated with the down-regulation of many Krüppel-type zinc finger genes (14, 18, 22); thus, it is conceivable that constitutive expression of BCL-6, caused by chromosomal rearrangements, interferes with normal B cell differentiation, thereby contributing to the abnormal lymph node architecture typifying DLCL.

Given that DLCL accounts for ~80% of NHL mortality (4), the identification of a specific pathogenetic lesion has important clinicopathologic implications. Lesions in BCL-6 may help in identifying prognostically distinct subgroups of DLCL. In addition, because a therapeutic response can now be obtained in a substantial fraction of cases (4), a genetic marker specific for the malignant clone may be a critical tool for the monitoring of minimal residual disease and early diagnosis of relapse (29).

REFERENCES AND NOTES

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11. A phage cDNA library constructed from RNA of the Bjab lymphoma cell line was screened with 1 x 10⁶ plaques by plaque hybridization with the Sac 4.0 probe that had been ³²P-labeled by random priming [A. P. Fernberg and B. Vogelstein, Anul. Biochem. 132, 6 (1983)]. The longest insert of the four phages isolated was subcloned into the pGEM-TZf(+)(Promega) and sequenced on both strands.
Isolation of the Cyclosporin-Sensitive T Cell Transcription Factor NFATp

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Nuclear factor of activated T cells (NFAT) is a transcription factor that regulates expression of the cytokine interleukin-2 (IL-2) in activated T cells. The DNA-binding specificity of NFAT is conferred by NFATp, a phosphoprotein that is a target for the immunosuppressive compounds cyclosporin A and FK506. Here, the purification of NFATp from murine T cells and the isolation of a complementary DNA clone encoding NFATp are reported. A truncated form of NFATp, expressed as a recombinant protein in bacteria, binds specifically to the NFAT site of the murine IL-2 promoter and forms a transcriptionally active complex with recombinant c-Fos and c-Jun. Antiserum to tryptic peptides of the purified protein or to the recombinant protein fragment react with T cell NFATp. The molecular cloning of NFATp should allow detailed analysis of a T cell transcription factor that is central to initiation of the immune response.

Nuclear factor of activated T cells is an inducible DNA-binding protein that binds to two independent sites in the IL-2 promoter (1, 2). NFAT is a multisubunit transcription factor (3) consisting of at least three DNA-binding polypeptides, the preexisting subunit NFATp (4–6) and homodimers or heterodimers of Fos and Jun family proteins (6–9). NFATp is present in the cytosolic fraction of unstimulated T cells (3–7); after T cell activation, it is found in nuclear extracts and forms DNA-protein complexes with Fos and Jun family members at the NFAT sites of the IL-2 promoter (3, 5–9). NFATp has also been implicated in the transcriptional regulation of other cytokine genes, including the genes for granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-4, and IL-5, and the tumor necrosis factor (TNF) (10).

NFATp is the target of a Ca2+-dependent signaling pathway initiated at the T cell receptor (3, 4, 6, 7, 11–13). The rise in intracellular free Ca2+ in activated T cells results in an increase in the phosphatase activity of the Ca2+- and calmodulin-dependent phosphatase calcineurin (14). NFATp is a substrate for calcineurin in vitro (4, 6) and is thought to be dephosphorylated by calcineurin in activated T cells, resulting in its translocation from the cytoplasm to the nucleus (13). Cyclosporin A (CsA) and FK506, which act as a complex with their respective intracellular receptors to inhibit the phosphatase activity of calcineurin (15), block the dephosphorylation of NFATp (4) and the appearance of NFAT in nuclear extracts of stimulated T cells (2, 3, 7, 12). This mechanism may account for the sensitivity of cyclosporin and IL-2 to other cytokine genes (10, 13) and thus for the profound immunosuppression caused by CsA and FK506 (13).

NFATp was purified from the C7.1W2 cell line (16), a derivative of the murine T cell clone Ar-3 (17), by ammonium sulfate fractionation followed by successive chromatography on a heparin-agarose column and an NFAT oligonucleotide affinity column (18). A silver-stained SDS gel of the purified protein showed a major broad band migrating with an apparent molecular size of ~120 kD (Fig. 1, top). We have shown that this band contains a DNA-binding phosphoprotein that is dephosphorylated by calcineurin to yield four sharp bands migrating with apparent molecular sizes of ~110 to 115 kD (6). NFATp DNA-binding activity was demonstrable in protein eluted from the SDS gel and renatured (4), and more than 90% of the activity recovered from the gel comigrated with the ~120-kD band (Fig. 1, lane 7). The faster migrating complexes formed with proteins of slightly smaller molecular size (lanes 8 to 11) most likely derive from partial proteolysis. The purified protein binds to the NFAT site with the appropriate specificity and forms a DNA-protein complex with recombinant Fos and Jun (6).

To confirm that the 120-kD protein was the preexisting subunit of the T cell transcription factor NFAT, we used antiserum to tryptic peptides derived from the 120-kD protein (18). When one such antiserum (to peptide 72) was included in the binding reaction, it “supershifted” the NFATp-DNA complex formed by the cytosolic fraction of unstimulated T cells (Fig. 2, lane