DNA-Binding Factors of B Lymphoid Cells Are Susceptible to Limited Proteolytic Cleavage during Nuclear Extract Preparation

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DNA-binding proteins that interact with the 3' end of the mouse μ immunoglobulin heavy chain gene were identified by the electrophoretic mobility shift assay. Complexes of distinctly different mobilities were formed by extracts prepared from B lymphoid lines representing different stages of maturation. The apparent stage-specific differences are shown to be due to proteolytic events that occurred during extract preparation.

The electrophoretic mobility shift assay is a sensitive method used in many laboratories to identify factors which specifically bind to DNA sequences. Complexes formed when nuclear factors bind to a DNA fragment migrate with reduced mobility relative to the noncomplexed DNA fragment (4, 5). This assay has been used extensively in studies of the regulatory regions of the immunoglobulin genes (1, 6, 9, 17, 18, 20-24, 26). We have also been using the assay to examine immunoglobulin gene sequences located at the 3' end of the μ heavy chain gene for potential regulatory regions involved in transcription termination (11, 19). Because studies of rRNA transcription have indicated that DNA-binding proteins play a role in termination of transcription of those genes (8), it was of interest to determine whether proteins could be identified which bind specifically to sequences near the termination site(s) in the μ - δ gene. Four factor-binding sites have been identified; they will be described later in detail (manuscript submitted). Here I report an important observation made during these experiments which indicates that some of the DNA-protein complexes detected by the electrophoretic mobility shift assay result from alterations of DNA-binding factors which occur during extract preparation.

B lymphomas were used as representatives of B lymphocytes, and hybridomas and myelomas were used as representatives of activated B lymphocytes. Nuclei were prepared by homogenizing washed cells in a buffer consisting of 25 mM Tris hydrochloride, 25 mM KCl, 7.5 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% or 0.1% Nonidet P-40, and 30% sucrose, pH 7.5. The nuclei were pelleted by centrifugation at $1,700 \times g$ for 15 min, and nuclear proteins were extracted with 0.4 M KCl-10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9)-1.5 mM $MgCl_2$ -0.1 mM EDTA-0.5 mM dithiothreitol-0.5 mM PMSF-5% glycerol-0.3 M sucrose for 30 min at 4°C. The extracts were dialyzed for 5 h against 20 mM HEPES (pH 7.9)-75 mM KCl-0.1 mM EDTA-0.5 mM dithiothreitol-0.2 mM PMSF-20% glycerol. After centrifugation at $12,000 \times g$, the extracts were frozen in liquid nitrogen and stored at -80°C. For the binding assay, 3 to 5 μ g of protein was incubated with 0.2 to 2 ng of radiolabeled DNA and 1 to 8 μ g of poly(dI-dC) in a 20- μ l volume for 30 min at 20°C (20). The reaction mixtures were analyzed on 4% polyacrylamide gels cast in 25 mM Tris borate-0.5 mM EDTA, pH 8.2. Incubation of nuclear extracts with a DNA fragment isolated from the 3' end of the μ gene (fragment A, Fig. 1E) produced complexes that migrated with reduced

mobility (Fig. 1A). The pattern of complexes observed differed depending upon whether the extract was prepared from lymphoma cell lines or from hybridoma and myeloma cell lines. Two bands showing retarded mobility were observed when the DNA fragment was incubated with the lymphoma extracts (Fig. 1A, lanes 1 and 2), whereas three additional, more rapidly migrating complexes were observed when the fragment was incubated with hybridoma or myeloma extracts (Fig. 1A, lanes 4 to 6). These results suggested that activated B lymphocytes have different DNA-binding factors that interact with a DNA sequence located at the 3' end of the μ gene. To determine whether the change in DNA-binding factors was induced by activation of B lymphocytes, I made use of the ability of the B lymphoma X16c8.5 to undergo activation events when treated with the mitogen lipopolysaccharide (LPS) (12). Incubation of the DNA fragment with nuclear extract prepared from the mitogen-treated lymphoma produced complexes similar to those produced by the hybridoma and myeloma extracts (Fig. 1A, lane 3).

Similar results were obtained with a DNA fragment located approximately 550 base pairs (bp) downstream of the μ_m2 exon (fragment C, Fig. 1E). A single complex of low mobility was observed when the fragment was incubated with lymphoma extracts (Fig. 1B, lanes 1 and 2), and two additional, more rapidly migrating complexes were observed when the fragment was incubated with hybridoma, myeloma, or LPS-treated X16c8.5 extracts (Fig. 1B, lanes 3 to 6).

These results suggested that there was an activation-dependent alteration in the DNA-binding factors that interact with DNA sequences located at the 3' end of the μ gene. However, additional experiments suggested an alternative interpretation of the data. First, analogous results were obtained with a variety of other DNA fragments that formed specific complexes. These fragments included two additional fragments isolated from the 3' end of the μ gene (data not shown), a fragment isolated from the enhancer region of the μ gene (fragment C, Fig. 1C, lanes 1 to 4), and a fragment isolated from the promoter region of the *I-A β* gene of the d haplotype (Fig. 1D, lanes 1 to 4). All of these DNA fragments showed similar types of DNA-protein complex formation when extracts from the two cell types were compared; that is, the lymphoma extracts produced a limited number of low-mobility bands, whereas the hybridoma and myeloma extracts produced multiple bands of greater mobility. Moreover, examination of the complexes formed by the fragment

FIG. 1. Electrophoretic mobility shift assay. End-labeled DNA fragments were incubated with nuclear extracts and analyzed on 4% polyacrylamide gels. The stars mark the noncomplexed DNA fragments. (A) Analysis of the μ_m 2 exon fragment. A 154-bp fragment extending from a *RsaI* site within the μ_m 2 exon to a *HincII* site located 3' of the exon (lanes 1, 2, and 3) or a 203-bp fragment extending from the *PvuII* site within the exon to a *RsaI* site located 3' of the exon (lanes 4, 5, and 6) was used. The 154-bp fragment and the 204-bp fragment contain 18 and 35 bp of polylinker sequence, respectively. Both fragments contain the same binding site. The nuclear extracts were prepared from the B cell lymphomas 38C-13 (lane 1), X16c8.5 (lane 2), and X16c8.5 cultured for 46 h with 10 μ g of LPS per ml; from the hybridoma D2 (lane 4); and from the myelomas MOPC 104E (lane 5) and MPC 11 (lane 6). (B) Analysis of a DNA fragment isolated from a region 3' of the μ_2 membrane exon. A 124-bp fragment extending from a *HaeIII* to the *HindIII* site located 547 bp 3' of the μ_2 membrane exon was used. The nuclear extracts were from 38C-13 (lane 1), X16c8.5 (lane 2), X16c8.5 cultured for 46 h with 10 μ g of LPS per ml (lane 3), MOPC 104E (lane 4), D2 (lane 5), D2 extract prepared from cells which were hypotonically lysed (lane 6), D2 extract prepared in the presence of pepstatin (lane 7), and D2 extract prepared in the presence of leupeptin (lane 8). (C) Analysis of the immunoglobulin H (IgH) enhancer. A 194-bp *PstI-HinI* DNA fragment from the IgH enhancer region was used. The nuclear extracts were from 38C-13, D2, X16c8.5, and X16c8.5 cultured for 46 h with LPS (lanes 1 to 4, respectively). (D) Analysis of a *I-A_B* promoter fragment. A 320-bp fragment isolated from the promoter region of the *I-A_B* gene of the d haplotype (A. Celada, M. Shiga, M. Imagawa, J. Kop, and R. Maki, manuscript submitted for publication). Nuclear extracts are as described in panel C. (E) Map of the μ heavy chain gene. The solid blocks on the horizontal line represent exons, the diamonds indicate the locations of the polyadenylation sites, and the solid blocks below the line labeled A, B, and C show the approximate locations of the fragments used in panels A, B, and C, respectively.

containing the *I-A_B* promoter region suggested a likely explanation for the differences observed between the extracts. A discrete band of retarded mobility was observed when this fragment was incubated with lymphoma extracts, whereas a smeared band pattern was seen when the fragment was incubated with the hybridoma extract or the extract isolated from LPS-treated X16c8.5 (Fig. 1D, lanes 1 to 4). This observation suggested that extracts prepared from activated B cells may contain larger amounts of proteases and raised the possibility that the additional, more rapidly migrating complexes formed between the other DNA fragments and factors present in the activated B cell extracts might result from proteolytic activity present in the extract. Indeed, DNA methylation protection experiments indicated that there were no differences in the footprints of the multiple complexes formed between the hybridoma extract and fragment A (Fig. 2).

Several experiments were performed to determine whether the observed difference reflected real differences between the DNA-binding proteins of the two cell types or whether the differences arose during extract preparation or factor binding. A second method of extract preparation was used that employed hypotonic cell lysis (3) rather than non-ionic detergent cell lysis. The rationale behind using this approach was that the hypotonic method of cell lysis may be less likely to release proteases sequestered in lysosomes or

other membrane-enclosed structures. Incubation of DNA fragments with hybridoma extract prepared by this method produced a pattern nearly identical to the complex pattern produced by the lymphoma extract (Fig. 1B, lane 6). Similar results were obtained when three other DNA fragments were assayed with these extracts (data not shown). This finding indicated that the majority of the smaller forms of the DNA-binding proteins present in extracts prepared from detergent-lysed cells were not present in intact hybridoma cells and strongly suggested that limited cleavage of DNA-binding proteins occurred during extract preparation. This was verified by preparing extracts from detergent-lysed hybridoma cells in the presence of pepstatin A, an inhibitor of aspartic proteases, or leupeptin, an inhibitor of cysteine proteases and some serine proteases. Inclusion of 10 μ g of pepstatin A per ml in the lysis and extraction buffers and 1 μ g/ml in the dialysis buffer had little or no effect on the complexes observed, whereas inclusion of the same concentrations of leupeptin changed the hybridoma pattern of complexes such that it resembled the lymphoma pattern of complexes (Fig. 1B, lanes 7 and 8). Similar results were observed when the three other DNA fragments isolated from the 3' end of the μ gene were assayed (data not shown). Active proteases were apparently not present in stored extracts, since the addition of leupeptin to a hybridoma extract-binding reaction did not affect the band pattern.

FIG. 2. Methylation protection analysis of the μ_m2 exon fragment-factor complex. (A) Non-coding strand analysis. D2 nuclear extract (lanes 1 to 4) and lymphoma WEHI 231 nuclear extract, which produces the same mobility shift pattern as do 38C-13 and X16c8.5 (lanes 5 to 6), were incubated with the 154-bp fragment, treated with dimethylsulfate, and electrophoresed on 4% polyacrylamide gels. Free and complexed DNA fragments were eluted from the gel, treated with piperidine, and analyzed on an 8% polyacrylamide sequencing gel. The doublet bands of complex 1 and of complex 2 (Fig. 1A) were combined for these analyses. Lanes: M, *Msp*I-digested pBR322 marker fragments; 1 and 5, noncomplexed DNA fragment; 2, complex 3; 3, complex 2; 4 and 6, complex 1. Numbers at the left indicate the lengths (in nucleotides) of the marker fragments. Arrows indicate the positions of bands which were underrepresented in the complexed DNA. No other underrepresented bands were observed. (B) Coding strand analysis. Preparative binding reactions were carried out with D2 extract and the 203-bp fragment. Free DNA and complexed DNA were prepared as described in panel A and analyzed on a 6% sequencing gel. Lanes: M, *Msp*I-digested pBR322 marker fragments; 7, free DNA; 8, complex 3; 9, complex 2; 10, complex 1. (C) Sequence surrounding residues protected from methylation. The G residues protected from methylation by complex formation are highlighted with asterisks. The μ_m polyadenylation signal is underlined.

Moreover, binding studies conducted with mixed extracts also indicated that little protease activity remained after the extract had been stored (data not shown).

These results indicate that limited proteolytic cleavage of DNA-binding proteins can occur during extract preparation and suggest that some of the DNA-binding factors of B lymphoid cells that have been thought to be cell type or stage specific may, in fact, result from differences in the proteases present in the cells being compared (1, 16–18). Our results indicate that the amount of protease activity is more abundant in hybridomas and myelomas than in lymphomas, a result concordant with previous studies showing that B lymphoid cells have increased levels of protease activity after mitogen-induced activation (13–15). Although the hypotonic method of cell lysis, the most commonly used procedure, decreased the amount of the more rapidly migrating complexes, the hybridoma extracts prepared by this procedure still showed a low, but easily discernible, level of these complexes (Fig. 1B, lane 6). Presumably, minor variations in experimental procedures could alter the relative

amount of proteolytic cleavage that occurs during extraction such that the cleaved binding proteins which form the smaller complexes become more or less abundant. Addition of the protease inhibitor leupeptin proved to be an effective way of preventing limited cleavage of the four DNA-binding proteins examined. Even with long autoradiography exposures, the major, more rapidly migrating complexes previously observed in extracts prepared from detergent-lysed hybridoma cells were not observed. A few other minor bands were seen with extracts prepared in the presence of leupeptin. These bands may arise from the action of other proteases not inhibited by PMSF or leupeptin which may be present in low amounts in these cell types. While it is possible that some of the DNA-binding factors which produce the more rapidly migrating complexes preexist in these cells and play a role, the results presented here suggest that caution should be exercised in interpreting stage-specific DNA-binding factors in B lymphoid cells. These results also raise the possibility that some of the multiple bands commonly seen in electrophoretic shift assays using extracts from other cell types might arise during extract preparation.

These results also provide potentially important information about the structure of these DNA-binding proteins. That is, these proteins appear to have a domain structure, and the DNA-binding domain maintains its binding properties in the absence of other domains. This is reminiscent of the properties of several well-characterized regulatory molecules, including the prokaryotic LexA protein, the yeast Gln4 protein, and steroid-type receptor molecules (2, 7, 10).

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