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Lipopolysaccharide-Unresponsive Mutant Pre-B-Cell Lines Blocked in NF-κB Activation

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NF-κB activation is a crucial late step in the induction of immunoglobulin κ light-chain gene expression in pre-B cells by lipopolysaccharide (LPS). We have analyzed NF-κB activation in three independent mutant lines of 70Z/3 pre-B cells which are unresponsive to LPS. All three variant cell lines failed to activate NF-κB when induced with LPS or the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate. However, all three cell lines contained functional NF-κB, as revealed by detergent treatment of cytoplasmic extracts. Moreover, cycloheximide induced limited activation of NF-κB comparable to that in wild-type 70Z/3 pre-B cells in two of the three variant lines. These results indicate that the mutations blocking κ gene induction in these variant 70Z/3 pre-B-cell lines affect NF-κB activation.

The κ genes in 70Z/3 pre-B cells are activated and transcribed in response to bacterial lipopolysaccharide (LPS), interleukin-1 (IL-1), gamma interferon (IFN-γ), and various physiological inducers which mimic different intracellular second messengers (6, 13, 18, 20; see also the review in reference 9). LPS, IL-1, PMA (12-*O*-tetradecanoylphorbol 13-acetate), and cyclic AMP analogs activate NF-κB transcription factor binding to the B motif in the κ enhancer (11, 14, 16, 18, 19). This factor exists in the cytoplasm of cells in an inactive state complexed to a labile inhibitor called IκB (2, 3). NF-κB activation is postulated to occur through protein kinase-mediated phosphorylation of the labile IκB inhibitor (3, 16, 17, 19). Distinct intracellular signalling pathways, involving either protein kinase C (activated by LPS or PMA) or cyclic AMP-dependent protein kinases (activated by IL-1), appear to converge at the activation of the NF-κB transcription factor (18, 19). NF-κB is also activated by protein synthesis inhibitors, presumably through depletion of the labile IκB inhibitor (2, 3, 17, 20). IFN-γ induction of κ transcription occurs through a different molecular mechanism which does not involve NF-κB activation (4, 10).

We have been studying LPS-unresponsive genetic variants of 70Z/3 pre-B cells to further define the molecular events and processes in κ light-chain gene activation. Here we report the characterization of a new LPS-unresponsive cell line called 70Z/3B, along with findings on κ induction and NF-κB activity in two previously reported variants 70Z/3 cell lines, 1.3E2 and 1B11-15 (12, 21). All three of these variant pre-B-cell lines failed to initiate κ expression because of a block in NF-κB activation.

LPS-unresponsive mutant 70Z/3 cell lines also do not respond to other κ inducers which activate NF-κB. We first analyzed the induction of κ light chains in 70Z/3B and the other two LPS-unresponsive lines treated with LPS, IL-1, NZB-F (i.e., an activity in the serum of young NZB mice [9, 10]), and IFN-γ (Table 1). Induction of cell surface κ expression was assayed by flow cytometry with fluorescein isothiocyanate-labeled goat anti-κ (10). Wild-type 70Z/3 cells treated with these inducers were 77 to 90% κ positive after 24 h of induction. All three LPS-unresponsive variant lines failed to increase κ expression in response to LPS, IL-1, or NZB-F. Uninduced variant lines had <5% κ-positive cells, and this result was not changed by any of the three inducers. Since LPS and IL-1 activate NF-κB through independent cell surface receptors and different intracellular signalling pathways (6, 15, 18, 19), the concomitant loss of responsiveness to both inducers suggests that the mutations in the variant cells affect either the κ gene or some common step in κ gene induction. The mechanism of κ induction of NZB-F is not known, but it likely involves NF-κB activation (10).

IFN-γ induced κ expression in wild-type 70Z/3 cells and 1.3E2 variant cells but not in 70Z/3B or 1B11-15 variant cells. IFN-γ treatment resulted in >80% κ-positive wild-type 70Z/3 cells and 1.3E2 variant cells. The level of κ-positive 70Z/3B and 1.B11-15 variant cells (<5%) was unchanged by IFN-γ induction. Accordingly, the new 70Z/3B variant line resembles the previously reported 1B11-15 line (12) in that it is defective in both the NF-κB- and the IFN-γ-stimulated pathways for κ induction.

The rearranged 70Z/3B κ gene is not defective. To determine if the rearranged 70Z/3B κ gene might be defective, we constructed cell hybrids between 70Z/3B variant cells and 4T00 (a line of MPC-11 myeloma cells which constitutively expresses NF-κB [17]). The κ light chain induced in 70Z/3 cells by LPS is readily distinguished from the larger full-length κ light chain made in MPC-11 cells (8, 20). 70Z/3B × 4T00 hybrid cell (Fig. 1, lane 2) expressed the MPC-11 κ light chain along with an new κ light chain (with the same mobility as the LPS-induced 70Z/3 κ light chain) not present in either uninduced 70Z/3B variant pre-B cells (lane 1) or 4T00 myeloma cells (lane 3). This finding establishes that the 70Z/3B κ gene can be activated and expressed in response to NF-κB. We could not distinguish whether transcription of the 70Z/3B κ gene was initiated by the constitutively expressed NF-κB factor from the myeloma cells or by the NF-κB factor from the pre-B cells activated by an element in myeloma cells which turns on NF-κB (1). Nonetheless, since

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TABLE 1. Induction of κ light-chain expression in wild-type and variant 70Z/3 pre-B cells

Cell line	Induction of κ expression[a] by:			
	LPS	IL-1	NZB-F	IFN-γ
70Z/3	+	+	+	+
1.3E2	−	−	−	+
1B11-15	−	−	−	−
70Z/3B	−	−	−	−

[a] Conditions for κ induction by different inducers and flow cytometry assays for κ-expressing cells were as previously described (10). −, <5% κ-positive cells; +, 77 to 90% κ-positive cells. Uninduced wild-type 70Z/3 cells had <5% κ-positive cells.

the 70Z/3B κ gene can be activated by NF-κB, this variant pre-B-cell line must be defective in a process or factor required for κ gene activation.

Variant cells contain functional NF-κB. Next, we analyzed 70Z/3B and the other two variant lines for NF-κB by using deoxycholate–Nonidet P-40 detergent treatment of uninduced cell extracts (2, 3). Nuclear and cytoplasmic extracts prepared from wild-type 70Z/3 and each of the three variant cell lines were examined for NF-κB binding in a gel mobility shift assay with a labeled DNA probe from the κ enhancer containing the NF-κB-binding site (16, 17). The results obtained with wild-type 70Z/3 and variant 70Z/3B cytoplasmic extracts are shown in Fig. 2. Equivalent NF-κB-binding activity was seen in detergent-treated cytoplasmic extracts from uninduced wild-type 70Z/3 and variant 70Z/3B cells (compare lanes 5 and 8). The mobility of the shifted band from the detergent-treated 70Z/3B extract was indistinguishable from the seen in the detergent-treated wild-type 70Z/3 cytoplasmic extract and in the nuclear extract from wild-type 70Z/3 cells induced with LPS (lanes 3, 5, and 8). The detergent-activated complexes also were abolished by a duplex competitor DNA oligonucleotide composed of the NF-κB motif (lanes 6 and 9). These features confirm that the binding activity in 70Z/3B extracts involves NF-κB. Detergent treatment of cytoplasmic extracts from the remaining two mutant 70Z/3 lines gave results similar to those shown for 70Z/3B (results not shown). Detergent-treated nuclear extracts from uninduced wild-type cells or from any of the variant lines did not contain significant amounts of NF-κB (results not shown). These results establish that all three variant lines contain covert cytoplasmic NF-κB which is indistinguishable in binding assays from the LPS-activated factor in the wild-type 70Z/3 cell line.

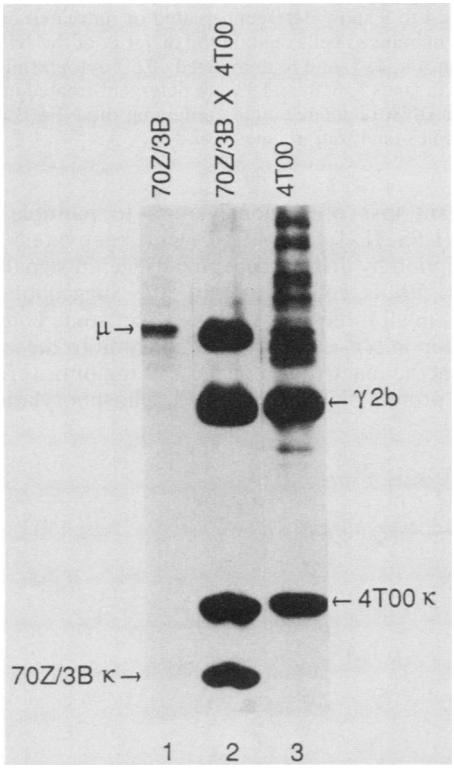

FIG. 1. Activation of 70Z/3 κ expression by fusion of 70Z/3 and 4T00. Somatic cell hybrids were generated by fusing thymidine kinase-negative 70Z/3B pre-B cells to hypoxanthine phosphoribosyltransferase-negative 4T00 myeloma cells (immunoglobulin γ2b, κ). Cytoplasmic extracts from cells labeled for 2 h with [^{35}S] methionine were precipitated with rabbit antisera specific for murine μ, γ2b, and κ chains, reduced, and analyzed by polyacrylamide gel electrophoresis on an 11% polyacrylamide gel. Six independent hybrid lines analyzed gave results identical to those for the one hybrid line shown here. Lanes: 1, 70Z/3; 2, hybrid 70Z/3B × 4T00; 3, 4T00. The more rapidly migrating κ chain (denoted 70Z/3B κ) has the mobility of the κ light chain induced by LPS in wild-type 70Z/3 cells (8, 20).

NF-κB is not activated in variant cells treated with LPS or PMA. The findings suggest that the variant cells are defective in the activation of NF-κB. To test this, we analyzed NF-κB activity in nuclear extracts prepared from wild-type and variant cells induced with LPS, PMA, or cycloheximide. These inducers were chosen because they act at different points in NF-κB activation. Figure 3 compares NF-κB-binding activities in gel mobility shift assays between wild-type 70Z/3 nuclear extracts and variant 70Z/3B nuclear extracts. In wild-type 70Z/3 cells, both LPS (lane 3) and PMA (lane 4) showed strong induction of NF-κB, while cycloheximide produced a slight induction of NF-κB (lane 5). Cycloheximide is a relatively inefficient inducer of NF-κB in comparison with LPS or PMA (17, 20). In variant 70Z/3B cells, neither LPS (lane 7) nor PMA (lane 8) induced NF-κB binding. However, cycloheximide treatment of 70Z/3B cells produced moderate activation of NF-κB binding (lane 9) comparable to that seen in cycloheximide-treated wild-type cells (lane 5). The cycloheximide concentration which activated NF-κB binding also stimulated comparable increases in κ transcription (i.e., two- to threefold) in run-on transcription assays in isolated nuclei (5, 20) from treated 70Z/3 and 70Z/3B cells (results not shown). Cycloheximide (but not LPS or PMA) also induced a modest activation of NF-κB binding in variant 1B11-15 cells (result not shown). The 1.3E2 variant line exhibited a low basal level of apparent NF-κB binding, even without induction, which was not detectably increased by any inducer, including cycloheximide (results not shown). The apparent failure of cycloheximide to increase NF-κB binding in these latter cells may reflect the inability of the gel mobility shift assay to detect small increases in NF-κB binding. These results confirm that all three variant cell lines are defective in NF-κB activation. Cycloheximide, which presumably activates NF-κB directly by causing turnover of the IκB inhibitor (2, 3), apparently bypasses the activation defects in the 70Z/3B and 1B11-15 variant cell lines.

Conclusions. The three unresponsive mutant 70Z/3 cell lines described here all appear likely to be defective in intracellular processes or activities involved in the inactivation of IκB and the release of active NF-κB. This conclusion is supported by the demonstration of functional NF-κB

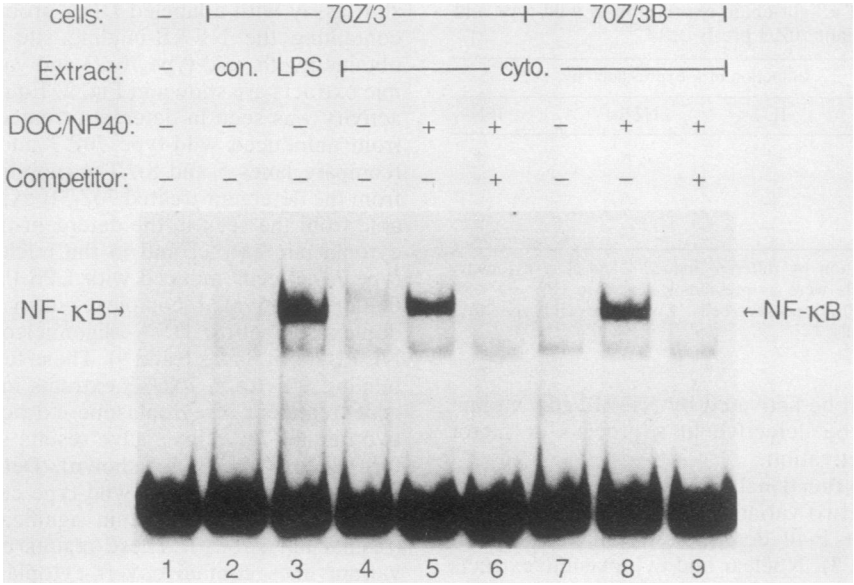

FIG. 2. Activation of NF-κB binding by detergent treatment of 70Z/3 and 70Z/3B cell extracts. Nuclear and cytoplasmic extracts were prepared as described by Dignam et al. (5). Electrophoretic gel mobility shift assays were performed on nuclear and cytoplasmic cell extracts incubated with a 70-nucleotide-long *Dde-Hae*III DNA fragment (16) endlabeled with polynucleotide kinase. Lanes 2 and 3 show untreated nuclear extracts from uninduced (con.) and LPS-induced (LPS) 70Z/3 cells. Lanes 4 to 9 show detergent-treated or untreated cytoplasmic extracts (cyto.) from uninduced 70Z/3 and 70Z/3B cells. Cytoplasmic extracts from uninduced cells contain the majority of the NF-κB (2, 3). Lanes: 1, no extract; 2, uninduced 70Z/3; 3, 70Z/3 induced with LPS at 10 μg/ml for 4 h; 4, 5, and 6, uninduced 70Z/3 cytoplasmic extracts; 7, 8, and 9, uninduced 70Z/3B cytoplasmic extracts. The samples for binding assays in lanes 5, 6, 8, and 9 were detergent-treated in vitro with 1% deoxycholate and 0.8% Nonidet P-40 (DOC/NP40) (2, 3). A duplex competitor DNA oligonucleotide containing the NF-κB motif (i.e., 5′-GCTGGGGACTTTCC-3′ [4]) was added at 100 ng per binding assay to the samples analyzed in lanes 6 and 9.

activity in all three variant cell lines by in vitro detergent treatment of cytoplasmic extracts and by cycloheximide-activated NF-κB binding and κ transcription comparable to that in wild-type 70Z/3 cells in two of the variant lines. The mutations in these independently derived variant pre-B cells may affect a common step in NF-κB activation. The 1B11-15 and 1.3E2 mutant lines have been reported to be in the same complementation group for LPS induction (12, 21). The concomitant loss of responsiveness to multiple different inducers (LPS, IL-1, and NZB-F) which activate NF-κB and κ transcription by distinct receptor-triggered signalling pathways (18, 19) is consistent with the suggestion that the mutations in all three variant lines affect some common late step. A step in NF-κB activation common to these different inducers is the inactivation of the IκB inhibitor (presumably through protein kinase-mediated phosphorylation). Re-

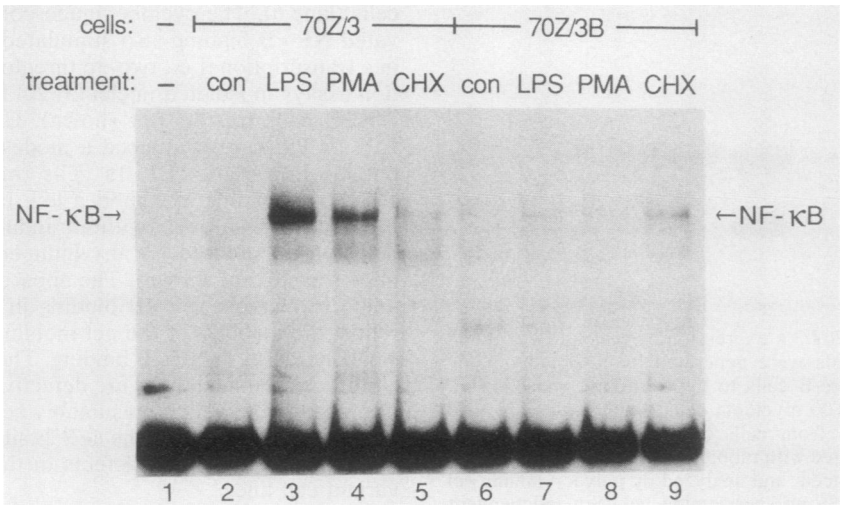

FIG. 3. Activation of NF-κB in LPS-, PMA-, and cycloheximide (CHX)-treated 70Z/3 and 70Z/3B cells. DNA factor-binding reactions were carried out as described in the legend to Fig. 2 with equivalent concentrations of 70Z/3 and 70Z/3B nuclear extracts. Lanes: 1, no extract; 2, uninduced 70Z/3 (con); 3, 70Z/3 induced with LPS at 10 μg/ml for 4 h; 4, 70Z/3 induced with PMA at 50 ng/ml for 1 h; 5, 70Z/3 induced with cycloheximide at 10 μg/ml for 4 h; 6, uninduced 70Z/3B; 7, 70Z/3B induced with LPS at 10 μg/ml for 4 h; 8, 70Z/3B induced with PMA at 50 ng/ml for 1 h; 9, 70Z/3B induced with cycloheximide at 10 μg/ml for 4 h.

cently, it was reported that the addition of the catalytic subunits of protein kinase C or A led to the activation of NF-κB in cytoplasmic extracts of wild-type 70Z/3 cells (19). This finding suggests that a single regulatory factor in NF-κB activation (i.e., IκB) may be phosphorylated by different protein kinases in a manner analogous to that of the CREB transcription factor (7). Further studies on these mutant pre-B-cell lines examining in vitro activation by the active subunits of protein kinases should resolve the nature of the activation defect(s) in these cells and help elucidate the molecular mechanisms in NF-κB activation.

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