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Maintenance of NF-κB Activity Is Dependent on Protein Synthesis and the Continuous Presence of External Stimuli

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The activation of NF-κB-like activities (called NF-κB) by tumor necrosis factor alpha (TNFα) and the phorbol ester phorbol 12-myristate 13-acetate (PMA) were compared. High levels of NF-κB activity were found 2 to 4 min after TNFα addition to human HL60 cells and lasted for at least 3 h, although the half-life of active NF-κB was less than 30 min. Inactive NF-κB, however, was relatively stable. NF-κB activation by TNFα was initially cycloheximide insensitive, but maintenance of NF-κB activity required ongoing protein synthesis and continuous stimulation by TNFα. Thus, the cells did not remain in an activated state without stimulation. In HL60 cells, NF-κB induction by PMA required 30 to 45 min and was completely dependent on de novo protein synthesis, while PMA (and interleukin-1) induced NF-κB activity rapidly in mouse 70Z/3 cells via a protein synthesis-independent mechanism. The NF-κB-like activities obtained under each condition behaved identically in methylation interference and native proteolytic fingerprinting assays. The NF-κB-like factors induced are thus all very similar or identical. We suggest that cell-specific differences in the protein kinase C-dependent activation of NF-κB may exist and that TNFα and PMA may induce expression of the gene(s) encoding NF-κB.

NF-κB (for reviews, see references 14, 15, and 23) was discovered as a B-cell-specific transcription factor that bound to an enhancer sequence located in the J-C intron of the κ light chain (22, 30, 31). NF-κB was shown either to be constitutively active, as in mature B lymphocytes (30, 31) and in monocytes and macrophages (16), or to be present in an inactive complex with an inhibitor protein, IκB (1). Inactive NF-κB could be activated by a posttranslational event involving stimulation of cells with bacterial lipopolysaccharide, phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) (31), which induce protein kinase C (5, 27, 34), or cytokines such as tumor necrosis factor alpha (TNFα), TNFβ, or interleukin-1 (IL-1) (19, 28). Active NF-κB can also be obtained from the NF-κB/IκB complex by treatment with sodium deoxycholate or formamide (1, 2) or by phosphorylation of IκB by using different protein kinases (12). Thus, activation of NF-κB apparently involves dissociation of IκB from its cytoplasmic complex with NF-κB and nuclear translocation of the active transcription factor (2).

Active NF-κB consists of two molecules of a DNA-binding subunit of 50 kDa associated with two molecules of non-DNA-binding 65-kDa protein. The 65-kDa protein is required for inactivation by IκB (3). The gene encoding the DNA-binding protein was recently isolated and shown to be identical to KBF1. The amino-terminal half of the 105- to 107-kDa open reading frame encodes the 50-kDa DNA-binding subunit and shows a high homology to the v-*rel* oncogene, its cellular homolog c-*rel*, and the *Drosophila* maternal morphogen dorsal. The exact function of the carboxy-terminal part of the open reading frame is still unclear (13, 21, 25a).

NF-κB recognizes the 11-bp sequence GGGGACTTTCC and some slightly variant sequences, which were found in several primate virus enhancers and cellular genes (reviewed in references 15 and 23). NF-κB is involved in transcriptional activation of, for example, the κ light chain, the interleukin-2 receptor α gene (6, 24), and the β-interferon gene (33) and in the activation of human immunodeficiency virus gene expression (11, 16, 26). Potential NF-κB binding sites are also present in the 5′-flanking regions of a number of other genes (33), where they are also likely to be involved in the transcriptional regulation of gene expression.

We are especially interested in the mechanism of NF-κB activation by TNFα. We showed that two different TNF receptors exist and that these receptors have slightly different affinities for TNFα (7, 18) or TNFβ (19). The degree of binding of TNFα and TNFβ to both TNF receptors correlated well with the amount of activation of NF-κB. High levels of active NF-κB were seen upon binding of TNFα to less than a few hundred TNF receptors, representing maximally 20 to 25% of all TNF receptors on HL60 and HEp2 cells, and were maximal 2 to 4 min after addition of TNFα (19). Both TNF receptors mediate activation of NF-κB by TNFα (17).

In this report, we analyze the mechanisms of NF-κB activation by TNFα and PMA in more detail. We show that two phases of NF-κB activation could be distinguished in HL60 cells. During the first phase, which is independent of de novo protein synthesis, NF-κB is obtained from a preformed NF-κB/IκB complex. In the second phase, the pool of inactive preexisting NF-κB might be exhausted and active NF-κB is obtained by de novo protein synthesis. The first phase of NF-κB activation is obtained in HL60 cells after stimulation by TNFα but not by PMA, while the second phase was seen with both TNFα and PMA. In addition, we show that cells contain NF-κB activity only during treatment with TNFα.

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MATERIALS AND METHODS

Reagents. Stock solutions of PMA (2 mM in dimethyl sulfoxide; Fluka) and cycloheximide (10 mg/ml in H_2O; Fluka) were used. Recombinant TNFα (18) and IL-1 were obtained from E. Hochuli (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and P. Lomedico (F. Hoffmann-La Roche Ltd., Nutley, N.J.), respectively. Antiserum to TNFα was a gift of M. Brockhaus (F. Hoffmann-La Roche Ltd., Basel, Switzerland). The serum was used at a 1:100 dilution.

Cell culture. The human promyelocytic cell line HL60, the human epithelial cell line HeLa, and the mouse pre-B lymphocytic cell line 70Z/3 were obtained from the American Type Culture Collection and maintained over many passages in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum or 10% (vol/vol) horse serum. 70Z/3 cells were cultivated in the presence of 50 nM mercaptoethanol (Fluka). All cells were used after reaching near confluence or a density of 1×10^6 to 1.5×10^6 cells per ml of culture medium.

Preparation of nuclear extracts and EMSA. Nuclear extracts were prepared essentially as described previously (10). HL60 cells were harvested by centrifugation for 5 min at $1,500 \times g$ and were washed once with phosphate-buffered saline. About 2×10^7 cells were resuspended with 500 μl of a hypotonic lysis buffer (buffer A [10]). After 20 min, the cells were homogenized by 20 strokes with a loose-fitting Dounce homogenizer. Nuclei were collected by centrifugation for 4 min at 6,500 rpm in a microfuge (~$4,000 \times g$), and the proteins were extracted with 3 packed pellet volumes of high-salt buffer (buffer C [10]). After 60 min, the samples were centrifuged as described above. The high-salt extracts were diluted with 3 volumes of low-salt buffer D (10) containing 1% Nonidet P-40 and were used immediately for electrophoretic mobility shift assays (EMSAs) or kept frozen. EMSA was performed as described previously (30). About 5,000 cpm of a ^{32}P-end-labeled *Dde*I-*Hae*III fragment of the κ light-chain enhancer containing the NF-κB binding site was used per assay. A restriction fragment mutated in the NF-κB binding site but otherwise identical to the wild-type fragment (22) was used as a negative control.

Native proteolytic fingerprinting of active NF-κB in nuclear extracts and methylation interference assays. Nuclear extracts of mouse bone marrow macrophages were prepared as described above either without addition of protease inhibitors or in the presence of a cocktail of the following inhibitors: 10 mM benzamidine, 100 U of aprotinin per ml, 10 μM leupeptin, 1 μM pepstatin, 1 mM *o*-phenanthroline, and 1 mM phenylmethylsulfonyl fluoride. Mouse macrophage nuclear extract (3 μl) was mixed with 3 μl of nuclear extracts derived from different cell lines. After 30 min at room temperature, the mixture was applied to EMSA.

Methylation interference assays were performed as described previously (20).

RESULTS

The pool of preexisting and inactive NF-κB was rapidly exhausted in HL60 cells when protein synthesis was inhibited. How stable is active NF-κB and how does the level of active NF-κB change with time after addition of TNFα? TNFα was added to HL60 cells, samples were taken after various times, and the amount of active NF-κB in nuclear extracts was determined by EMSA with an oligonucleotide containing the intact NF-κB binding site of the κ light chain. As a control, a fragment mutated in the NF-κB binding site but otherwise identical to the wild-type oligonucleotide was used to identify nonspecifically binding proteins. Roughly similar levels of active NF-κB were seen upon incubation of HL60 cells with TNFα for 0.5 to at least 3.5 h (Fig. 1A, lanes 2 to 8; Fig. 1B, solid line). When cells were first incubated for 30 min with TNFα prior to cycloheximide addition and incubation was continued for various periods of time, the amount of active NF-κB declined rapidly, with a half-life of less than 30 min. Active NF-κB was virtually absent after incubation for more than 1.5 h with cycloheximide (Fig. 1A, lanes 9 to 15; Fig. 1B, broken line). Thus, active NF-κB is unstable.

Rapid activation of NF-κB was independent of ongoing protein synthesis in HL60 cells. No decrease in the amount of active NF-κB in nuclear extracts was observed when cells were pretreated with cycloheximide for 30 or 60 min prior to 30 min of incubation with TNFα, while some decrease could be seen after 2 h of preincubation (Fig. 1C, lanes 2 to 4 and 9 to 12). When cycloheximide pretreatment was followed by incubation with TNFα plus cycloheximide for more than 1 h, then as expected a drastic decrease in the amount of active NF-κB was seen (Fig. 1C, lanes 5 to 9), which resulted from the rapid turnover of active NF-κB (see above). Cycloheximide alone did not activate NF-κB to a significant degree in HL60 cells (Fig. 1C, lane 1), while NF-κB activation by cycloheximide was observed in 70Z/3 cells, as shown by others (see below). Thus, the cytosolic pool of preexisting NF-κB is rapidly exhausted after TNFα stimulation of HL60 cells, and maintenance of constant levels of active NF-κB over longer times requires de novo protein synthesis. Cytosolic extracts of unstimulated HL60 cells contained variable amounts of active NF-κB (not shown), while the presence of active NF-κB in nuclear extracts was strictly dependent on cell stimulation. Deoxycholate treatment of cytosolic fractions increased the amount of active NF-κB, directly confirming the existence of a cytosolic pool of inactive NF-κB in HL60 cells (not shown).

Maintenance of constant levels of active NF-κB required continuous stimulation of HL60 cells by TNFα. Roughly constant levels of NF-κB activity were seen for at least 3.5 h after addition of TNFα, and maintenance of this level required de novo protein synthesis in later stages (see above). Is the continuous stimulation by TNFα needed for maintenance of NF-κB activity? To answer this question, cells were incubated with TNFα in the presence or absence of a neutralizing antiserum to TNFα. This antiserum (Fig. 2, lane 4) but not rabbit control serum (Fig. 2, lane 3) prevented TNFα-mediated NF-κB activation when added together with TNFα. To test the potential requirement of continuous stimulation by TNFα, HL60 cells were first incubated with TNFα, followed 1 h later by addition of rabbit control serum or the neutralizing antiserum to TNFα. After an additional 50 min of incubation, cells were harvested and NF-κB activity in nuclear extracts was determined. Only nuclear extracts of cells treated with the antiserum to TNFα were devoid of active NF-κB (Fig. 2, lanes 5 and 6). Thus, in addition to ongoing protein synthesis (see above), continuous stimulation of cells by TNFα is required to maintain high levels of NF-κB activity.

Activation of NF-κB by PMA was slow in HL60 cells but rapid in 70Z/3 cells. Activation of NF-κB was very rapid and long lasting in HL60 cells when TNFα was present. NF-κB activity was already seen after 2 min of incubation with TNFα, was maximal after 5 min of incubation, and lasted for at least 3 h of incubation with TNFα (Fig. 3A, lanes 1 to 7). In contrast, activation of NF-κB by PMA required 30 min to

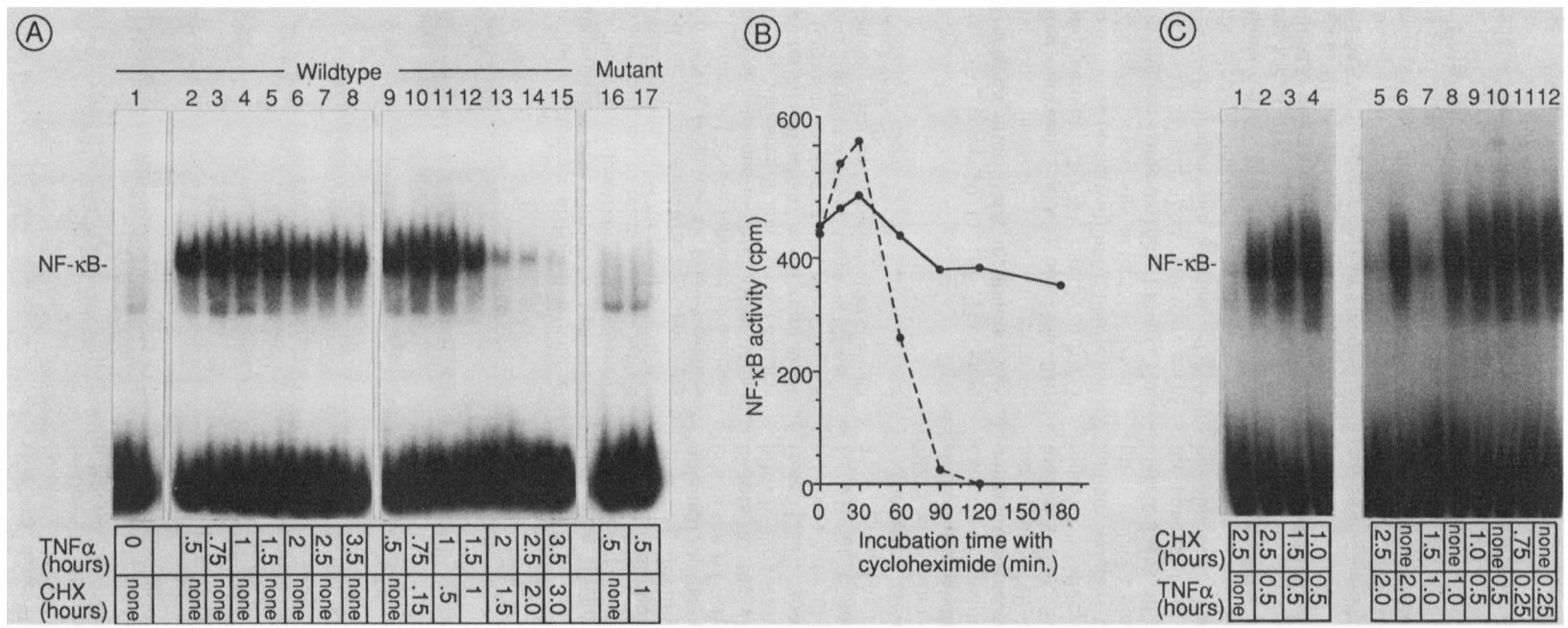

FIG. 1. Demonstration that rapid activation of NF-κB by TNFα is not inhibited by cycloheximide treatment but maintenance of NF-κB activation requires de novo protein synthesis. HL60 cells were incubated at 37°C with 10 nM TNFα. At this TNFα concentration, receptor saturation is reached (18). Samples were split after 30 min of incubation with TNFα. One half of the cells was incubated further without addition (A, lanes 1 to 8, 16; B, ——). To the other half, cycloheximide (CHX) was added to 20 μg/ml and incubation was continued for the indicated times (A, lanes 9 to 15 and 17; B, – – –). In another experiment, cells were preincubated for the indicated times with cycloheximide (20 μg/ml) and TNFα was added at the indicated times prior to cell harvesting (C, lanes 1 to 12). Samples were rapidly cooled in ice water, and NF-κB activity was measured in nuclear extracts by EMSA, using an oligonucleotide containing the wild-type binding site for NF-κB from the κ light chain (A, lanes 1 to 15) (30) or, as a control, an inactive mutated binding site that does not bind NF-κB (A, lanes 16 and 17) (22). Nuclear extracts obtained from equal cell aliquots were used in each lane. The position of the complexes between NF-κB and the DNA fragment is indicated (NF-κB). (B) For quantitation of the results presented in panel A, pieces of the gel containing the protein-DNA complexes were cut out and radioactivity was measured by Cerenkov counting.

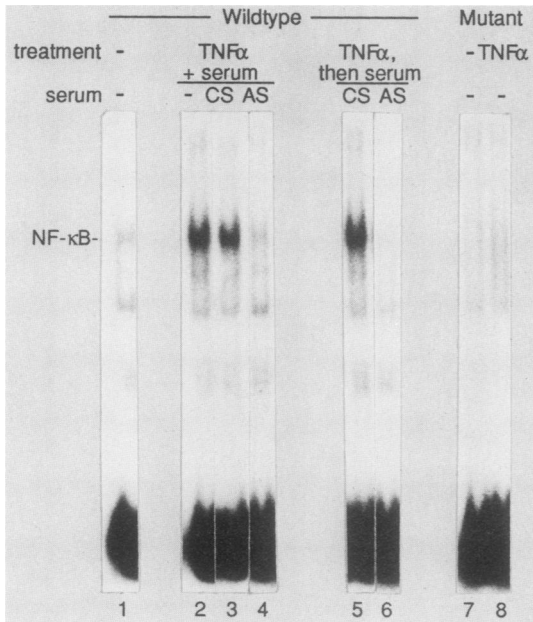

FIG. 2. Demonstration that maintenance of NF-κB activity requires continuous stimulation of HL60 cells with TNFα. Cells were incubated for 1 h at 37°C in the absence (lane 1) or presence (lane 2) of 5 pM TNFα, which is just sufficient to reach maximal NF-κB activation (19). The cells were also incubated for 1 h at 37°C with a mixture of 5 pM TNFα and control antiserum (CS; lane 3) or 5 pM TNFα and an antiserum to TNFα (AS; lane 4). Parallel cultures were first incubated for 1 h with 5 pM TNFα, followed by addition of control antiserum (lane 5) or antiserum to TNFα (lane 6), and incubation was continued for an additional 50 min. Nuclear extracts were prepared and assayed for NF-κB activity as described in the legend to Fig. 1. DNA fragments containing the intact NF-κB binding site (Wildtype; lanes 1 to 6) or a mutant fragment (Mutant; lanes 7 and 8) were used. Samples derived from equivalent amounts of cells were analyzed in each case.

1 h of incubation (Fig. 3A, lanes 10 to 16). The slow appearance of NF-κB activity after PMA incubation was specific for HL60 cells, since in 70Z/3 cells activation of NF-κB by PMA required between 5 and 15 min (Fig. 3B, lanes 10 and 11). IL-1 also activated NF-κB rapidly in 70Z/3 cells (Fig. 3B, lanes 1 to 6), while no activation by TNFα was found (not shown). 70Z/3 cells lacked any detectable levels of TNF receptors in binding experiments (not shown). The induced binding activities in HL60 and 70Z/3 cells were seen with use of the intact NF-κB binding site but not a DNA fragment containing a mutated binding site (Fig. 3A, lanes 8, 9, 17, and 18; Fig. 3B, lanes 7, 8, 15, and 16), thus confirming their NF-κB-like binding specificity.

NF-κB induction by PMA was completely dependent on ongoing protein synthesis in HL60 cells but not in 70Z/3 cells. Induction of NF-κB by PMA differed not only in kinetics in HL60 and 70Z/3 cells but also in cycloheximide sensitivity. A more precise analysis of the time course of NF-κB activation by PMA was performed. Active NF-κB was first detected 45 min after PMA addition to HL60 cells, was maximal after 1 h of incubation, decreased between 1 and 2 h of incubation, and then increased again and was detected for at least 4.5 h after PMA addition (Fig. 4A, lanes 2 to 11). The transient decrease was consistently observed (not shown). In contrast to NF-κB activation by TNFα (see above), no protein synthesis-independent activation of NF-κB by PMA was seen in HL60 cells. Simultaneous addition of cycloheximide and PMA to HL60 cells almost completely abolished the PMA-induced activation of NF-κB (Fig. 4A, lanes 12 to 19). However, when cells were first incubated with PMA for 30 min before addition of cycloheximide, NF-κB activation was observed only for up to 1.5 h of incubation with PMA, matching the initial stage of NF-κB activation seen with PMA only (Fig. 4A; compare lanes 5 to 7 with lanes 20 to 24). The later stage of NF-κB activation, after the transient decrease seen with PMA only, was not observed (Fig. 4A; compare lanes 8 to 11 with lanes 25 and 26). In 70Z/3 cells, preincubation for 30 min with cycloheximide prior to 30 min of incubation with PMA or IL-1 plus cycloheximide did not influence NF-κB activation (Fig. 4B; compare lanes 2 and 3 with lanes 5 and 6). However, only part of the NF-κB activation resulted from the stimulation with IL-1 or PMA, since cycloheximide alone also activated NF-κB in 70Z/3 cells (Fig. 4B, lanes 1 and 4). Thus, NF-κB activation by PMA is strictly dependent on ongoing protein synthesis in HL60 but not 70Z/3 cells.

The active NF-κB-like proteins obtained from HL60 and 70Z/3 cells after treatment with different stimuli showed identical methylation interference patterns. Methylation interference assays were performed by using PMA-induced NF-κB from mouse 70Z/3 cells, a condition of NF-κB activation similar to that originally described by Sen and Baltimore (31) for this cell line, and the TNFα-induced NF-κB from human HL60 cells. Only one DNA strand was analyzed. Identical patterns of guanidine contacts were obtained for the two factors. Methylation of three of the four G's in the NF-κB binding site interfered with binding of the factors to the DNA (Fig. 5). Thus, the TNFα-induced NF-κB-like factors induced here behave similarly and differ from H2TF1, another NF-κB-related protein, which closely interacts with all four G residues of the DNA sequence (4). NF-κB-like factors from PMA-stimulated 70Z/3 cells and TNFα-stimulated HL60 cells may therefore be homologous.

The active NF-κB-like proteins obtained from HL60 and 70Z/3 cells after treatment with different stimuli showed similar proteolytic fingerprints. Total lysates and nuclear extracts of unstimulated mouse bone marrow macrophages contain a protease that generates a shorter form of mouse macrophage NF-κB, which is still capable of specific binding to DNA (9a). To test whether the NF-κB-like proteins induced by different stimuli in HL60 and 70Z/3 cells were similar, nuclear extracts of each cell line were incubated prior to DNA binding with extracts of unstimulated mouse macrophages. If the active NF-κB-like proteins represent the same protein, then identical proteolytic fragments should be obtained. The mouse macrophage extract used was virtually devoid of endogenous NF-κB activity (Fig. 6A, lanes 5 and 10). A faster-migrating complex between DNA and NF-κB was seen upon incubation of nuclear extracts of TNFα-stimulated HL60 cells with the macrophage extract (Fig. 6A, lanes 3 and 4). The formation of the faster-migrating band was inhibited by a cocktail of protease inhibitors, indicating that this product is indeed derived from NF-κB by proteolysis (Fig. 6A, lanes 8 and 9). For unexplained reasons, the amount of radiolabeled DNA fragment present in the complex between proteolytic product and DNA was higher than that between intact NF-κB and the DNA (Fig. 6A and B). A small amount of proteolytic fragment was observed after addition of macrophage extract to nuclear extracts of unstimulated HL60 cells (Fig. 6A, lanes 1 and 2). This product may either be derived from the macrophage extract itself (Fig. 6A; compare lanes 2 and 5) or

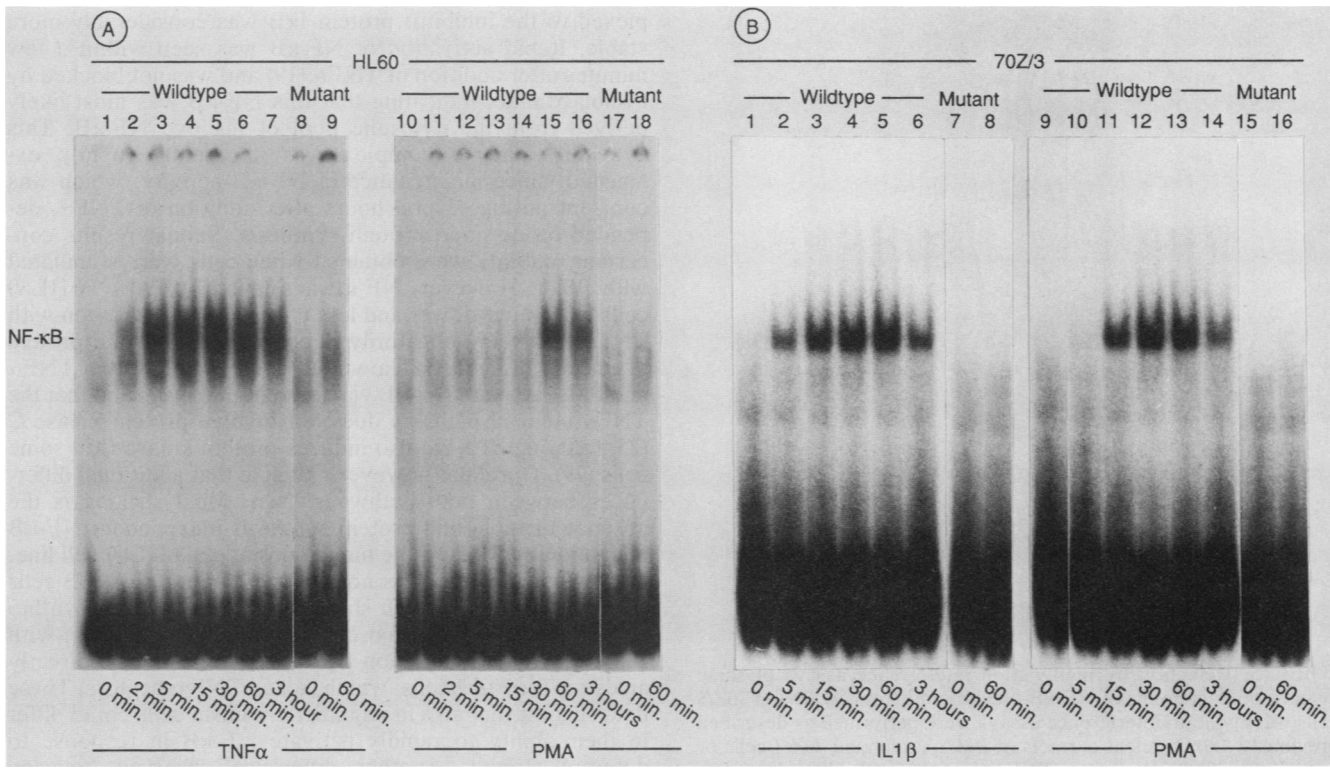

FIG. 3. Demonstration that the kinetics of NF-κB activation by PMA is slow in HL60 but not in 70Z/3 cells, while TNFα and IL-1 activate NF-κB rapidly. HL60 (A) or 70Z/3 (B) cells were incubated at 37°C with 10 nM TNFα (A, lanes 1 to 9), 40 nM PMA (A, lanes 10 to 18; B, lanes 9 to 16), or 5 ng of IL-1 per ml (B, lanes 1 to 8). Samples were taken at the indicated times and rapidly cooled to 0°C. NF-κB activity was measured in nuclear extracts as described in the legend to Fig. 1, using either a DNA fragment containing the intact NF-κB binding site (Wildtype) or an inactive binding site (Mutant).

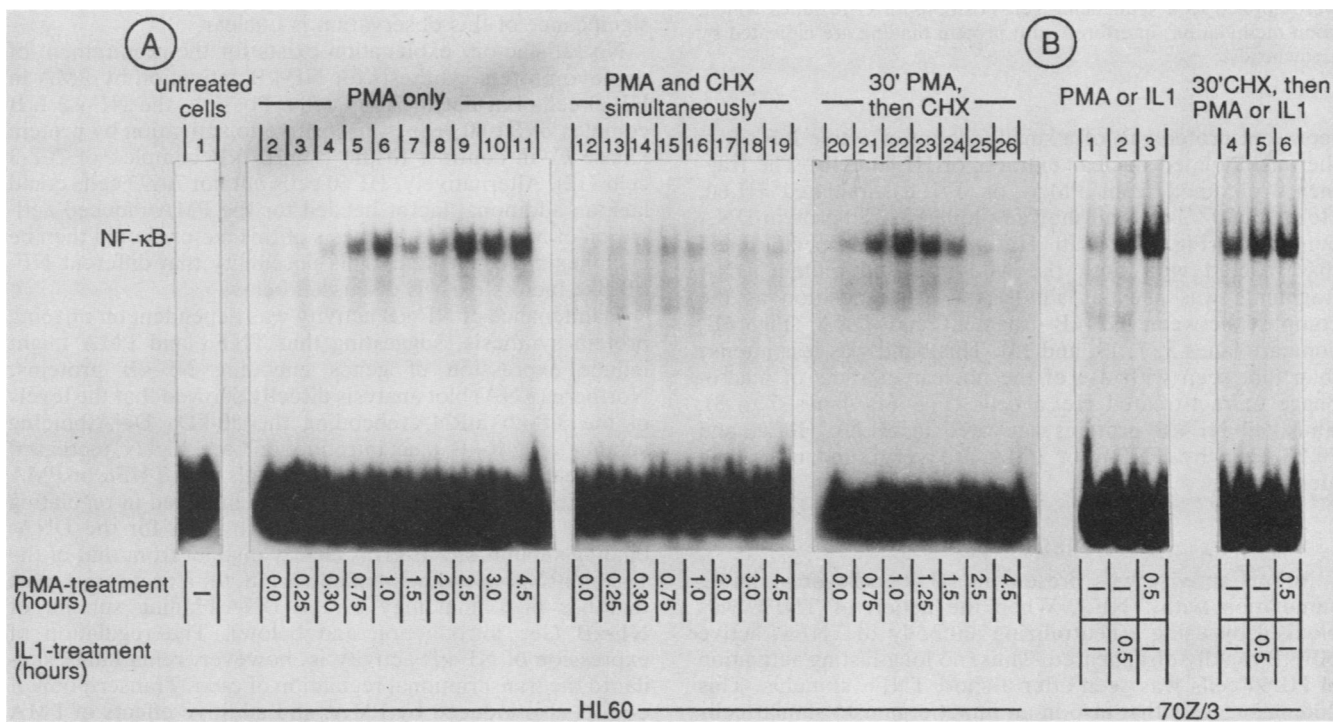

FIG. 4. Demonstration that in HL60 but not 70Z/3 cells, activation of NF-κB by PMA is completely dependent on ongoing protein synthesis and cycloheximide alone does not activate NF-κB. HL60 (A) or 70Z/3 (B) cells were incubated at 37°C with either cycloheximide (CHX), PMA, IL-1, or combinations of these substances as indicated. Samples were then rapidly cooled, and nuclear extracts were prepared and assayed for NF-κB activity as described in the legend to Fig. 1.

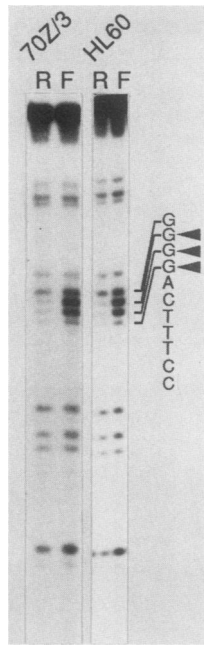

FIG. 5. Detection by methylation interference assays of same guanine contact points of NF-κB-like proteins from HL60 and 70Z/3 cells. Methylation interference assays were performed as described previously (20). Nuclear extracts of PMA-stimulated 70Z/3 cells or partially purified NF-κB from TNFα-treated HL60 cells prepared as described by Kawakami et al. (20) were applied to EMSA, using a partially methylated DNA fragment. Pieces of the EMSA gel containing the free DNA fragment or the DNA/NF-κB complex were cut out; the DNA was extracted and chemically degraded by the Maxam-Gilbert method. Reaction products of the free DNA (F) and the DNA isolated from the retarded DNA-protein complexes (R) were applied to a sequencing gel. Positions of G residues which, upon methylation, interfered with protein binding are indicated by arrowheads.

represent proteolysis of a small amount of active NF-κB in the unstimulated nuclear extracts of HL60 cells. The fragments obtained from PMA- or TNFα-stimulated HL60, HeLa, or 70Z/3 cells all showed similarly sized protein-DNA complexes (Fig. 6B). With HeLa cells, a nonspecific band that reacted with both the wild-type and mutant DNA fragments was seen at almost the same position as the complex between NF-κB fragment and DNA (Fig. 6B; compare lanes 5, 7, 13, and 14). This band was less intense than that seen with use of the nuclear extracts of macrophage extract-treated HeLa cells (Fig. 6B, lanes 5 to 8). Thus, all NF-κB proteins activated in HL60, HeLa, and 70Z/3 cells by TNF-α or PMA are very similar or even identical.

DISCUSSION

NF-κB activity was present in HL60 cells only during stimulation with TNFα. When the action of TNFα was blocked by using a neutralizing antibody to TNFα, active NF-κB rapidly disappeared. Thus, no long-lasting activation of HL60 cells was seen after a short TNFα stimulus. This finding suggests that also in an intact organism similar cells may only transiently respond to short TNFα stimulation with NF-κB activation and increased expression of genes controlled by the action of NF-κB. Active NF-κB showed a half-life of less than 30 min, while inactive NF-κB complexed to the inhibitor protein IκB was considerably more stable. Rapid activation of NF-κB was seen within a few minutes after addition of TNFα (19) and was not blocked by cycloheximide, indicating that this NF-κB was most likely derived from the cytosolic pool of inactive NF-κB. This cytosolic inactive complex was apparently rapidly exhausted, since maintenance of NF-κB activity, which was constant during several hours after addition of TNFα, depended on de novo protein synthesis. Similar results concerning stability were obtained when cells were stimulated with PMA. However, NF-κB activation by PMA in HL60 cells was much slower and less effective than that seen with TNFα and was obligatorily dependent on ongoing protein synthesis. It has been shown previously (25, 28) that TNFα and PMA activate NF-κB via different pathways and that the TNFα-induced pathway does not involve protein kinase C (25), although TNFα also induces protein kinase C in some cells (29). Our data, however, indicate that additional differences between both pathways exist. Most striking is the absence of rapid and protein synthesis-independent NF-κB activation by PMA in the human promyeloid HL60 cell line. However, this effect was not observed in mouse 70Z/3 cells (a pre-B-cell line), which showed rapid and protein synthesis-independent activation of NF-κB upon stimulation with PMA or IL-1. Stimulation by TNFα could not be directly analyzed for 70Z/3 cells, which lacked TNF receptors. These data suggest that also in an intact organism cells could differ in their ability to rapidly activate NF-κB in response to different stimuli. Another difference observed between HL60 and 70Z/3 cells concerned cycloheximide-induced NF-κB activation. Cycloheximide treatment activated NF-κB in 70Z/3 cells, and the activations by PMA or IL-1 and cycloheximide were additive, indicating different activation pathways as shown before (28, 31, 32). Cycloheximide alone did not induce NF-κB activity in HL60 cells. The significance of this observation is unclear.

No satisfactory explanation exists for the requirement of de novo protein synthesis for NF-κB activation by PMA in HL60 cells but not in 70Z/3 cells. Possibly the NF-κB/IκB complex of HL60 cells is insensitive to activation by protein kinase C, in contrast to the NF-κB/IκB complex of 70Z/3 cells (12). Alternatively, HL60 cells but not 70Z/3 cells could lack an additional factor needed for the PMA-induced activation of NF-κB. The synthesis of this factor should then be cycloheximide sensitive. This possibility that different NF-κB-like factors exist is discussed below.

Maintenance of NF-κB activity was dependent on ongoing protein synthesis, suggesting that TNFα and PMA might induce expression of genes encoding NF-κB proteins. Northern (RNA) blot analysis directly showed that the levels of the 3.8-kb mRNA encoding the 50-kDa DNA-binding subunit of NF-κB (see introduction) are highly increased after treatment of HL60 and HeLa cells with TNFα or PMA (25a). This could mean that NF-κB is involved in regulating its own expression. The size of the mRNA for the DNA-binding subunit of NF-κB is clearly distinct from that of the 10-kb mRNA encoding human c-*rel* (8, 9), a proto-oncogene that has high homology to the DNA-binding subunit of NF-κB (see introduction and below). The regulation of expression of NF-κB activity is, however, remarkably similar to the transcriptional regulation of c-*rel*. Transcription of c-*rel* is also induced by PMA, and additive effects of PMA and cycloheximide on the levels of mRNA were observed. Cycloheximide, however, in contrast to PMA, acts to enhance the posttranscriptional stability of the c-*rel* mRNA and does not induce its transcription (9).

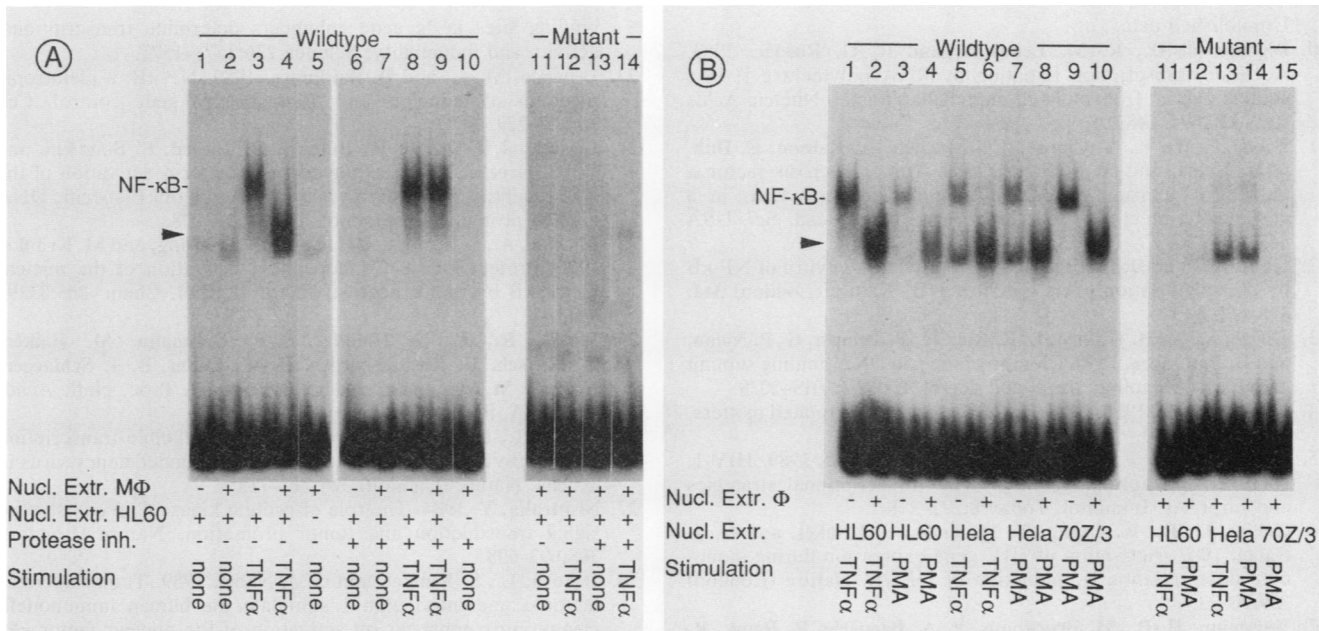

FIG. 6. Identical native proteolytic fingerprints of NF-κB-like proteins activated under different conditions. Nuclear extracts of unstimulated mouse macrophages (Nucl. Extr. MΦ) contain a protease capable of generating a shorter DNA-binding form of NF-κB and are themselves virtually devoid of endogenous NF-κB activity. Cells were incubated without any addition (none) or with TNFα or PMA. Nuclear extracts (Nucl. Extr.) of equivalent cell samples were then treated where indicated with macrophage extracts, in the absence or presence of a cocktail of protease inhibitors (Protease inh.). NF-κB activation was determined by EMSA, using a DNA fragment containing the wild-type NF-κB binding site (Wildtype) or a mutated inactive binding site (Mutant) as described in the legend to Fig. 1. Positions of the complex between DNA and intact NF-κB and the proteolytic fragment (arrowhead) are indicated.

Was the same NF-κB protein induced in all experiments, or were different NF-κB-like proteins activated? The NF-κB activity induced by TNFα in HL60 cells is true NF-κB: the partial protein sequence of the DNA-binding subunit of NF-κB isolated from TNFα-stimulated HL60 cells (25a) was identical to that of the NF-κB subunit from two other human cells (21, 25a). The NF-κB activities induced by TNFα and PMA in HL60 cells were indistinguishable in native proteolytic fingerprinting. In addition, the PMA-induced NF-κB activity of 70Z/3 cells and TNFα-induced NF-κB activity in HL60 cells showed identical methylation interference patterns. These data, of course, cannot exclude the existence of multiple NF-κB-like proteins, but they drastically reduce the number of potential candidates. One obvious candidate for an NF-κB-like protein is the c-*rel* product. NF-κB and c-*rel*/v-*rel* are highly homologous (see introduction), give similar methylation interference patterns for NF-κB binding sites, and can even form heterodimers that still bind DNA (21). Participation of c-*rel* in addition to NF-κB or instead of NF-κB in generating the NF-κB activity detected in the experiments described here, such as the PMA-induced NF-κB activation in HL60 cells, is not very likely. The apparently identical molecular masses in each case of the DNA-protein complexes and of their proteolytic fragments suggest but do not prove the activity of only one type of protein-DNA complex. In addition, TNFα and PMA drastically increased the mRNA levels for the DNA-binding subunit of NF-κB in HL60 cells (see above). We therefore suggest that NF-κB-like proteins induced by PMA and TNFα in HL60 and HeLa cells may be identical to NF-κB and homologous to mouse NF-κB from PMA-activated 70Z/3 cells. More work will be needed to directly verify this hypothesis.

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