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NF-κB: A family of inducible and differentially expressed enhancer-binding proteins in human T cells

(DNA–protein crosslinking/interleukin 2 receptor/human immunodeficiency virus/T-cell activation/cytoplasmic inhibitor IκB)

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ABSTRACT **A sensitive DNA–protein crosslinking approach has been used to characterize four inducible T-cell proteins (50 kDa, 55 kDa, 75 kDa, and 85 kDa) that specifically bind to κB enhancer elements. Partial proteolytic mapping revealed a distinct cleavage pattern for three of these proteins. These polypeptides are sequestered as inactive precursors in the cytosol of unstimulated T cells but can be converted into active forms *in vivo* by phorbol ester stimulation or *in vitro* by detergent treatment. The induction of these proteins by phorbol ester results in a strikingly biphasic pattern of nuclear expression with the 55-kDa and 75-kDa species appearing within minutes, whereas the 50-kDa and 85-kDa species appear only several hours after cellular stimulation. These data suggest that NF-κB-binding activity may not correspond to a single polypeptide but rather a family of at least four inducible and differentially regulated DNA–binding proteins that are expressed with distinct kinetics in human T lymphocytes.**

Eukaryotic enhancers are cis-acting genetic elements that stimulate RNA polymerase II-directed gene expression in a position- and orientation-independent fashion (1–3). One prototypical enhancer, the κB element, is present in many cellular and viral genes, including those encoding the κ immunoglobulin light chain (4, 5), the α subunit of the interleukin 2 receptor (IL-2Rα) (6), interleukin 2 (7–9), granulocyte–macrophage colony-stimulating factor (10, 11), and the human immunodeficiency virus type 1 (HIV-1) (12). The κB enhancer serves as a recognition site for a nuclear factor(s), designated NF-κB, that is constitutively expressed in mature B cells (4) and can be induced in T cells by such agents as phorbol esters (13, 14), tumor necrosis factor α (15, 16), and the Tax protein of the human T-cell leukemia virus type 1 (HTLV-I) (17–19). This inducible DNA–binding activity appears to be regulated primarily at a posttranslational level through interactions with a cytoplasmic inhibitor termed IκB (20–23). NF-κB has recently been purified from Namalwa B cells (24) and HeLa cells (22) as a 50- to 51-kDa protein that stimulates *in vitro* transcription from the HIV-1 5′-long terminal repeat (24). Together, these studies have suggested that NF-κB activity is confined to a single polypeptide that activates the expression of multiple genes in eukaryotic cells (25).

In this report, we have used an *in situ* DNA–protein crosslinking approach to demonstrate that multiple κB enhancer elements specifically interact with at least four different inducible DNA–binding proteins. We provide evidence that these four proteins exist as latent cytoplasmic precursors in unstimulated T cells and are converted to active nuclear forms with biphasic kinetics after phorbol ester treatment. These data suggest that NF-κB activity comprises not one but a family of inducible DNA–binding proteins that are differentially regulated in human T lymphocytes.

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MATERIALS AND METHODS

Cell Lines and Nuclear Extract Preparation. Jurkat human T cells were grown in RPMI 1640 medium/7.5% fetal bovine serum supplemented with penicillin and streptomycin. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml), and extracts were prepared at various times after stimulation as described (26, 27) with the following modifications: (*i*) buffer A (27) contained 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.3 M sucrose, and 0.5 mM phenylmethylsulfonyl fluoride and (*ii*) the addition of buffer B (27) was omitted. Cytoplasmic extracts were prepared from the 100,000 × *g* supernatant as described (20, 27).

DNA–Protein Binding and UV-Crosslinking. Short, photoreactive (28) κB duplexes were prepared as described (29). The effect of detergents on cytoplasmic κB-binding activity was determined by addition of deoxycholate (DOC) and either Nonidet P-40 as described (20) or the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate (CHAPSO) (30) to the binding mixture, before PAGE. For *in situ* DNA–protein crosslinking, polyacrylamide slabs from gel-retardation assays (29) were placed on a 300-nM UV light source (Fotodyne; 7000 mW/cm^2) and irradiated for 20 min. DNA–protein complexes were excised and analyzed by discontinuous SDS/7.5% PAGE under reducing conditions.

Peptide Mapping. For partial peptide mapping, DNA–protein adducts were generated from preparative-scale (20-fold) crosslinking reactions containing a wild-type IL-2Rα κB probe or a functional IL-2Rα κB mutant (CT → TC at positions −260 and −259; ref. 15) that yielded significantly higher crosslinking efficiencies. Adducts (≈500 cpm) excised from SDS/polyacrylamide gels were either digested with *N*-chlorosuccinimide *in situ* (31) or electroeluted and subjected to partial enzymatic (Asp-N or Arg-C) proteolysis (32). Adduct cleavage products retaining covalently bound ^{32}P-labeled DNA were analyzed on 10% polyacrylamide/SDS gels.

RESULTS AND DISCUSSION

Recent attention has focused on the role of NF-κB in the expression of inducible genes involved in T-cell growth (33). We and others have demonstrated that a κB motif (GG-

Abbreviations: CHAPSO, 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate; DOC, deoxycholate; HIV-1, human immunodeficiency virus type 1; HTLV-I, human T-cell leukemia virus type 1; IL-2Rα, interleukin 2 receptor α subunit; PMA, phorbol 12-myristate 13-acetate.

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GAATCTCCC; −266 to −256) within the IL-2Rα promoter gene is required for full induction of IL-2Rα gene expression by PMA (6), tumor necrosis factor α (15, 16), and HTLV-I Tax (17–19). *In vitro* DNA–binding studies have confirmed that these same T-cell activation signals induce the expression of one or more NF-κB-like DNA–binding activities (6, 15–19). Consistent with the possible binding of more than one protein (6, 17), gel-retardation assays performed with nuclear extracts from PMA-stimulated Jurkat T cells and a radiolabeled IL-2Rα κB probe revealed the formation of two discrete DNA–protein complexes, designated B1 and B2 (Fig. 1*A*, lane 1). The inclusion of either wild-type κB (lane 2) or κB-deleted (lane 3) sequences in competition experiments confirmed the κB-specific nature of these inducible nucleoprotein interactions.

To characterize the inducible DNA–binding protein(s) involved in the formation of B1 and B2, PMA-induced nuclear extracts were incubated with a 5′-bromo-2′-deoxyuridine-substituted IL-2Rα κB probe, fractionated into the B1/B2 complexes by gel retardation, and then subjected to UV irradiation *in situ*. We have observed that crosslinking of these complexes within the polyacrylamide matrix (J.A.M., unpublished data) significantly increases the specific signal relative to performing this photochemical reaction in solution (29). Subsequent SDS/PAGE of the crosslinked DNA–protein adducts excised from these irradiated gels revealed the presence of two κB-specific proteins distinctive to the B1 complex and two additional proteins shared between B1 and B2 complexes (Fig. 1*B*). Consistent with the molecular mass (50–51 kDa) reported previously for human NF-κB (22, 24), the more rapidly migrating B2 complex contained two polypeptide species with sizes of ≈50 and 55 kDa (lane 2). In sharp contrast, the more slowly migrating B1 complex contained two additional polypeptide components ≈75 kDa and 85 kDa in size (lane 1). Partial peptide maps of these adducts (Fig. 1*C*), produced by cleavage with Asp-N (lanes 5–8), Arg-C (lanes 9–12), or *N*-chlorosuccinimide (lanes 13–16), suggested that the primary structures of the 85-kDa, 75-kDa, and 50/55-kDa proteins are distinct. However, the consistent 3- to 6-kDa difference observed for the major proteolytic fragments derived from the 50-kDa and 55-kDa proteins raises the possibility that these polypeptides may be closely related. Although the potential contribution of posttranscriptional (e.g., alternative mRNA splicing) and posttranslational (e.g., phosphorylation, controlled proteolysis) events to the expression of these four polypeptides remains unknown, these findings suggest that the IL-2Rα κB enhancer may be regulated by complex interactions with not one but multiple κB-specific inducible host factors.

We next examined whether any of these polypeptides reacted with other cellular or viral κB enhancers. Radiolabeled probes corresponding to the κB elements present in the interleukin 2 gene (7–9), the long terminal repeat of the type I human immunodeficiency virus (HIV-1_A κB and HIV-I_B κB; ref. 12), the κ immunoglobulin gene (4, 5), and the H-2K class I major histocompatibility complex gene (H-2K κB; ref. 34–36) each supported the formation of specific DNA–protein complexes that comigrated with the B1 and/or B2 complexes detected with the IL-2Rα κB enhancer (J.A.M., unpublished data). To identify the proteins interacting with these various κB enhancers, UV-crosslinking studies were performed. As shown in Fig. 2, the predominant adducts detected with the HIV-1_A (lane 6), HIV-1_B (lane 8), and immunoglobulin (lane 10) κB probes were indistinguishable from three of the factors (p55, p75, and p85) captured with the IL-2Rα κB enhancer (lane 1), whereas the fourth factor (p50)

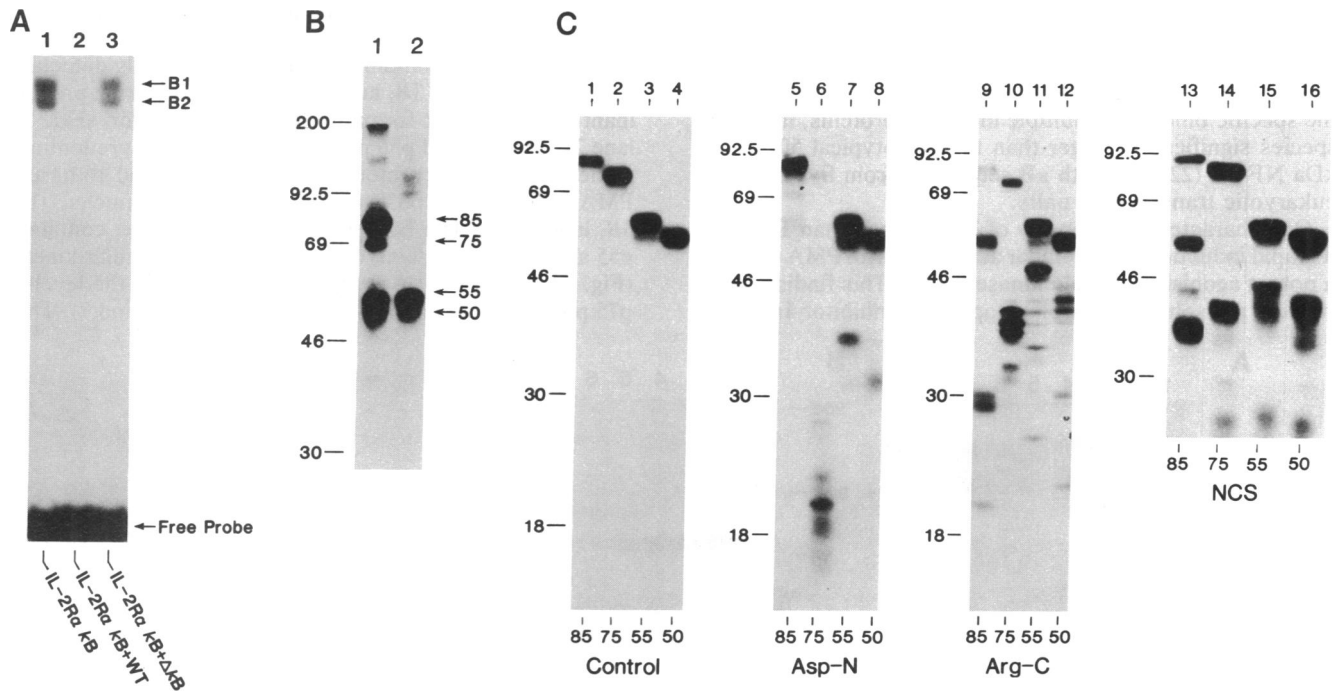

FIG. 1. (*A*) PMA induction of κB enhancer-binding proteins. Nuclear extracts (26) from Jurkat T cells stimulated for 16 hr with PMA (50 ng/ml) were incubated with a 5-bromo-2′-deoxyuridine-substituted IL-2Rα κB oligonucleotide probe (−275 to −249; ref. 17) and the resultant ^{32}P-labeled DNA–protein complexes were resolved on 5% polyacrylamide gel (lane 1). Wild-type (wt) κB (−275 to −249; lane 2) and κB-deleted (−281 to −243; lane 3) IL-2Rα sequences (100-fold molar excess) were included in competition reactions. (*B*) *In situ* crosslinking of κB-specific proteins to the IL-2Rα κB enhancer. Nucleoprotein complexes B1 and B2 were resolved by gel retardation, UV irradiated *in situ*, excised as separate gel slices, and analyzed directly on 7.5% polyacrylamide/SDS gel under reducing conditions. These four κB-specific species were also found in extracts from PMA-stimulated HeLa and uninduced Raji B cells (J.A.M., unpublished data). (*C*) Peptide mapping of κB-specific binding proteins. Cross-linked adducts (lanes 1–4) were subjected to partial cleavage with endoproteases Asp-N (37°C, lanes 5–8) and Arg-C (25°C, lanes 9–12) for 4 hr at an enzyme/substrate ratio of 1:25. Alternatively, adducts were digested *in situ* with 15 mM *N*-chlorosuccinimide (NCS) (lanes 13–16) as described (31). Cleavage products were analyzed as in *B*.

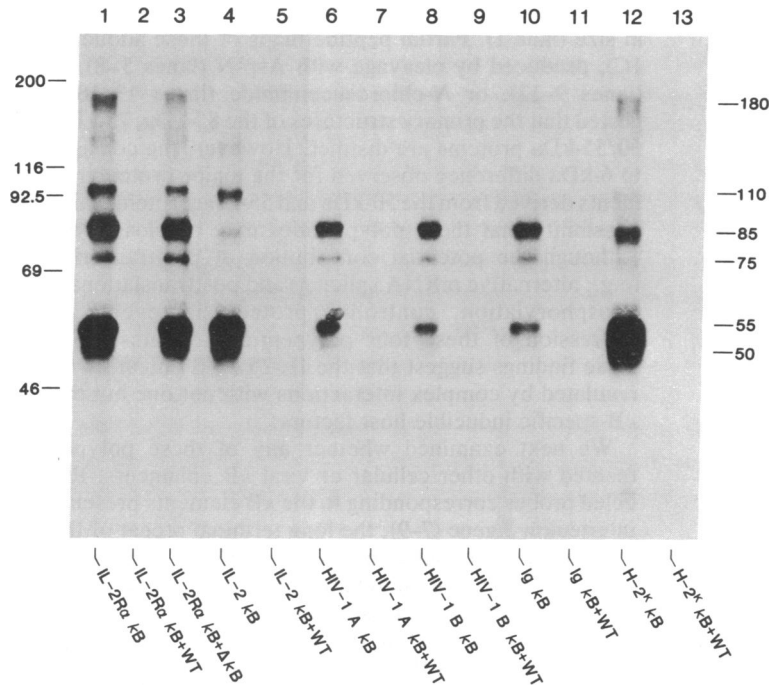

FIG. 2. UV-crosslinking analysis of DNA–protein complexes formed with various κB enhancer elements. The nuclear extracts described in Fig. 1*A* were mixed with 27-base-pair (bp) ^{32}P-labeled probes containing centrally placed κB elements in the presence or absence of homologous competitior oligonucleotides (100-fold molar excess) as indicated. Sequences of the 27-bp oligonucleotides were as follows: IL-2Rα, CAA CGG CAG GGG AAT CTC CCT CTC CTT; interleukin 2, ACA AAG AGG GAT TTC ACC TAC ATC CAT; HIV-1_A, TTG CTA CAA GGG ACT TTC CGC TGG GGA; HIV-1_B, TTT CCG CTG GGG ACT TTC CAG GGA GGC; κ immunoglobulin, TCA ACA GAG GGG ACT TTC CGA GAG GCC; H-2K, CCA GGG CTG GGG ATT CCC CAT CTC CAC. Protein–DNA complexes were resolved by gel retardation and UV-crosslinked *in situ*. Nucleoprotein complexes from each lane were analyzed on 7.5% polyacrylamide/SDS gel.

was not readily apparent. In contrast, DNA–protein complexes formed with the interleukin 2 (lane 4) and H-2K (lane 12) κB probes contained p50 but lacked significant amounts of p75. The prominent 110-kDa and/or 180-kDa species formed with the IL-2Rα, interleukin 2, and H-2K probes were likely derived from the crosslinking of two κB-specific proteins on the same probe (J.A.M., unpublished data). It should be noted that the extent of crosslinking of each protein species may vary due to the positioning of the photoreactive 5-bromo-2′-deoxyuridine in the different κB enhancer sequences. Notwithstanding, these data provide evidence for the specific binding of multiple inducible proteins, including species significantly larger than the prototypical 50- to 51-kDa NF-κB (22, 24), with κB sequences from five different eukaryotic transcription units.

One characteristic property of NF-κB in human T cells is its rapid induction after cellular activation with PMA (13, 14), a potent agonist of protein kinase C (37). This finding likely reflects phosphorylation of cytoplasmic inhibitor IκB (23), disassembly of the cytoplasmic complex that normally sequesters NF-κB, and transport of this factor to the nucleus (20–22). To compare whether p50, p55, p75, and p85 were induced with similar or different kinetics, nuclear extracts were prepared from Jurkat T cells stimulated with PMA for 20 min to 48 hr. Without PMA, no nucleoprotein complexes were demonstrable by gel retardation (Fig. 3*A*, lane 1); nor were any κB-specific polypeptides captured by crosslinking (Fig. 3*B*, lane 1). However, within 20 min after PMA addition, the p75 and p55 κB-binding activities were readily detectable in the nucleus (Fig. 3*B*, lane 2) in a form mediating predominantly B1 complex formation by gel retardation (Fig. 3*A*, lane 2). The p75 and p55 proteins remained the predominant nuclear κB-specific proteins until between 4 and 16 hr after PMA addition when p85 and p50 appeared in the nucleus (Fig. 3*B*, lanes 4, 5). The B2 gel-retardation complex, containing p55 and p50 binding activities, emerged with similar kinetics (Fig. 3*A*, lanes 4–7). As p85 and p50 appeared, the levels of p75 progressively diminished (Fig. 3*B*, lanes 6 and 7). Thus,

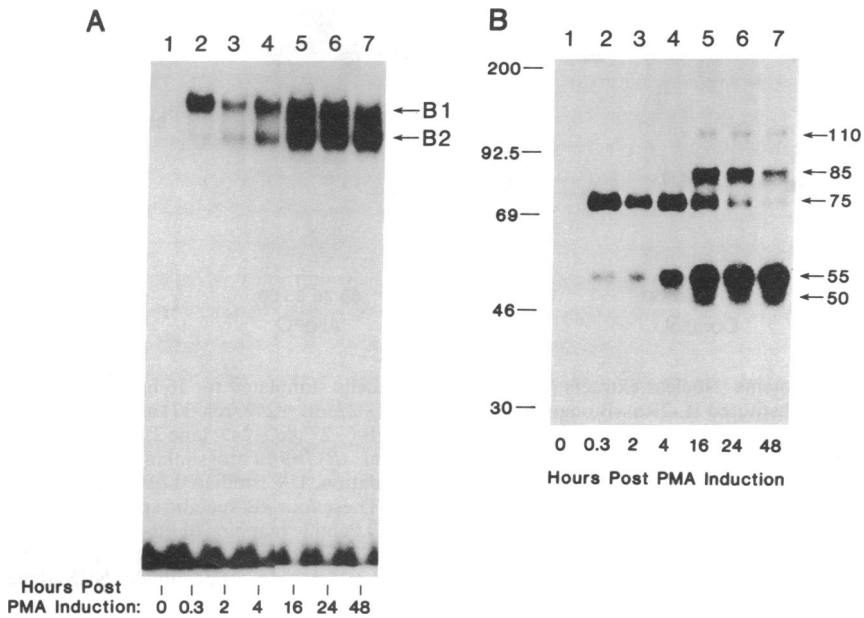

FIG. 3. (*A* and *B*) Time course for PMA induction of κB enhancer-binding proteins. Nuclear extracts (12–18 μg of nuclear protein) (27) prepared at the times indicated, were incubated with IL-2Rα probe (see Fig. 1 legend) and analyzed by gel retardation (*A*). After UV irradiation, gel slices at positions corresponding to B1 and B2 complexes from each lane were excised and analyzed together on 7.5% polyacrylamide/SDS gel under reducing conditions (*B*).

the induced nuclear expression of these NF-κB-like polypeptides in Jurkat T cells after phorbol ester activation is temporally regulated in a distinctly biphasic manner.

As noted above, the 50- to 51-kDa NF-κB protein has been shown to exist in an inactive form in the cytosol in association with an inhibitor termed IκB (20–23). This complex can be disrupted *in vitro* by treatment of cytoplasmic extracts with mild denaturants, such as DOC, thus allowing the detection of cytosolic NF-κB in gel-retardation assays (20–23). To investigate which of the four inducible NF-κB-like activities that we observed in nuclear extracts from PMA-stimulated T cells might be regulated by IκB, we examined the κB-specific proteins released by DOC treatment of cytoplasmic extracts from unstimulated Jurkat T cells. As shown in Fig. 4*A*, gel-retardation assays revealed DOC-dependent formation of a DNA–protein complex (compare lanes 2 and 3) from these resting T-cell cytoplasmic extracts that comigrated with the B1 nucleoprotein complex obtained with PMA-induced nuclear extracts (lane 1). Crosslinking of this DOC-induced B1 complex revealed three prominent photoreactive adducts, including the p75 and p55 proteins that rapidly appeared in the nucleus after PMA stimulation *in vivo* (Fig. 4*B*, lane 2). A prominent band at 180 kDa that may correspond to an oligomeric complex of p55 and p75 (J.A.M., unpublished data) was also identified. Thus, both p55 and p75 are preformed proteins sequestered in the cytoplasm of Jurkat T cells in a DOC-releasable manner.

The conspicuous absence of p50 and p85 in these DOC-treated cytoplasmic extracts raised the possibility that the late nuclear expression of these proteins in PMA-stimulated cells might be controlled at the level of transcription or translation. In this regard, although cycloheximide almost completely blocked the late induction of p50 and p85, actinomycin D, emetine, puromycin, and anisomycin only modestly inhibited their appearance (J.A.M., unpublished data). We thus reasoned that these proteins might also be at least in part preformed but perhaps sequestered in a qualitatively different manner than p55 and p75. This hypothesis was confirmed by treatment of cytoplasmic extracts with DOC in combination with the zwitterionic detergent CHAPSO. As shown in Fig. 4*C*, under conditions of 1% DOC/5% (wt/vol) CHAPSO, the p50 and p85 proteins were released from cytoplasmic extracts in conjunction with p55 and p75 (lane 2). In contrast, lower concentrations of CHAPSO resulted in the selective release of p55 and p75 only (lane 1). These findings thus confirm the preformed nature of all four of these κB-binding species in unstimulated Jurkat T cells but emphasize that these proteins appear sequestered in the cytoplasm as two distinct pairs (p75/p55 and p85/p50), each of which exhibits a markedly different pattern of kinetic induction after PMA stimulation (Fig. 3*B*). It is possible that two different cytoplasmic inhibitors (38) may mediate these sequestration processes. In addition, the late induction of p50 and p85 may be regulated in part through *de novo* protein synthesis.

CONCLUSIONS

We have used a sensitive DNA–protein cross-linking approach to identify a family of DNA-binding proteins (p50, p55, p75, and p85) that participate in sequence-specific interactions with a variety of κB enhancer elements. Proteolytic mapping of these factors reveals a distinct cleavage pattern for p85, p75, and p55/p50, suggesting that they are distinctive proteins. In light of biochemical evidence for a protein that associates with NF-κB but fails to bind DNA (22), the possibility that combinations of these factors form stable complexes *in vivo* cannot be excluded. Preliminary studies suggest that p75 corresponds to a DNA-binding species also capable of engaging p55 by means of a specific protein–protein interaction (data not shown). As well, p85 may be identical to HIVEN86A, an 86-kDa polypeptide

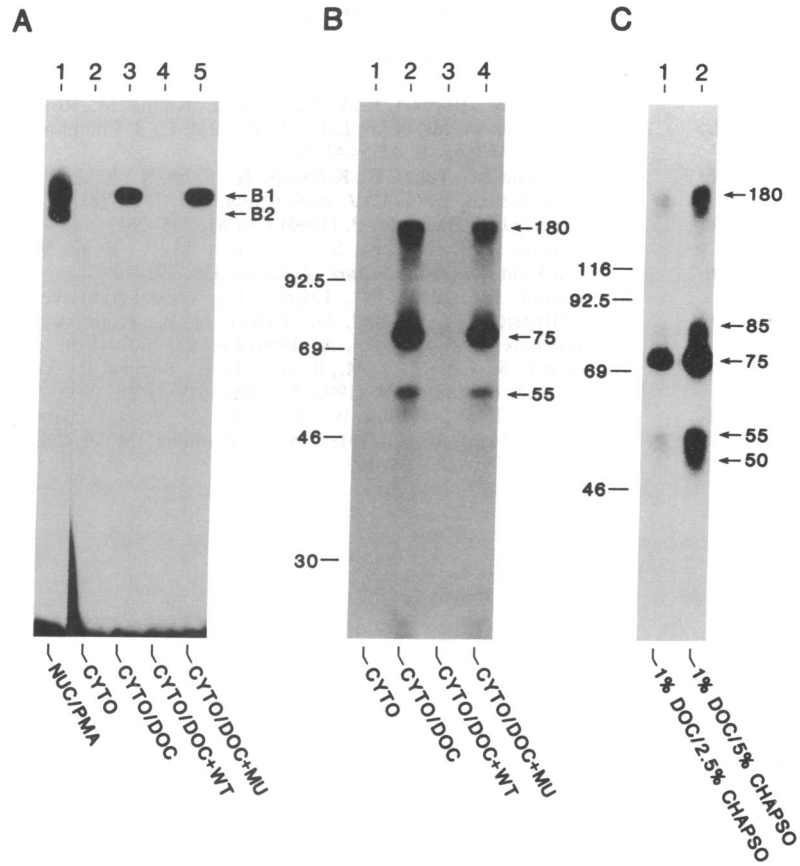

FIG. 4. (*A*) DOC-induced release of cytoplasmic NF-κB activity in unstimulated Jurkat human T cells. Cytoplasmic extracts (CYTO) were incubated with ^{32}P-labeled IL-2Rα κB probe with (lanes 3–5) or without (lane 2) DOC (0.6% wt/vol) and Nonidet P-40 (1.2% vol/vol) as described (20), and the resultant nucleoprotein complexes were resolved on a 5% polyacrylamide gel. Competitor DNA fragments corresponding to unlabeled wild-type κB (WT, lane 4) and κB-deleted (MU, lane 5) IL-2Rα sequences were added at 100-fold molar excess relative to ^{32}P-labeled DNA. Positions of nucleoprotein complexes formed with nuclear extracts from PMA-stimulated Jurkat cells (lane 1) are denoted at right. (*B*) *In situ* crosslinking of DOC-dependent cytoplasmic proteins (CYTO) to the IL-2Rα κB enhancer. Nucleoprotein complexes shown in lanes 2–5 of *A* (or their equivalent in competition experiments) were irradiated *in situ*, excised, and analyzed on 8.75% polyacrylamide/SDS gels. Positions and relative molecular sizes of the three major photoreactive adducts and ^{14}C-labeled protein standards are given in margins. (*C*) Effect of CHAPSO on cytoplasmic release of κB-binding activity. Cytoplasmic extracts were incubated with photoreactive IL-2Rα κB probes in 1% DOC and either 2.5% (lane 1) or 5.0% (lane 2) CHAPSO. Nucleoprotein complexes resolved by gel retardation were crosslinked *in situ* and analyzed on 7.5% polyacrylamide/SDS gel. The appearance of p85 and p50 is evident at CHAPSO/DOC ratios of 5:1.

previously shown to bind the κB enhancers present in the HIV-1 long terminal repeat (39) and IL-2Rα gene (6).

Like the 50- to 51-kDa NF-κB factor (22, 24), these four proteins appear bound to a cytosolic inhibitor(s) that can be released *in vitro* by treatment with detergents. Remarkably, nuclear translocation of these four factors after PMA stimulation occurs with distinctly biphasic kinetics. Specifically, the 55-kDa and 75-kDa polypeptides, which are released in unison by DOC, appear together in the nucleus within minutes after PMA induction. In contrast, the 50-kDa and 85-kDa proteins, which require different detergent conditions for their cytoplasmic release, are induced with greatly delayed kinetics. It therefore seems likely that the inducible activities of these "early" and "late" factors are temporally controlled by distinct mechanisms. In view of their biphasic induction kinetics, it will be of considerable interest to determine whether these four κB enhancer-binding proteins exert differing functional effects on the expanding number of cellular and viral genes recognized to be under NF-κB control.

Note Added in Proof. Kieran *et al.* (40) and Ghosh *et al.* (41) have recently reported the molecular cloning of human and murine NF-κB cDNAs and have found that the corresponding proteins share homology with the v-*rel* oncogene product. We have demonstrated that the p50, p55, p75, and p85 crosslinked species are specifically immunoprecipitated by anti-v-Rel antibodies and that p85 is comprised of the human c-*REL* protooncogene product bound to the κB enhancer (42).

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