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In vivo control of NF-κB activation by IκBα

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The transcription factor NF-κB is stored in the cytoplasm in complexes with the inhibitor protein IκBα. It has been shown *in vitro* that dissociation of IκBα from these complexes results in active NF-κB. In this report we show that lipopolysaccharide (LPS)-induced activation of B or pre-B cells results in loss of IκBα from NF-κB complexes *in vivo*. Many liberated NF-κB dimers reached the nucleus, where increased c-*rel*, p65 and p50 were detected by immunoblotting and by DNA binding assays. Some liberated dimers were retained in the cytoplasm, however, through binding to newly synthesized IκBα, a finding which strongly suggests (i) that the LPS-induced signal causes dissociation of complexes rather than preventing their association and (ii) that dissociation results from modification of IκBα and not of c-*rel* or p65. No effect of LPS treatment was detected on p105 or p100, which also retain *rel* family members in the cytoplasm. Quite unexpectedly, we also found that in unstimulated cells there is a constant ongoing process of degradation and replacement of complexed IκBα. We propose that this turnover results in the low level of active NF-κB presumably necessary even in the unstimulated cell, and that the high rate of synthesis of IκBα provides the ability to turn off NF-κB activity rapidly as soon as the activating signal ceases.

Key words: c-*rel*/IκBα/NF-κB/transcription factor/regulatory complexes

Introduction

Nuclear factor-κB (NF-κB) (Sen and Baltimore, 1986a) is a transcription factor that plays a crucial role in the regulation of numerous genes, including many of those involved in immune or inflammatory responses (for review, see Grilli *et al.*, 1993). As originally described, NF-κB was a heterodimer composed of a p50 and p65 subunit (Baeuerle and Baltimore, 1989; Ghosh and Baltimore, 1990), but it is now known that there are five different proteins that can participate in dimer formation. In addition to p50 and p65, these are p52, c-*rel* and *rel* B (also called I-*rel*). All are related over a stretch of ~300 amino acids to the c-*rel* oncogene and to the *Drosophila* morphogen *dorsal* (Ballard *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Neri *et al.*, 1991; Nolan *et al.*, 1991; Ruben *et al.*, 1991, 1992a; Schmid *et al.*, 1991; Ryseck *et al.*, 1992). DNA-binding dimers can be homodimers (except for *rel* B) or many (or perhaps all) of the possible heterodimers [see above references plus Inoue *et al.* (1991), Urban *et al.* (1991), Hansen *et al.* (1992) and Perkins *et al.* (1992)]. Transcriptional activation is due chiefly to p65, c-*rel* and *rel* B (Bull *et al.*, 1990; Inoue *et al.*, 1991; Schmitz and Baeuerle, 1991; Ballard *et al.*, 1992; Ruben *et al.*, 1992b; Ryseck *et al.*, 1992) although under certain conditions p50 homodimers can also transactivate (Fujita *et al.*, 1992).

NF-κB activity is constitutive in B cells and in some monocyte cell lines, but in most other cells it is very low or undetectable. However, NF-κB activity can be rapidly induced in most cells by treatment with any of a variety of agents, e.g. phorbol esters, tumor necrosis factor (TNF) or hydrogen peroxide (for review see Grilli *et al.*, 1993). The fact that in most cases this induction does not require protein synthesis (Sen and Baltimore, 1986b; Hohmann *et al.*, 1991) led to the discovery that NF-κB is stored in inactive form in the cytoplasm (Baeuerle and Baltimore, 1988). Storage of c-*rel*- and p65-containing dimers is accomplished through binding to one of several inhibitor proteins.

One of the inhibitors is the high molecular weight precursor to p50 (Rice *et al.*, 1992). Both p50 and p52 are synthesized as inactive cytoplasmic precursors (p105 and p100, respectively), of which the N-terminal halves constitute the active DNA-binding form (Bours *et al.*, 1990, 1992; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Meyer *et al.*, 1991; Neri *et al.*, 1991; Schmid *et al.*, 1991). A significant fraction of p105 is bound to c-*rel* or p65, and these p105/c-*rel* and p105/p65 dimers are strictly cytoplasmic and unable to bind DNA. Processing of p105 to p50 occurs slowly (Fan and Maniatis, 1991), but gradually leads to the appearance of p50/p65 and p50/*rel* dimers (Rice *et al.*, 1992). Whether the rate of processing is influenced by stimulation of the cell is not yet clear. In a similar manner, p100, the precursor of p52, also retains c-*rel* and p65 in the cytoplasm (Mercurio *et al.*, 1993).

The most extensively studied inhibitor of the *rel* family proteins is IκBα, the product of the *MAD-3* gene (Haskill *et al.*, 1991) and the human homolog of chicken pp40 (Davis *et al.*, 1991) and rat RL/IF-1 (Tewari *et al.*, 1992). *In vitro*, it binds efficiently to c-*rel*, p65 and *rel* B and prevents DNA binding by their homo- or heterodimers (Zabel and Baeuerle, 1990; Kerr *et al.*, 1991; Wulczyn *et al.*, 1992). It also binds to p50 homodimers but does not prevent their binding to DNA (Beg *et al.*, 1992). Cytoplasmic IκBα complexes are thought to be major targets in signal transduction pathways. It is known that phosphorylation of IκBα *in vitro* prevents its binding to *rel* and to p50/p65 (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990; Kerr *et al.*, 1991) and it is presumed that phosphorylation regulates association *in vivo* as well. Whatever the precise mechanism, the working hypothesis is that stimulation of the cell results in loss of IκBα from cytoplasmic complexes, transport of *rel* family proteins to the nucleus, followed by DNA binding and gene activation. Consistent with this scenario, stimulation of cells with agents such as TNFα results in the rapid disappearance

of IκBα, as monitored by immunoblotting (Brown *et al.*, 1993; Sun *et al.*, 1993).

In this paper we examine IκBα-containing complexes directly, by immunoprecipitation. We show that lipopolysaccharide (LPS) stimulation of murine B and pre-B cells results in loss of IκBα from all pre-existing c-*rel*- and p65-containing dimers, in accordance with the working model. Some but not all of the liberated c-*rel* and p65 translocates to the nucleus and exhibits DNA-binding activity. The remaining c-*rel* and p65 are captured by newly synthesized IκBα and retained in the cytoplasm, a result which strongly suggests that complexes dissociate because of modification of IκBα, not of c-*rel* or p65. We also examine IκBα complexes in unstimulated cells and find a quite unexpected dynamism. While c-*rel* and p65 are stable proteins, complexed IκBα turns over with a half-life of 30−60 min in a variety of cell lines. We propose that this turnover results in the low level of active NF-κB that is presumably necessary even in the unstimulated cell. In addition, we suggest that the high rate of synthesis of IκBα relative to that of *rel* family members provides the ability to turn off NF-κB activity rapidly as soon as the activating signal ceases.

Results

Activation of cells results in dissociation of IκBα

To test the effect of cellular stimulation on IκBα, we determined the response of the murine B cell line WEHI 231 and of the murine pre-B cell line 70Z/3 to bacterial LPS, an effective activator of these cells (Sen and Baltimore, 1986b). Cells were grown in ^{35}S-containing medium for 1 h, and then in non-radioactive medium with or without LPS. We asked whether [^{35}S]IκBα would be lost from *rel* family complexes during incubation of the cells in LPS.

To examine the 70Z/3 cells, cytoplasmic extract was immunoprecipitated with both anti-c-*rel* and anti-p65 (see Figure 1 for description of antisera). The washed precipitate was released from antibody by incubation in excess competing peptides and the eluted (c-*rel*- and p65-containing) material was reprecipitated as indicated in Figure 2A. In cells chased for 90 min without LPS, both anti-c-*rel* (lane 1) and anti-p65 (lane 2) precipitated [^{35}S]IκBα, as expected, and anti-IκBα precipitated ^{35}S-labeled IκBα, c-*rel* and p65 (lane 3). In LPS-treated cells, however, neither anti-c-*rel* (lane 4) nor anti-p65 (lane 5) precipitated [^{35}S]IκBα, and anti-IκBα precipitated only a trace of [^{35}S]c-*rel* or p65 (lane 6). Thus, LPS treatment of the 70Z/3 pre-B cells resulted in loss of [^{35}S]IκBα from c-*rel*- and p65-containing complexes. At the same time, the amount of precipitable cytoplasmic c-*rel* and p65 declined somewhat after LPS treatment (compare lane 1 with lane 4, and lane 2 with lane 5). This suggests that the c-*rel* and p65 released from IκBα left the cytoplasm, where they had constituted a significant fraction of total c-*rel* and p65.

There are two additional points to be made about this experiment. First, while the amount of [^{35}S]c-*rel* and [^{35}S]p65 precipitable by anti-IκBα after LPS treatment was very low, it was not zero (lane 6). We wondered whether this low level represented incomplete dissociation of ^{35}S-labeled complexes or whether it reflected binding of newly synthesized (and hence non-radioactive) IκBα to c-*rel* and p65. To test this, LPS treatment was carried out in the presence of cycloheximide. The result was that precipitation of [^{35}S]c-*rel* or [^{35}S]p65 by anti-IκBα was abolished (lane 10). Thus, synthesis of IκBα proceeds in the presence of LPS, and new IκBα is able to associate with previously synthesized [^{35}S]c-*rel* or [^{35}S]p65.

Second, this experiment suggests that LPS treatment for 90 min has little effect on processing of p105 and/or p100. In the untreated cells, both the c-*rel* precipitate (lane 1) and the p65 precipitate (lane 2) contained a protein(s) migrating

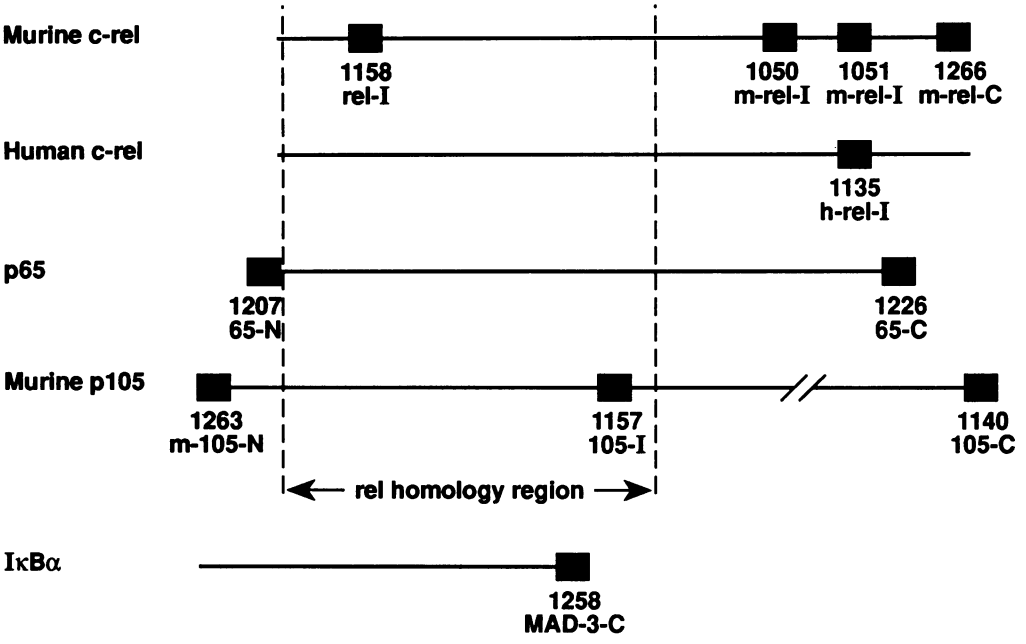

Fig. 1. Antisera. The diagram shows the peptide antisera used in these experiments. In the text each is referred to generically (e.g. anti-c-*rel*, anti-p65, etc.), then by its specific number (e.g. #1158, #1266, #1207, etc.), then by its description (e.g. m-*rel*-I, for murine c-*rel* internal; m-*rel*-C, for murine c-*rel* C-terminal; or 65-N, for p65 N-terminal). Those sera without an 'm' or 'h' in their descriptive name recognize murine and human proteins. Each serum is specific for its protein and does not cross-react with other family members.

at the position expected of p105 and p100 (which co-migrate under our conditions). This protein(s) was also present, in about the same amounts, after LPS treatment (lanes 4 and 5). Thus, of the known inhibitors that bind to c-*rel* and p65, the major effect of LPS is on IκBα. Of course we cannot rule out the possibility that longer treatment or a different stimulus might also accelerate processing, as reported for phorbol ester treatment of HeLa cells (Mercurio *et al.*, 1993).

To test whether these results are unique to 70Z/3 cells, a similar experiment was performed with WEHI 231 cells, where levels of the proteins of interest are much higher. With one exception, results were much like those seen with 70Z/3 cells. As above, WEHI 231 cells were grown in ^{35}S-containing medium, then in non-radioactive medium with and without LPS. Cytoplasmic extracts were immunoprecipitated with anti-c-*rel*, precipitates were eluted with peptide, and eluates were reprecipitated as shown in Figure 2B. In extracts from the untreated cells, anti-c-*rel* precipitated [^{35}S]IκBα (lane 1) and anti-IκBα precipitated [^{35}S]IκBα and [^{35}S]c-*rel* (lane 2), just as in the 70Z/3 experiment. LPS treatment resulted in the loss of [^{35}S]IκBα from the *rel* precipitate (lane 4), but, unlike the result with 70Z/3 cells, anti-IκBα was still able to precipitate considerable [^{35}S]c-*rel* (lane 5). Thus, while LPS caused the loss of [^{35}S]IκBα, replacement by new unlabeled IκBα must have been so rapid that much of the temporarily liberated [^{35}S]c-*rel* remained precipitable by anti-IκBα. As above, this interpretation was confirmed by treating with LPS in the presence of cycloheximide. Under those conditions, not only did [^{35}S]IκBα dissociate from c-*rel* (lane 7), but also anti-IκBα was unable to precipitate [^{35}S]c-*rel* (lane 8). Thus, as in 70Z/3 cells, synthesis of IκBα continued in the presence of LPS, and newly synthesized molecules were able to associate with pre-existing but newly liberated [^{35}S]c-*rel*. This strongly suggests (i) that the LPS signal affects IκBα complexes rather than free IκBα, i.e. the signal causes disruption of complexes rather than preventing their formation, and (ii) that disruption results from some change to IκBα, not to c-*rel*, since [^{35}S]c-*rel* remained able to bind to new inhibitor molecules. In addition, this experiment shows that translocation to the nucleus is not a necessary consequence of every dissociation event. If new IκBα is synthesized at a high enough rate [and 90 min exposure to at least some activating agents is sufficient to increase the level of IκBα mRNA significantly (Haskill *et al.*, 1991; Brown *et al.*, 1993; Sun *et al.*, 1993)] dissociated *rel* and/or p65 may be efficiently recaptured and retained in the cytoplasm.

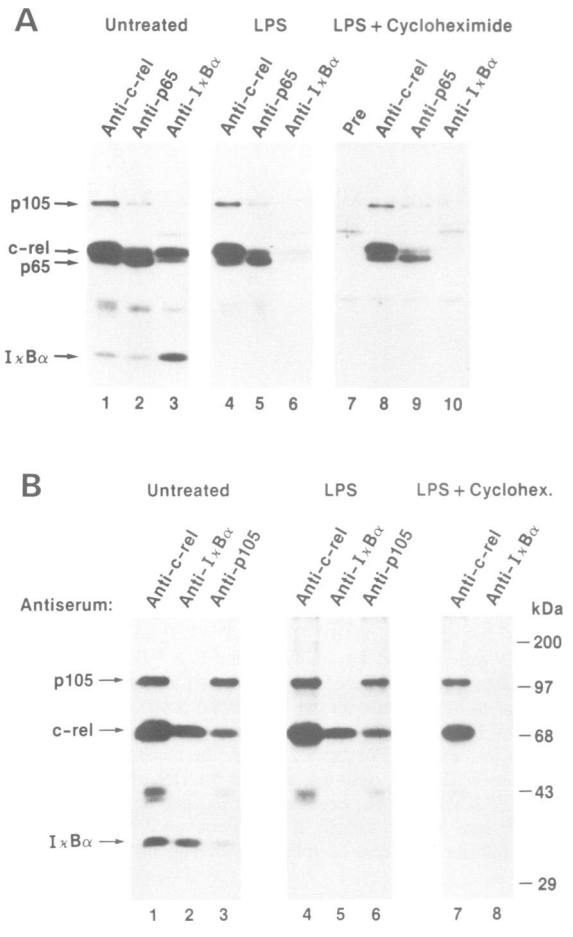

Fig. 2. LPS results in loss of IκBα. (**A**) 70Z/3 cells were grown for 1 h in medium containing [^{35}S]methionine and [^{35}S]cysteine (each at 100 μCi per ml). Excess unlabeled methionine and cysteine were then added, and the cells were diluted 10-fold into complete medium containing LPS (15 μg/ml), LPS plus cycloheximide (20 μg/ml) or no additives. After 90 min, cytoplasmic extracts were immunoprecipitated with a combination of anti-c-*rel* (#1051, m-*rel*-I) and anti-p65 (#1226, 65-C). The precipitates were eluted with excess peptides, and the eluates were reprecipitated with anti-c-*rel* (#1050, m-*rel*-I), anti-p65 (#1207, 65-N) or anti-IκBα (#1258, MAD-3C), as indicated, and analyzed by SDS–PAGE. Each lane contains the precipitate from ~10^7 cells. The film was exposed for 30 days. (**B**) WEHI 231 cells were treated as in (A). Cytoplasmic extracts were immunoprecipitated with anti-c-*rel* (#1050, m-*rel*-I). Precipitates were eluted with peptide, and eluates were reprecipitated with anti-c-*rel* (#1051, m-*rel*-I), anti-IκBα (#1258, MAD-3-C) or anti-p105 (#1263, m-105-N), as indicated. Each lane contains the preciptate from ~10^7 cells. The film was exposed for 3 days.

We wondered whether the [^{35}S]IκBα that disappears from c-*rel* complexes in response to LPS could be found as free molecules. This was tested by exhaustively precipitating the cytoplasmic extract from LPS-treated WEHI 231 cells with anti-c-*rel* and anti-p65, and then looking for [^{35}S]IκBα in the supernatant. None was detectable (data not shown), suggesting that it had been degraded. The same result was obtained with two different IκBα antibodies, neither of which was directed at a region with a potential phosphorylation site.

As with the 70Z/3 cells, there was no obvious effect of LPS treatment on processing of pre-existing p105. A high molecular weight protein(s) coprecipitated with c-*rel* in both untreated and LPS-treated cells (lanes 1 and 4); presumably it represents a combination of p105 and p100. When the *rel*-containing complexes were reprecipitated with anti-p105, bands of similar intensity were seen in treated (lane 6) and untreated (lane 3) cells.

LPS treatment increases nuclear c-rel and p65

The preceding experiments showed that LPS treatment resulted in loss of IκBα from c-*rel*-containing cytoplasmic complexes. We next tested whether dissociation outpaced replacement sufficiently to allow some liberated complexes to reach the nucleus. 70Z/3 cells were treated with LPS, and nuclear and cytoplasmic extracts were assayed by immunoblotting (Figure 3A). In untreated cells there was little nuclear p50, p65 or c-*rel*, but the level of all three increased significantly upon LPS treatment. Most p50 and p65 remained in the cytoplasm even after treatment

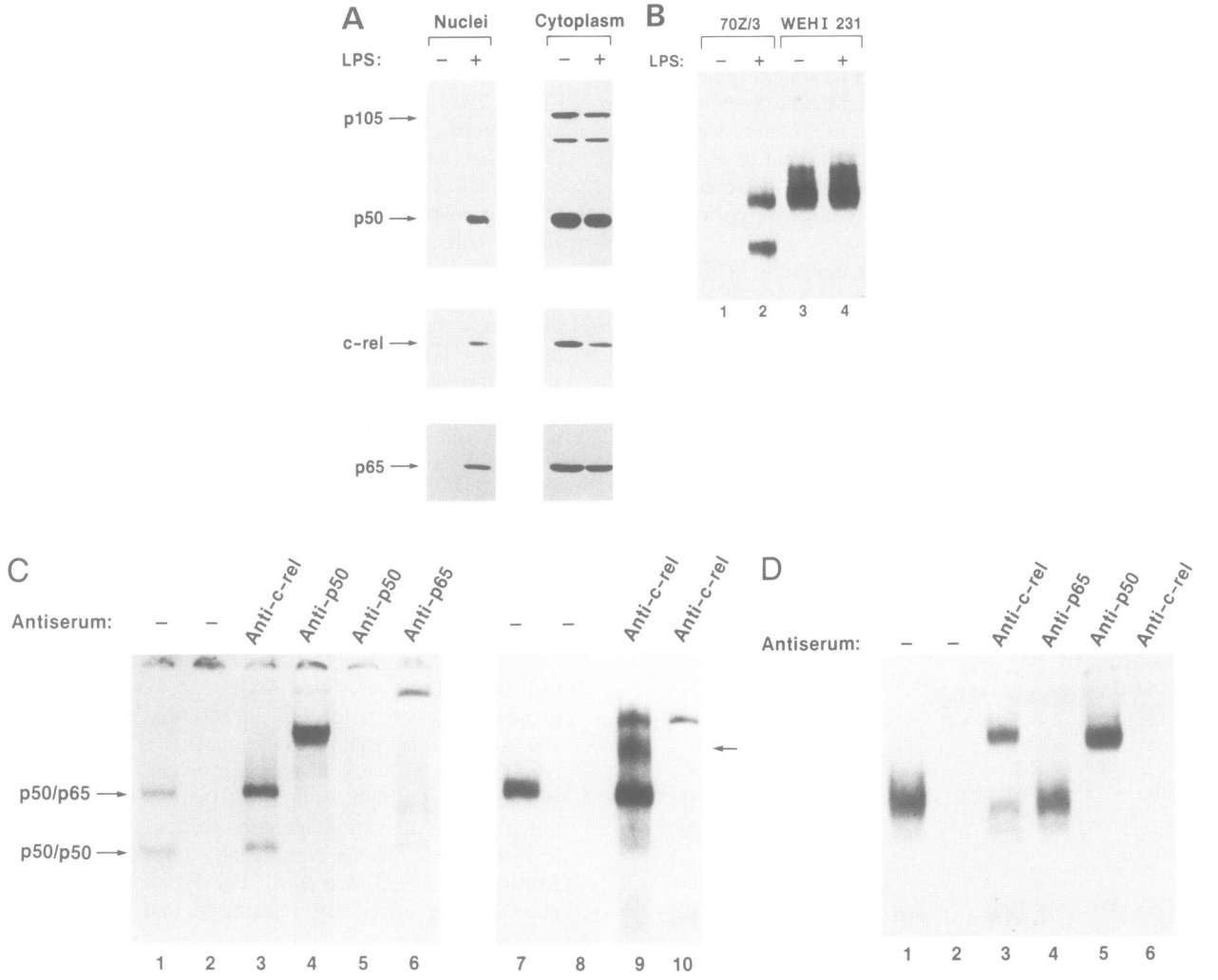

Fig. 3. Effect of LPS on nuclear c-*rel* and p65 and on DNA-binding activity. (**A**) 70Z/3 cells were treated with LPS (15 μg/ml) for 85 min or were left untreated. Nuclear and cytoplasmic extracts were assayed by immunoblotting using anti-c-*rel* (#1050, m-*rel*-I), anti-p65 (#1226, 65-C) or anti-p50 (#1157, 105-I). Each lane contains extract from 2.6×10^6 cells (*rel*, p65) or 1.4×10^6 cells (p50). (**B**) 70Z/3 and WEHI 231 cells were treated with LPS (10 μg/ml) for 90 min. Nuclear extracts from treated and untreated cells were assayed for ability to bind to a ^{32}P-labeled oligonucleotide containing the murine kappa chain intronic κB site. Unbound probe migrated to the bottom of the gel and is not shown in the figure. (**C**) 70Z/3 cells were treated with LPS, and nuclear extracts were assayed as in (B). Prior to incubation with the ^{32}P-labeled probe, samples were incubated with anti-c-*rel* (#1051, m-*rel*-I, lane 3; or #1266, m-*rel*-C, lane 9), anti-p50 (#1263, m-105-N; lane 4) or anti-p65 (#1226, 65-C; lane 6). Samples in lanes 5 and 10 contained ^{32}P-labeled probe and antiserum, but no nuclear extract. For lanes 2 and 8, the incubation mixture included a 40-fold molar excess of unlabeled κB oligonucleotide. (**D**) Nuclear extract from WEHI 231 cells was assayed for DNA-binding activity using a ^{32}P-labeled oligonucleotide containing the IL-6 κB site. Prior to addition of the ^{32}P-labeled probe, extracts were incubated with anti-c-*rel* (#1266, m-*rel*-C; lane 3), anti-p65 (#1226, 65-C; lane 4) or anti-p50 (#1263, m-105-N; lane 5). The sample in lane 6 contained ^{32}P-labeled probe and anti-c-*rel*, but no nuclear extract. For lane 2, the incubation mixture included a 200-fold molar excess of unlabeled κB oligonucleotide.

(reflecting not only IκBα-containing complexes, but those with p105 and p100 as well), but the amount of c-*rel* is low enough in these cells that transport to the nucleus diminished the cytoplasmic level noticeably. With WEHI 231 cells the results were similar, except that even untreated cells had some nuclear c-*rel* and p50. The levels of nuclear c-*rel* and p65 were increased appreciably by LPS treatment (data not shown).

LPS-induced DNA binding activity

We next tested whether the increased level of nuclear *rel* family members would be reflected in increased DNA binding activity. 70Z/3 and WEHI 231 cells were treated with LPS for 90 min, and nuclear extracts were assayed for their ability to bind to the κB site from the intron enhancer of the murine kappa light chain gene. In agreement with results of others (Sen and Baltimore, 1986b), there was significantly more binding activity in LPS-stimulated than in unstimulated 70Z/3 cells (Figure 3B, lanes 1 and 2). In contrast, WEHI 231 cells had a high level of activity in unstimulated cells [as has been reported previously (Sen and Baltimore, 1986b; Inoue *et al.*, 1991)]. This constitutive level was so high that it did not increase significantly upon LPS treatment (lanes 3 and 4), in spite of the fact that immunoblot analysis had shown increased nuclear c-*rel* and p65 in LPS-treated cells.

To determine the composition of the DNA binding complexes, their reactivity with various *rel* family antisera was tested. Both of the prominent bands produced by the 70Z/3 extracts were shifted by anti-p50 (Figure 3C, lane 4), while only the upper band was shifted by anti-p65 (lane 6). Anti-c-*rel* had little or no effect on the bound complexes (lane 3). In a second experiment using a different anti-c-*rel* serum, however, a small amount of shifted material was seen

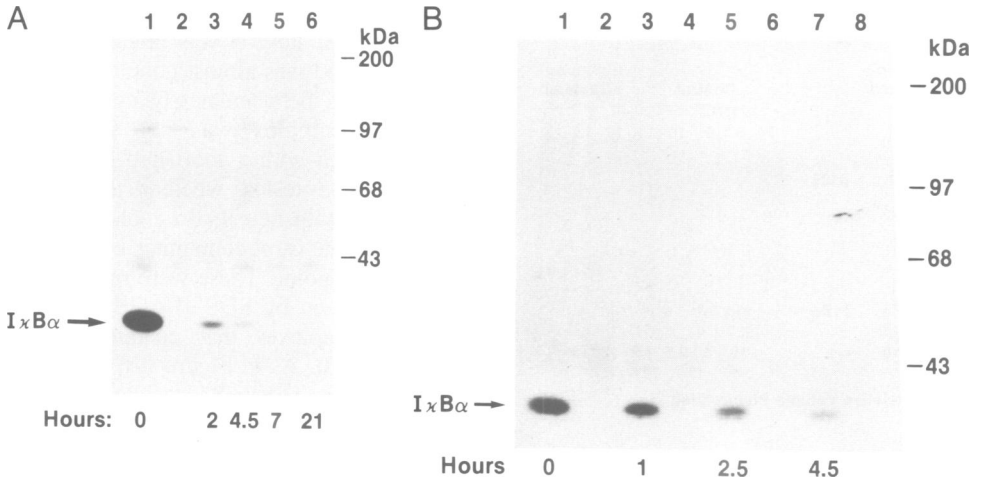

Fig. 4. IκBα has a short half-life. (**A**) WEHI 231 cells were grown for 1 h in medium containing [^{35}S]methionine and [^{35}S]cysteine (each at 100 μCi per ml). Cells were pelleted, resuspended in non-radioactive complete medium, and incubated at 37°C for the indicated times. Whole cell extracts were adjusted to 1% SDS and 0.5% β-mercaptoethanol and boiled for 5 min. After diluting at least 4-fold with TNT-E buffer, aliquots were immunoprecipitated with anti-IκBα (#1258, MAD-3-C). The sample shown in lane 2 contained competing peptide 1258. Each lane contains precipitate from ~3 × 10^6 cells and the film was exposed for 6 days. (**B**) Jurkat cells were treated as in A, except that the ^{35}S-containing medium was not removed following the labeling period. Instead, excess unlabeled methionine and cysteine were added, and cells were diluted 5-fold into complete medium. To reduce background on the gel, immunoprecipitation was performed in two steps. Lysate (obtained as in A) was precipitated with anti-IκBα (#1258, MAD-3-C). Precipitates were washed with TNT buffer, boiled in 1% SDS for 5 min, diluted at least 4-fold with TNT and then reprecipitated with fresh anti-IκBα. Samples in lanes 2, 4, 6 and 8 contained competing peptide 1258 during the second precipitation. Each lane contains precipitate from ~9 × 10^6 cells, and the film was exposed for 7 days.

(lane 9, arrow). Thus, the upper band must be composed predominantly of a p50/p65 heterodimer, but may also contain some c-*rel*/p50, while the lower band is either a p50/p50 homodimer or a p50/p52 heterodimer (or both).

In contrast, c-*rel* in WEHI 231 nuclear extracts bound very well to κB-containing probes. A significant fraction of the total bound complex was shifted by anti-c-*rel* (Figure 3D, lane 3), while little or none was shifted by anti-p65 (lane 4). All of it was shifted by anti-p50 (lane 5). Thus WEHI 231 extracts contain a prominent p50/c-*rel* heterodimer, as well as other unidentified p50-containing complexes (with *rel* B? p52?). There was no change in this pattern with extracts from LPS-treated cells: the nuclear p65 detected by immunoblot analysis did not contribute significantly to the total DNA-binding activity (data not shown).

In summary, LPS treatment of 70Z/3 and WEHI 231 cells resulted in loss of IκBα from all pre-existing c-*rel*- or p65-containing complexes. Most, but not all, of the liberated c-*rel* and p65 was transported to the nucleus in 70Z/3 cells, where they were detectable both by immunoblotting and by DNA binding assays. In WEHI 231 cells some of the liberated c-*rel* and p65 reached the nucleus, but most appeared to be bound to newly synthesized IκBα and retained in the cytoplasm.

IκBα has a short half-life

The preceding experiments showed that disruption of IκBα complexes occurred upon activation of the cell. We wondered whether dissociation also occurs at a lower but significant rate in the unstimulated cell. This question was prompted by the observations that in pre-B cells cycloheximide alone results in both nuclear κB binding activity (Sen and Baltimore, 1986b) and transcription of the kappa light chain gene (Wall *et al.*, 1986). Both of these studies suggest that there is a short-lived protein inhibitor of kappa gene expression. If the labile protein is IκBα, then dissociation of IκBα complexes may be a frequent occurrence and not limited to periods of cellular activation.

To investigate the stability of IκBα, WEHI 231 cells were grown in medium containing [^{35}S]methionine and [^{35}S]cysteine for 1 h and then in non-radioactive medium for varying times. Total cell extract was boiled in SDS, precipitated with anti-IκBα and analyzed by SDS−PAGE. The intensely labeled protein band seen in the pulse-labeled sample (Figure 4A, lane 1) was nearly gone after 2 h of chase (lane 3) and continued to decline thereafter (lanes 4−6). The same result was obtained with three different IκBα antibodies directed at different regions of the protein. This result shows that in these murine B cells total cellular IκBα turns over very rapidly. From multiple experiments we estimate a half-life of ~30 min.

To determine whether this result is unique to B cells, in which NF-κB is active constitutively, similar experiments were performed with the murine pre-B cell line 70Z/3 (propagated in either serum-free or serum-containing medium), the human plasmacytoma cell line Sultan, the human T cell line Jurkat and the human epithelial cell line HeLa. The results showed that IκBα turns over rapidly in these cells as well. In all cases the half-life of IκBα was ~1 h (Figure 4B and data not shown).

Most IκBα is complexed to rel and/or p65

The preceding experiments measured the stability of total cellular IκBα. They leave open the possibility that there is a rapidly turning over pool of free IκBα, while complexed IκBα is quite stable. To determine the relative amounts of free versus complexed IκBα, we examined 70Z/3 and Jurkat lysates from which all c-*rel* and p65 had been removed. ^{35}S-labeled cytoplasmic extracts were exhaustively immunoprecipitated with anti-c-*rel* and anti-p65, and the amount of precipitable [^{35}S]IκBα in the supernatant was compared with that in non-depleted lysates. The result was

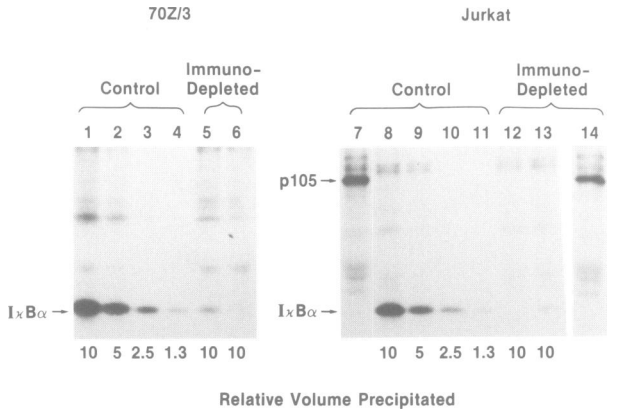

Fig. 5. Most IκBα exists in complexed form. 70Z/3 or Jurkat cells were grown in ^{35}S-containing medium for 1 h. Cytoplasmic extracts were divided in half. One half was treated with excess anti-c-*rel* (#1050, m-*rel*-I; or #1135, h-*rel*-I) and excess anti-p65 (#1226, 65-C); the other half was treated with the same volume of an irrelevant antiserum. After discarding the precipitates, the supernatants were immunoprecipitated with anti-IκBα (#1258, MAD-3-C). To reduce the background this was done twice. The first precipitates were washed, boiled in 1% SDS, diluted and reprecipitated with fresh anti-IκBα. In order to quantify the amount of IκBα in the immunodepleted extracts, the intensity of the precipitated sample (lane 5 for 70Z/3, lane 13 for Jurkat) was compared with that in several different amounts of the control extract (lanes 1–4) for 70Z/3, lanes 8–11 for Jurkat). In both cases the immunodepleted samples contained IκBα at only ~15% of the level seen in the controls. Precipitations for lanes 6 and 12 were performed in the presence of competing IκBα peptide. Samples in lanes 7 and 14 were precipitated twice with anti-p105 (#1140, 105-C) instead of anti-IκBα. Since a strong p105 band remains in the immunodepleted sample (lane 14), the loss of most IκBα cannot be attributed to nonspecific effects of incubation with the c-*rel* and p65 antisera.

that most IκBα was bound to c-*rel* or p65; treating the lysate with anti-c-*rel* and anti-p65 removed about 85% of the total IκBα (Figure 5). We regard this as a minimum estimate of bound IκBα since additional molecules may be bound to p50 homodimers (Beg *et al.*, 1992; Zabel *et al.*, 1993), which were not removed in these experiments. In fact, in a similar study with WEHI 231 cells, the small amount of IκBα not precipitable by anti-c-*rel* and anti-p65 was precipitated by anti-p50 (data not shown). Thus, in a pre-B, a T cell and a B cell line, almost all IκBα is complexed rather than free.

Complexed IκBα also turns over rapidly

The observation that most IκBα is bound and that total IκBα has a short half-life necessarily implies that bound IκBα turns over rapidly. To test this prediction, a specific subset of c-*rel*-containing complexes was examined in a pulse–chase experiment. WEHI 231 cells were labeled, chased for various times and immunoprecipitated with anti-c-*rel*. The washed precipitates were released from antibody by incubation in excess competing peptide, and the eluted (c-*rel*-containing) material was reprecipitated with anti-p65 and analyzed by SDS–PAGE. Since each sample was precipitated sequentially with anti-c-*rel* and anti-p65, each lane of the resulting gel shows complexes that contain both c-*rel* and p65 (Figure 6A). In these complexes both [^{35}S]c-*rel* and [^{35}S]p65 were quite stable over the course of 22 h, suggesting that they associated with each other at or soon after synthesis, and that they remained associated throughout the experiment. In contrast, ^{35}S-labeled IκBα declined rapidly. The intense band seen in the pulse-labeled sample (Figure 6A, lane 1) was much reduced after a 2 h chase (lane 2) and was almost gone after 5 h (lane 3). Thus, the association between newly synthesized IκBα and the c-*rel*/p65 complex was not stable, for the [^{35}S]IκBα disappeared with a short half-life.

Next we tested whether the displaced [^{35}S]IκBα is replaced with new IκBα molecules. As in the preceding experiment, c-*rel*-containing complexes were collected at each time point. These were reprecipitated with anti-IκBα and analyzed by SDS–PAGE. Thus, the gel shows only those complexes that contained both c-*rel* and IκBα (Figure 6B). As in Figure 6A, pulse-labeled [^{35}S]c-*rel* was stable, while [^{35}S]IκBα declined rapidly. However, the fact that anti-IκBα precipitated c-*rel* even after [^{35}S]IκBα had almost disappeared (Figure 6B, lanes 4 and 5) shows that new unlabeled IκBα replaced the lost labeled molecules. This conclusion was confirmed by chasing in the presence of cycloheximide (Figure 6C). The result was that after a 90 min chase anti-IκBα was unable to precipitate [^{35}S]*rel*-containing complexes (compare the c-*rel* band in lanes 2 and 4). Thus, complexed IκBα is unstable. Even in unstimulated B cells it is frequently lost from c-*rel*- and p65-containing complexes and is replaced by a newer molecule.

There are two further predictions. First, in c-*rel*-IκBα complexes, the incorporation of ^{35}S by IκBα and that by c-*rel* should reflect not only their contents of methionine plus cysteine but also their differing half-lives. In fact, the intensity of the IκBα band in Figure 6B, lane 1, is significantly greater than that of c-*rel*. The labeling period in that experiment was 1 h (which is two half-lives of IκBα), so substantial turnover of newly synthesized molecules occurred during that time. When cells were labeled for only 10 min, the difference in incorporation between IκBα and c-*rel* was greatly magnified (to at least a factor of 5), as expected (data not shown).

Second, treatment with a protein synthesis inhibitor would be expected to increase nuclear c-*rel* and p65, as old IκBα decays but synthesis of new IκBα is blocked. To test this, WEHI 231 cells were incubated in cycloheximide for varying times, and nuclear and cytoplasmic extracts were analyzed by immunoblotting (Figure 6D). The results showed relatively little nuclear c-*rel* or p65 after 15 or 50 min of treatment, but a significantly increased level after 3 h in the presence of cycloheximide. There is a substantial amount of nuclear p50 in untreated cells, and this level also increased somewhat after 3 h treatment. (We have observed repeatedly that cycloheximide at 20 μg/ml does not inhibit protein synthesis completely; this may account for the lack of response after 50 min treatment.) Thus, cycloheximide blocks synthesis of most IκBα (Figures 2A and B and 6C) and results in increased levels of nuclear c-*rel*, p65 and p50. This is consistent with the model of unstable association of IκBα with *rel* family complexes.

The rapid turnover of bound IκBα is not unique to B cells. In a pulse–chase experiment with 70Z/3 pre-B cells we examined complexes containing IκBα and c-*rel* or p65. The result was qualitatively the same as that with B cells. A constant level of ^{35}S-labeled c-*rel* or p65 was associated with IκBα, but [^{35}S]IκBα declined with a half-life of ~1 h (see below). Thus, even in non-B cells IκBα complexes are dynamic. Newly synthesized IκBα remains associated with *rel* or p65 for a relatively short time, is lost and is

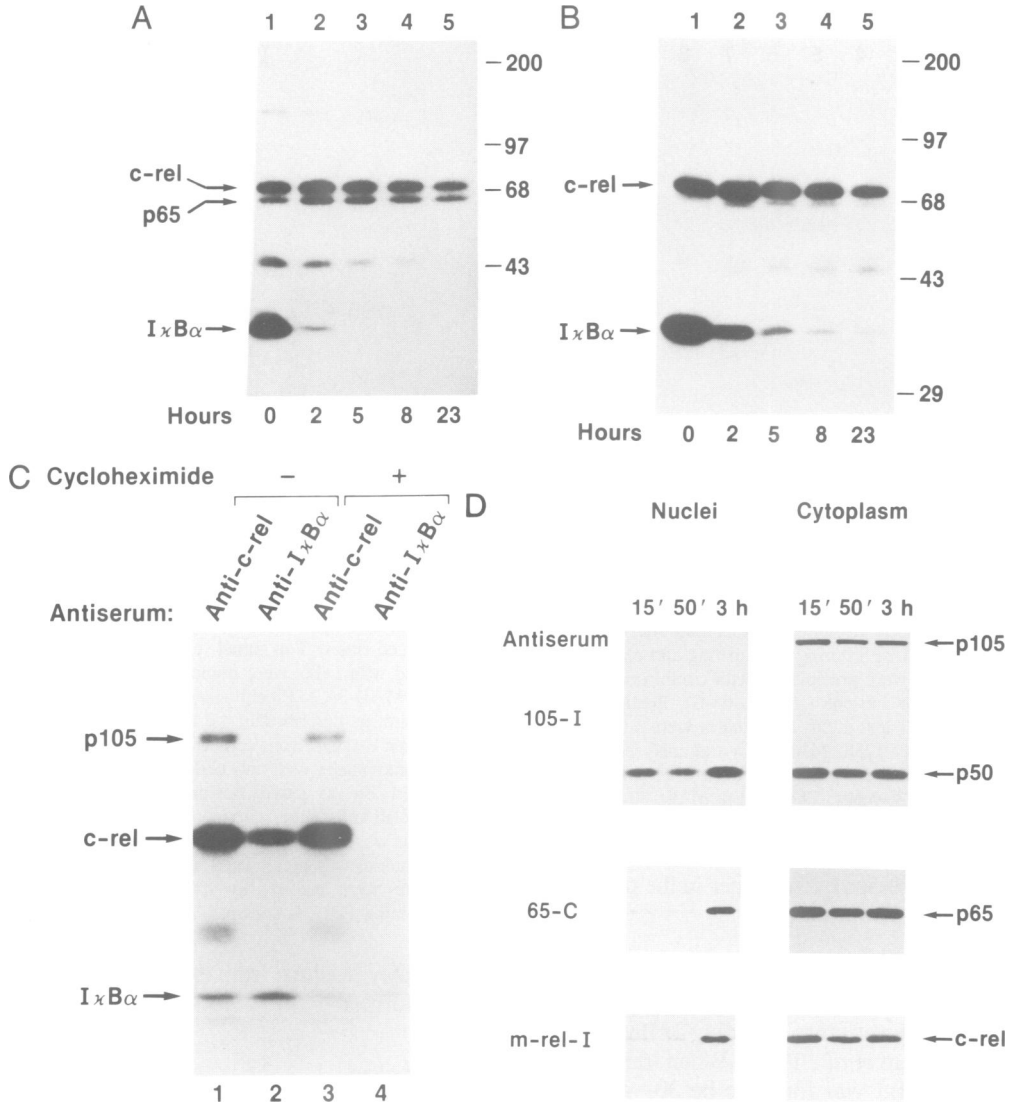

Fig. 6. Complexed IκBα turns over rapidly. (**A**) WEHI 231 cells were labeled and chased as detailed in the legend to Figure 5. Cells were lysed in TNT buffer, and lysates were immunoprecipitated with anti-c-*rel* (#1051, m-*rel*-I). Precipitates were washed and then released from antibody by incubation with excess competing peptide 1051. Eluates were reprecipitated with anti-p65 (#1226, 65-C) and analyzed by SDS−PAGE. (**B**) WEHI 231 cells were labeled and chased, and lysates were immunoprecipitated and eluted with anti-c-*rel* and peptide 1051, respectively, as in (A). Eluates were reprecipitated with anti-IκBα (#1258, MAD-3C) and analyzed by SDS−PAGE. (**C**) WEHI 231 cells were ^{35}S-labeled and chased for 90 min with (lanes 3 and 4) or without (lanes 1 and 2) cycloheximide at 20 μg/ml. Lysates were immunoprecipitated with anti-c-*rel* (#1050, m-*rel*-I), eluted with peptide 1050, and reprecipitated with anti-IκBα (#1258, MAD-3C). In parts A, B and C, each lane contains precipitate from ~10^7 cells and the films were exposed for 3−5 days. (**D**) WEHI 231 cells were incubated in complete medium plus cycloheximide (20 μg/ml) for 15 min, 50 min or 3 h. For each time, nuclear and cytoplasmic extracts were analyzed by immunoblotting with anti-c-*rel* (#1158, *rel*-I), anti-p65 (#1226, 65-C) or anti-p50 (#1157, 105-I). Each lane contains extract from ~5 × 10^5 cells.

replaced by a newer molecule. Since the decay rate mirrors the turnover rate of total IκBα, the lost molecule is most likely degraded.

IκBα turnover in the presence of LPS

The preceding experiments have shown that in unstimulated cells nearly all IκBα is complexed with *rel* family members and that it turns over rapidly (half-life 30−60 min). They also demonstrated that treatment with an activating agent accelerates this normal process: all pre-existing IκBα disappeared from *rel*-containing complexes by 90 min after addition of LPS. These findings predict that the turnover time of IκBα should be reduced in LPS-treated cells. To test this, 70Z/3 cells were incubated for 3 h with or without LPS, then ^{35}S-labeled for 45 min and chased in non-radioactive medium for varying times. (The LPS-treated cells also received LPS during the labeling and chase periods.) c-*rel*- and p65-containing complexes were precipitated, eluted with peptides and reprecipitated with anti-IκBα. The resulting gel therefore shows only those complexes containing IκBα and c-*rel* and/or p65 (Figure 7). The result was a noticeable decrease in the turnover time of bound [^{35}S]IκBα in LPS-treated cells. Its half-life in untreated cells was ~1 h (lanes 1−4), but it was considerably less than that in LPS-treated cells (lanes 5−8). Examining total rather than bound IκBα gave the same result (data not shown). Thus, the half-life of IκBα, already short in

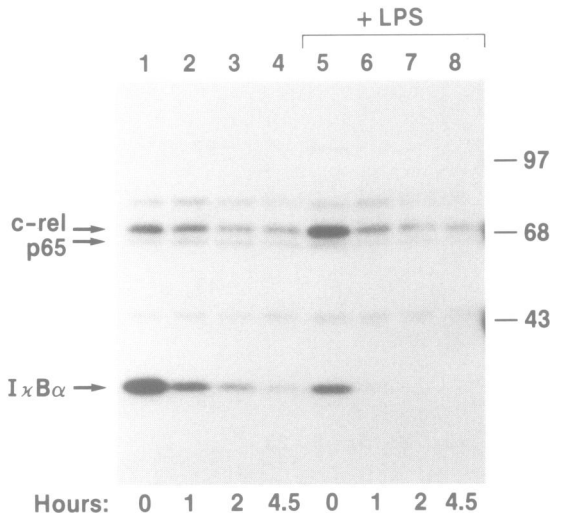

Fig. 7. Turnover of IκBα in the presence of LPS. 70Z/3 cells were grown with or without LPS (15 μg/ml) for 3 h, labeled in medium containing [^{35}S]methionine and [^{35}S]cysteine (100 μCi/ml each) for 45 min and then chased as described in the legend to Figure 4B. The LPS-treated cells also received LPS during the labeling and chase periods. Cytoplasmic extracts were precipitated with combined anti-c-*rel* (#1051, m-*rel*-I) and anti-p65 (#1226, 65-C). Precipitates were eluted with peptides 1051 and 1226, and eluates were reprecipitated with anti-IκBα (#1258, MAD-3-C) and analyzed by SDS−PAGE. Each lane contains the precipitate from ~2 × 10^7 cells, and the film was exposed for 3 weeks. The identity of the 80 kDa band is unknown; the protein at 45 kDa is most likely p50.

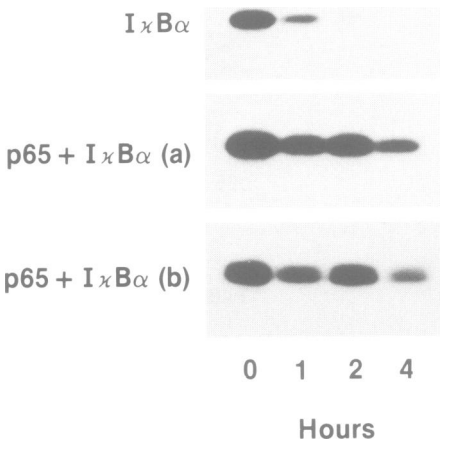

Fig. 8. Stability of IκBα in transfected cells. Human 293 cells were transfected with an expression vector encoding IκBα (Haskill *et al.*, 1991) with or without a second expression vector encoding p65 (Ruben *et al.*, 1992b). 48 h later, cells were grown in medium containing [^{35}S]methionine for 1 h and then chased in non-radioactive medium for the indicated times. **Top panel**: cytoplasmic extracts from cells transfected with IκBα were immunoprecipitated with anti-IκBα (#1258, MAD-3-C). Precipitates were boiled and reprecipitated with fresh serum, as described in the legend to Figure 4B. **Middle panel**: cytoplasmic extracts from cells cotransfected with IκBα and p65 were immunoprecipitated with anti-IκBα, boiled and then reprecipitated with fresh anti-IκBα, as above. **Bottom panel**: cytoplasmic extracts from cotransfected cells were immunoprecipitated with anti-IκBα. The precipitate was eluted from antibody with excess peptide, and the eluted material was precipitated with anti-p65 (#1226, 65-C). Precipitates were analyzed by SDS−PAGE. Only the portion of the gel containing IκBα is shown.

unstimulated cells, is decreased even further in the presence of an activating agent.

Stability of IκBα in transfected cells

Two recent papers examined the stability of IκBα in cells transfected with expression vectors encoding IκBα and/or p65 (Scott *et al.*, 1993; Sun *et al.*, 1993). When transfected alone, the half-life of IκBα was found to be 30−40 min. However, when cotransfected with p65, or when induced by overexpression of p65, IκBα was very stable, with a half-life of many hours. These results led to the conclusion that while free IκBα turns over rapidly, bound IκBα does not. Yet our experiments reported above showed that complexed IκBα is unstable. We have therefore tested whether this difference is attributable to experimental methodology or to the use of transfected versus non-transfected cells.

We transfected cells with IκBα DNA with or without p65 DNA, and we examined the stability of IκBα protein using the two-step immunoprecipitation protocols detailed above. The results are in complete agreement with those of Sun *et al.* (1993) and Scott *et al.* (1993). In a pulse−chase experiment, transfected IκBα had a half-life of less than 1 h and was only barely detectable after 2 h (Figure 8, top panel). In contrast, IκBα was much more stable when cotransfected with p65. This was true regardless of whether immunoprecipitation was performed with anti-IκBα alone (Figure 8, panel a), or with sequential anti-IκBα and anti-p65 (panel b). In a separate experiment we examined the stability of IκBα induced by overexpression of transfected p65. Just as shown by Scott *et al.* (1993), the induced IκBα was very stable, with no detectable decrease after a 7 h chase (data not shown). Thus, there is a real difference in the stability of IκBα in transfected versus non-transfected cells, presumably resulting from the sustained high level expression in the former.

Discussion

We report here on two key features of IκBα regulation of NF-κB in the unstimulated cell. First, we showed that essentially all IκBα is complexed to *rel* family members in the cytoplasm: there is little or no free IκBα. Second, in contrast to c-*rel* or p65, IκBα is unstable, turning over with a half-life of ~30 min (WEHI 231 B cells) to 60 min (various non-B cell lines). This implied that complexes themselves are unstable, with frequent dissociation and/or degradation of the IκBα—and we were able to demonstrate this directly. Synthesis of new IκBα is rapid enough that most liberated *rel* family dimers are efficiently captured by new IκBα and retained in the cytoplasm.

The experiments reported here also reveal aspects of IκBα regulation in the activated cell. First, in response to LPS, all previously synthesized IκBα was gradually lost from *rel*- and p65-containing complexes in both WEHI 231 and 70Z/3 cells. Since the lost IκBα was not detectable by immunoprecipitation, it was probably rapidly degraded. Dissociation of complexes was correlated with the appearance of *rel* family members in the nucleus. These results complement those from recent experiments using TNFα, where treatment resulted in maximal nuclear κB-binding activity after only a few minutes. The level of total cellular IκBα was monitored by immunoblotting and was found to decline drastically almost immediately, correlating

nicely with the rapid rise in DNA-binding activity (Brown *et al.*, 1993; Sun *et al.*, 1993). While this manuscript was being reviewed, two additional studies documented the loss of IκBα following treatment of cells with various activating agents, including LPS (Beg *et al.*, 1993; Cordle *et al.*, 1993).

Second, during the relatively slow LPS-induced process, some of the lost IκBα molecules were replaced with newly synthesized ones: even after 90 min of treatment, anti-IκBα still precipitated some previously synthesized c-*rel* and p65, even though all of the old IκBα had disappeared. Thus even during stimulation of the cell, not every dissociation event results in nuclear translocation. Rather, the high rate of IκBα synthesis results in modulation of the response to LPS and prevents an all-out migration to the nucleus. These competing processes of association and dissociation may be detectable only with a relatively weak activator such as LPS. With a strong signal such as TNFα (Hohmann *et al.*, 1990), the speed and extent of the reaction may preclude their observation, at least initially.

Third, the fact that some liberated dimers were able to associate with newly synthesized IκBα strongly suggests that the LPS-induced signal causes disruption of complexes rather than preventing their association, and that complexes are broken due to modification of IκBα, and not of the dimers.

We wish now to consider the unstimulated cell in more detail. First, why is there little or no free IκBα in the unstimulated cell? Excess inhibitor could interfere with response to an activating signal, so the advantage of this arrangement is evident. But how is the precise balance between NF-κB and IκBα maintained? Roughly coordinated rates of synthesis are probably involved, but it is also possible that free IκBα (i.e. never complexed, as distinguished from dissociated) is more unstable than complexed IκBα, just as indicated by the transfection experiments (Figure 8; Scott *et al.*, 1993; Sun *et al.*, 1993). It is not clear why cotransfected IκBα is so stable relative to the endogenous IκBα in the various cell lines we examined. However, if the transfected cells accurately reflect the normal *ratio* of the half-lives of complexed versus free IκBα, then free IκBα in non-transfected cells may survive for only a few minutes at most.

Why is complexed IκBα unstable? (i) Perhaps the association between *rel* family members and IκBα is not strong. In that case occasional dissociation events might result in free IκBα being recognized and degraded. We do not favor this explanation even though there is no direct evidence to rule it out. In our experience isolated IκBα-containing complexes are stable for days and survive multiple purification procedures. (ii) Perhaps complexed IκBα is inherently unstable due to some feature of its structure that marks it for rapid turnover. In that case we need not invoke a separate dissociation event, but only recognition and degradation. However, this theory requires a second mechanism to account for the response of IκBα to activating signals. (iii) Alternatively, perhaps the complexed molecule is stable until it is modified in some way by normal cellular processes. For example, perhaps the kinase that phosphorylates IκBα in an activated cell operates constitutively at a low level in the unstimulated cell. Phosphorylation of IκBα might result in its recognition and degradation, perhaps following its dissociation from the complex. This is an attractive hypothesis because it postulates a single mechanism to account for events in both the unstimulated and the activated cell, but there is as yet no evidence to support it. Whatever the basis for IκBα's instability in the cell lines examined here, there is no *a priori* reason to think that *all* cells have such unstable IκBα. For example, if the hypothetical kinase mentioned above has little or no constitutive activity, complexed IκBα might be more stable. On the other hand it is important to stress that the results we present are not limited to the WEHI 231 B cell line, but pertain to a pre-B, a T, a plasmacytoma and an epithelial cell line as well.

The cost of maintaining this system is considerable. A 1 h half-life for IκBα means that it will be replaced in half of all the complexes every hour, even in the absence of an activating signal. Looked at another way, if the half-life of IκBα-bound c-*rel* and p65 is ~8 h, IκBα is produced at at least 8 times the rate of their synthesis. What is the advantage to the cell of such a seemingly wasteful system? One possibility is that it generates the low level of NF-κB activity that is presumably necessary even in unstimulated cells. At a minimum, dividing cells must double their NF-κB content once per cycle, and active NF-κB is probably necessary to accomplish this [the genes for both p105 and c-*rel* have κB sites in their promoter regions (Hannink and Temin, 1990; Capobianco and Gilmore, 1991; Ten *et al.*, 1992; T.Kochel and N.R.Rice, unpublished)]. The continual turnover of IκBα may provide the opportunity for small amounts of liberated c-*rel*/p65/p50 to enter the nucleus. In contrast, if the complexes were very stable in the absence of an activation signal, there would be no active NF-κB for 'house-keeping' purposes.

A second possible advantage of the system follows from the high rate of IκBα synthesis, which may provide the ability to curtail NF-κB activity rapidly once the activating signal subsides. It has been suggested that IκBα is not only the major cytoplasmic inhibitor of NF-κB, but that it is also responsible for turning off active NF-κB in the nucleus (Zabel *et al.*, 1993). The hypothesis is that some excess IκBα molecules may enter the nucleus, bind to active NF-κB and release it from DNA. However, there can only be excess IκBα if its rate of synthesis exceeds that of the c-*rel* and p65 to which it binds. If their rates of synthesis were the same, all new IκBα would bind to new c-*rel*/p65: there would never be uncomplexed inhibitor, even in the presence of an activating agent. In fact, the rate of synthesis of IκBα considerably exceeds that of the c-*rel*/p65 to which it binds. We showed that under 'resting' conditions enough IκBα is made not only to bind new c-*rel*/p65, but also to replace old IκBα in pre-existing dimers. Under activating conditions, however, some or many of those pre-existing dimers will have left the cytoplasm for the nucleus, thus creating an imbalance between the amount of newly synthesized IκBα and its available targets. If some of the excess IκBα escapes degradation and enters the nucleus, it could inactivate some of the translocated dimers. Such a system ensures that maintenance of NF-κB activity depends on continued migration of liberated dimers from cytoplasm to nucleus, i.e. on the continued presence of the activating signal. If the stimulus abates and no new dimers reach the nucleus, the nuclear IκBα can turn off the response very quickly. Thus, even before the increased transcription of the IκBα gene that is induced by activating agents (Haskill *et al.*, 1991; Tewari *et al.*, 1992; Brown *et al.*, 1993; Sun *et al.*, 1993), the

system is very responsive to changes in the environment. The important point is that it requires that IκBα and its target p65 and c-*rel* molecules be synthesized at different rates. Thus, viewing this regulatory sytem as a whole, storage of active transcription factor in the cytoplasm enables instantaneous gene activation in response to cell stimulation. Constitutive synthesis of excess inhibitor enables instantaneous damping of activity upon cessation or diminution of stimulation.

Materials and methods

Cells and reagents

Mouse cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, and, in the case of 70Z/3, 50 μM 2-mercaptoethanol. The serum contained < 10 endotoxin units per ml by the limulus amebocyte lysate assay (Gibco). Human 293 cells were grown in DMEM plus 10% serum. Serum-free medium (Aim-V) was obtained from Gibco, *Escherichia coli* LPS from Sigma, and cycloheximide from Boehringer-Mannheim.

Antisera

All of the antisera were raised in rabbits against synthetic peptides coupled to keyhole limpet hemocyanin. The peptide sequences for #1050, 1051, 1135, 1140, 1157, 1207, 1226 and 1258 were published previously (Kochel *et al.*, 1991; Rice *et al.*, 1992). #1158 is NH_2-Val-Thr-Lys-Asn-Asp-Pro-Tyr-Lys-Pro-His-Pro-His-Asp-Leu-Val-Gly-Lys-Cys (residues 66–82 of murine c-*rel*), and #1266 is NH_2-Cys-Glu-Gln-Leu-Ser-Asp-Pro-Phe-Thr-Tyr-Gly-Phe-Phe-Lys-Ile (residues 573–586 of murine c-*rel*) (Bull *et al.*, 1990). #1263 is NH_2-Ala-Asp-Asp-Asp-Pro-Tyr-Gly-Thr-Gly-Gln-Met-Phe-His-Leu-Cys (residues 2–15 of murine p105) (Ghosh *et al.*, 1990). A cysteine residue at the N- or C-terminus of #1158, #1266 and #1263 was added to allow coupling to hemocyanin, as described by Liu *et al.* (1979). Peptides were purchased from Multiple Peptide Systems (San Diego, CA).

Metabolic labeling

Cells were washed in phosphate-buffered saline (PBS) and then resuspended in methionine- and cysteine-free medium containing 10% dialyzed fetal calf serum and [^{35}S]methionine and [^{35}S]cysteine (each at 50–100 μCi/ml). Cells were incubated at 37°C for 30–60 min. The dialyzed serum was omitted when 70Z/3 cells propagated in serum-free medium were labeled.

Immunoprecipitations

For whole cell extracts, cells were lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM sodium chloride, 2 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.25% SDS). For cytoplasmic extracts TNT-E was used (20 mM Tris, pH 7.5, 50 mM sodium chloride, 1% Triton X-100, 5 mM EDTA). Lysates were cleared by centrifuging at 10 000 *g* for 10 min. For single-cycle immunoprecipitations, lysate from ~10^7 cells was incubated overnight at 4°C with 10 μl antiserum (or 5 μl for 70Z/3 and Jurkat cells) in a total volume of 1 ml. Precipitates were collected on protein A–Sepharose (Pharmacia), washed in TNT-E, boiled in loading dye and electrophoresed on 10% SDS–polyacrylamide gels. Gels were soaked in 1 M sodium salicylate for 30 min, dried and autoradiographed at −70°C.

For two-cycle immunoprecipitations, lysate from ~10^8 cells was incubated with 100 μl antiserum (or 50 μl for 70Z/3 and Jurkat cells) in a total volume of ~5 ml and precipitates were collected on protein A–Sepharose. The washed precipitate was resuspended in about 0.5 ml TNT-E and incubated overnight at 4°C with 100 μg (or 50 μg) cognate peptide. The supernatant was collected, aliquoted, reprecipitated with other antisera and analyzed by SDS–PAGE as above.

Immunoblotting

Cell pellets were washed in PBS, then in hypotonic buffer (HB) (25 mM Tris, pH 7.4, 1 mM magnesium chloride, 5 mM potassium chloride) and then incubated in HB for 15 min on ice. An equal volume of HB containing 1% NP-40 was added, and nuclei were pelleted by centrifugation at 500 *g* for 5 min. The supernatant constitutes the cytoplasmic fraction. Nuclei were resuspended in 20 mM Tris, pH 8, 25% glycerol, 0.42 M sodium chloride, 1.5 mM magnesium chloride, vortexed vigorously and agitated at 4°C for ~30 min. Debris was pelleted at 900 *g* for 5 min; the supernatant constitutes the nuclear fraction. Equal cell equivalents of nuclear and cytoplasmic fractions were added to loading dye, boiled, electrophoresed on a 10% gel and transferred to Immobilon-P (Millipore). Filter strips were incubated in primary antibody for 30 min (1:1000 dilution for 1050 and 1226; 1:2000 for 1158 and 1157), then in peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim) at 1:10 000 for 30 min and then analyzed using Amersham's enhanced chemiluminescence system.

Cell fractionation for EMSA

10^8 cells were pelleted, washed in PBS and resuspended in 1 ml buffer A (10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% NP-40). After 10 min on ice, the lysate was centrifuged at 500 *g* for 10 min; the supernatant is the cytoplasmic fraction. The nuclear pellet was resuspended in 20 μl buffer B (25 mM HEPES, pH 7.6, 1 M KCl, 0.1 mM EDTA, 1 mM DTT) with frequent vortexing for 15 min at 4°C. After centrifuging at 20 000 *g* for 20 min, the supernatant was diluted with buffer C (25 mM HEPES, pH 7.6, 20% glycerol, 0.1 mM EDTA, 1 mM DTT) at a ratio of 1 part supernatant to 3.75 parts buffer C. This mixture constitutes the nuclear extract.

Oligonucleotides for EMSA

Oligonucleotides were synthesized by an Applied Biosystems synthesizer and were used without purification. The sequences are: murine intronic kappa chain κB site (underlined) (Sen and Baltimore, 1986a) with artificial flanking sequences, 5′-GAATTCCTGGGGGACTTTCCGAGAATTC-3′; the IL-6 promoter κB site (underlined), with its natural flanking sequences, 5′-TCAAATGTGGGATTTTCCCATGAGTCT-3′ (Libermann and Baltimore, 1990). After renaturing complementary strands, 200 ng DNA was labeled in 10 μl by incubation with [^{32}P]ATP and T4 polynucleotide kinase. This mixture was brought up to 200 μl with 10 mM Tris, pH 7.5, 1 mM EDTA and the probe was purified twice on a G-50 spin column.

Electrophoretic mobility shift assay (EMSA)

The binding reaction mixture was 10 mM HEPES, pH 7.5, 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 0.5 μg poly(dIC), 0.5 μg sonicated double-stranded salmon sperm DNA, ^{32}P-labeled oligonucleotide (= 1 ng, ~5000–10 000 c.p.m.) and nuclear extract (= 5 μg protein). The mixture was incubated at room temperature for 30 min. For supershift assays, the reaction mixture minus the probe was incubated with 1 μl antiserum for 15 min at room temperature. The ^{32}P-labeled oligonucleotide was then added and incubation continued for 30 min. Products were analyzed on 5% acrylamide gels made up in 0.5 × TBE and 0.001% NP-40.

Transfection experiments

Human 293 cells in 6 cm dishes were transfected by the calcium phosphate procedure with 4 μg plasmid DNA. 48 h later the cells were grown in medium containing [^{35}S]methionine (50 μCi/ml) for 1 h and then chased in non-radioactive medium. Individual dishes were harvested at various times thereafter. Cells were lysed in TNT-E and immunoprecipitated as described in the legend to Figure 8. For the top two panels in that figure each lane represents the precipitate from about 10% of a dish, while for the bottom panel, each lane represents the precipitate from ~50% of a dish.

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