NF-κB/Rel family members are physically associated phosphoproteins

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We performed radioimmunoprecipitation followed by serial immunoblots to show that, in the unstimulated Jurkat T cell line, the NF-κB/Rel family proteins, p80-c-Rel, p105-NF-κB, p65-NF-κB, p50-NF-κB and p36-IκBα, can be detected as complexes using antisera against c-Rel, p105-NF-κB or p65-NF-κB. p36-IκBα and p105, both known inhibitors of NF-κB function, can physically associate with NF-κB/Rel family members, but not with each other. *In vivo* and *in vitro* phosphorylation experiments demonstrated that NF-κB/Rel family members, including p105, c-Rel, p50, p65 (for the first time for p50 and p65) and p36-IκBα are also phosphoproteins. Phosphoserine and phosphothreonine residues were identified in these proteins isolated from unstimulated Jurkat cells. Both unphosphorylated and hyperphosphorylated forms of p36-IκBα were found in the complexes, suggesting that hyperphosphorylated IκBα is still capable of associating with the NF-κB/Rel family members. After stimulation with phorbol 12-myristate 13-acetate and phytohaemagglutinin for 10 min, p105-NF-κB and p50-NF-κB, but not p36-IκB, were highly phosphorylated. Phosphopeptide mapping of p105 showed that phorbol ester/phytohaemagglutinin stimulation may change p105 phosphorylation qualitatively.

INTRODUCTION

NF-κB was originally characterized as a multisubunit nuclear factor that binds a specific DNA motif in the κ light-chain enhancer to turn on κ-chain expression (Sen and Baltimore, 1986). It was later found to be ubiquitous; however, except in mature B-cells, monocytes and certain T-cell lines, NF-κB is usually inactive. Recent molecular cloning has identified many members of the NF-κB/Rel family of transcription factors including p50-NF-κB (also known as NFKB1) with its precursor p105-NF-κB, p65-NF-κB (RelA), p50B-NF-κB (also known as NFKB2, p55 or lyt-10) with its precursor p98-NF-κB, c-Rel, I-Rel (RelB in the mouse) and the *Drosophila* morphogen Dorsal [reviewed in Lenardo and Baltimore (1989), Baeuerle (1991), Gilmore (1991) and Blank et al. (1992)]. These proteins share structural and functional similarities in their N-termini, referred to as the Rel homology domains, which are essential for DNA-binding, nuclear localization and dimerization functions.

The most extensively characterized NF-κB complex contains p50 and p65. The inactive p50/p65 heterodimer is associated with an inhibitor molecule, IκB, and is localized in the cytoplasm (Baeuerle and Baltimore, 1988a,b; Zabel and Baeuerle, 1990). In response to a variety of stimuli such as mitogens, cytokines and viral infection, IκB may become phosphorylated and dissociate from the complex (Baeuerle and Baltimore, 1988a,b; Kerr et al., 1991). The active p50/p65 NF-κB then translocates to the nucleus, binds to the κB motif and functions as a transcriptional regulator. To date, most of the information has been obtained from gel-shift assays and *in vitro* studies. Detailed molecular mechanisms obtained from *in vivo* studies are not readily available. However, physical association among various family members and IκBs, and phosphorylation changes apparently play important roles.

C-Rel has been reported to be complexed with cellular proteins including p105-NF-κB, an unidentified 125 kDa protein and a 36–40 kDa inhibitor, MAD-3, recently renamed IκBα (Simek and Rice, 1988; Lim Tung et al., 1988; Morrison et al., 1989; Lim et al., 1990; Davis et al., 1990a,b; Kochel et al., 1991; Capobianco et al., 1992; Rice et al., 1992). Either cytoplasmic p105-NF-κB or p36-IκBα can physically associate with c-Rel or p65-NF-κB, and sequester these transcription factors in the cytoplasm (Neumann et al., 1992; Rice et al., 1992; Naumann et al., 1993). Although the inactive p105/c-Rel or p105/p65 complex can be further processed into a p50/c-Rel or a p50/p65 complex in stimulated cells (Rice et al., 1992), it is not clear whether p50 and p65 are contained in the c-Rel complex in unstimulated cells. The physical complexing among different members of the NF-κB/Rel family and numerous IκBs present a complex system which is subject to precise regulation, much of it apparently mediated by protein kinases and protein phosphatases.

In vitro studies showed that phosphorylation with protein kinase C, haem-regulated eIF2α kinase and cyclic AMP-dependent protein kinase, presumably through IκB molecules, results in activation of NG-κB (Baeuerle and Baltimore, 1988a,b; Ghosh and Baltimore, 1990; Kerr et al., 1991). c-Rel and v-Rel have been reported to be phosphorylated on serine and threonine residues (Simek and Rice, 1988; Mosialos et al., 1991). A recent report further suggested that c-Rel and p105 are both phosphorylated on serine, threonine and tyrosine residues in stimulated Jurkat cells (Neumann et al., 1992). However, it remains to be shown whether p65 and p50 are phosphoproteins and if phosphorylation of the family members is involved in regulation of NF-κB *in vivo*. In the present report, we demonstrate in unstimulated cells that p105-NF-κB, p65-NF-κB, p50-NF-κB, p80-c-Rel and p36-IκBα can all be detected in c-Rel immunoprecipitates. These physically associated proteins are all phosphorylated on serine and threonine residues. We also studied the changes in phosphorylation of NF-κB proteins and IκBα after phorbol 12-myristate 13-acetate (PMA) phytohaemagglutinin (PHA) stimulation.

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; PHA, phytohaemagglutinin; PVDF, poly(vinylidene) difluoride.

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MATERIALS AND METHODS

Western (immuno-) blot analyses

Crude cell lysates were analysed by Western (immuno-) blot as previously described (Li et al., 1987, 1993). The antiserum dilution typically used was 1:5000. In combined radioimmunoprecipitation and Western-blot analysis, the membrane was first exposed to X-ray film to detect radioactive proteins and then immunoblotted with different antisera in succession without removing the reactivity detected in the previous analysis. The reactivity was measured by an enhanced chemiluminescence detection system (Amersham) in which light emission is detected within seconds rather than the hours or days needed to detect ^{32}P- or ^{35}S-labelled proteins.

Metabolic labelling of cells

Jurkat cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Whittaker Bioproducts), 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were labelled with 0.1 mCi/ml (1000 Ci/mmol) [^{35}S]methionine (ICN) in methionine-free medium at a density of 5×10^6/ml for approx. 16 h, or with 0.5 mCi/ml [^{32}P]P_i (Dupont/New England Nuclear) in phosphate-free RPMI 1640 for 3 h. For activation experiments, cells were labelled with [^{32}P]P_i for 2 h with the addition of PHA (1 μg/ml) and PMA (50 ng/ml) for the last 10 min.

Immunoprecipitation

[^{35}S]Methionine- or [^{32}P]P_i-labelled cells were washed twice with PBS, lysed in RIPA buffer (20 mM Tris/HCl, pH 7.6, 2 mM EDTA, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 1% aprotinin, 70 μg/ml phenylmethanesulphonyl fluoride, 40 μg/ml Tos-Phe-CH_2Cl, 5 μg/ml Tos-Lys-CH_2Cl, 5 μg/ml leupeptin) containing, for ^{32}P-labelled cells, phosphatase inhibitors (5 mM NaF, 1 mM sodium vanadate, 0.5 mM sodium pyrophosphate). The lysates were clarified by centrifugation at 12000 *g* for 30 min and incubated with antisera in the presence or absence of competing synthetic peptides (1 μg for each μl of polyclonal antiserum). The immunocomplexes were collected with Protein A–Sepharose beads, washed, boiled and resolved by SDS/PAGE, electrophoretically transferred to poly(vinylidene difluoride) (PVDF) membrane (Millipore), and visualized by autoradiography. IκBα antiserum was raised against a C-terminal synthetic peptide corresponding to residues 300–317 of the MAD-3 sequence (Haskill et al., 1991). Anti-(c-Rel 1135) (kindly provided by N. Rice), anti-(p105N 1141), anti-(p105C 1140) and anti-(p65 1226) were generated as described by Rice et al. (1992) against synthetic peptides corresponding to residues of c-Rel 493–509, p105 2–15, p105 955–969 and p65 537–550 respectively. The sequences of the immunogenic peptides were specifically selected to avoid cross-reactivity with other members of the family.

In vitro phosphorylation

Jurkat cells were harvested, washed, lysed and the lysates were immunoprecipitated as described above. The washed immunocomplexes were incubated in 50 μl of phosphorylation buffer (10 mM Hepes, pH 7.5, 10 mM $MnCl_2$) containing 2 μl of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) at 37 °C for 15 min. The reactions were stopped by removing the supernatant and adding Laemmli loading buffer (Laemmli, 1970). After boiling, samples were subjected to SDS/PAGE (10% polyacrylamide), Western transfer and autoradiography. After the detection of ^{32}P-labelled proteins, the membrane was probed sequentially with different antisera.

Phosphoamino acid analysis

The radioactive proteins were located on PVDF membranes by autoradiography and the appropriate areas of the membrane excised for hydrolysis. The membrane slices were wet briefly in methanol, washed with 10 ml of distilled water and hydrolysed at 110 °C for 1.5 h in 6 M HCl. The hydrolysed samples were evaporated to dryness and subjected to one-dimensional thin-layer high-voltage electrophoresis as described by Farrar and Ferris (1989).

RESULTS

Specificities of antisera

We first performed Western-blot analysis to determine the specificity of each antiserum (Figure 1). Jurkat cell lysates were analysed with immune sera (lanes 1, 3, 5, 7, 9) or immune sera preincubated with competing synthetic peptides (lanes 2, 4, 6, 8, 10), against which the antisera were raised. In addition to the p80-c-Rel (Figure 1, lane 1, solid circle), the anti-c-Rel serum specifically detected several other proteins (compare lanes 1 and 2) with apparent molecular masses of 100, 64 and 45 kDa (p100, p64 and p45 respectively). These additional proteins could be either truncated c-Rel molecules or proteins containing sequences related to the synthetic peptide. As it is not intended in this report to distinguish between these two alternatives, we refer to all these as sequence-related proteins (arrowheads for those species also detected in immunoprecipitations shown in Figures 2 and 3a). The p105N antiserum (specific for an N-terminal p105

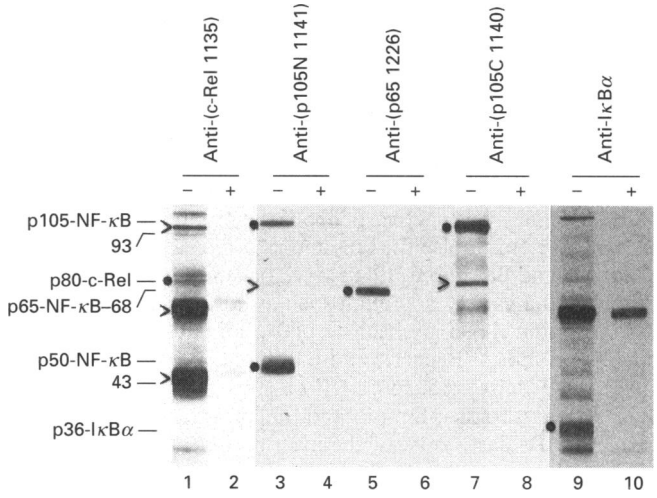

Figure 1 Antisera specificities analysed by Western blot using Jurkat extract

Total cell extract from Jurkat cells was resolved by preparative SDS/PAGE and transferred to PVDF membrane. The membrane was sliced into individual strips (80 μg of protein/strip) and incubated with the designated antisera with (lanes 2, 4, 6, 8, 10) or without (lanes 1, 3, 5, 7, 9) preincubation with the competing synthetic peptides (indicated by + or −). The expected proteins directly recognized by the antisera are indicated by solid circles. Sequence-related proteins recognized by the antisera are indicated by arrowheads; 93, 68 and 43 kDa markers are also indicated.

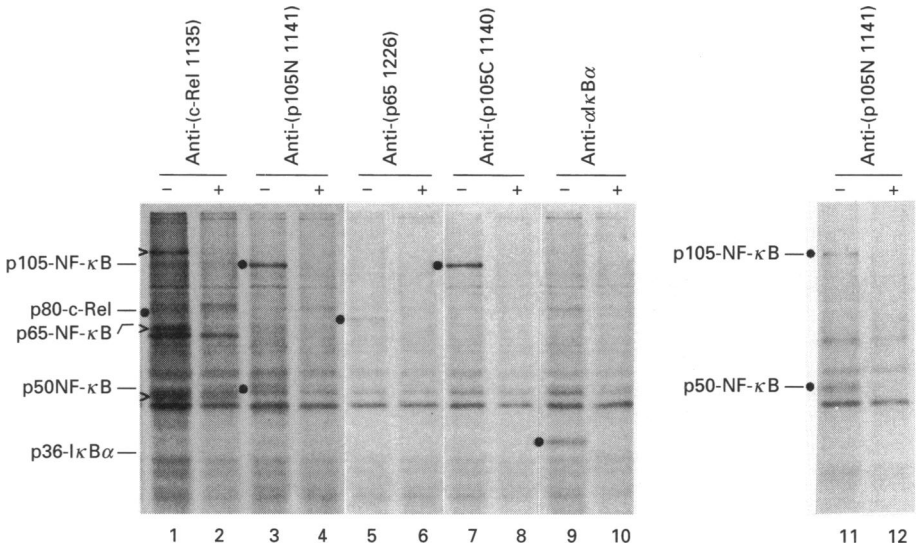

Figure 2 Antisera specificities analysed by immunoprecipitation

[^{35}S]Methionine-labelled Jurkat cells were lysed and boiled in RIPA buffer containing 0.5% SDS. The diluted (to a final SDS concentration of 0.1%) and cleared lysates were immunoprecipitated with the designated antisera with (lanes 2, 4, 6, 8, 10, 12) or without (lanes 1, 3, 5, 7, 9, 11) the presence of competing peptides (indicated by + or −). The washed immunoprecipitates were analysed by SDS/PAGE, fluorography and autoradiography. Lanes 11 and 12 show the same result, but with p50 better resolved, as in lanes 3 and 4. Solid circles and arrowheads mean the same as in Figure 1.

sequence), capable of reacting with both the NF-κB p50 and its precursor p105, detected p50, p105 (Figure 1, lane 3, solid circles) and, at a very low level, a 75 kDa sequence-related protein (Figure 1, lane 3, arrowhead). The p105C antiserum (specific for a C-terminal p105 sequence), on the other hand, specifically detected p105 (Figure 1, lane 7, solid circle) and a sequence-related protein also with a molecular mass of 75 kDa (Figure 1, lane 7, arrowhead), but not p50 which lacks the C-terminal portion of p105. The p65 antiserum detected only p65-NF-κB (Figure 1, lane 5, solid circle). Although the IκBα antiserum was reactive with multiple cellular proteins, it specifically reacted with p36-IκBα (Figure 1, lane 9, solid circle).

Radioimmunoprecipitation was also carried out after the [^{35}S]methionine-labelled cells were lysed by boiling in RIPA buffer containing SDS. This assay allows the detection of only the sequence-identical or -related proteins and not the physically associated proteins. Results (Figure 2) similar to those shown in the Western blots (Figure 1) were obtained showing that each antiserum reacted with the expected protein(s) (solid circles) and sequence-related proteins (arrowheads).

Physical associations of NF-κB/Rel family proteins

Having determined the specificity of each antiserum, we next wished to determine which other proteins might be coimmunoprecipitated by each antiserum. [^{35}S]Methionine-labelled Jurkat cells were lysed and immunoprecipitated, in the absence of SDS, using the same panel of antisera with (Figure 3, lanes 2, 4, 6, 8) or without (Figure 3, lanes 1, 3, 5, 7) the competing peptides. The immunocomplexes were subjected to SDS/PAGE, electrophoretically transferred to PVDF membrane, and visualized by autoradiography (Figure 3a). In c-Rel immunocomplexes, several proteins were precipitated only with the immune serum (Figure 3a, lane 1) and not with the serum preincubated with the competing peptide (Figure 3a, lane 2). In addition to p80-c-Rel (solid circle), p100, p64 and p45 (arrowheads, also identified in Figures 1 and 2, lanes 1), proteins with apparent molecular masses of 105, 65, 50 and 36 kDa (triangles on the right-hand side of lane 1, Figure 3) were detected. As the same c-Rel antiserum detected only the former proteins in boiled lysates (Figures 1 and 2, lanes 1), the latter polypeptides (triangles) are probably physically associated with c-Rel. For clarity of interpretation, all symbols are used consistently throughout the text. Solid circles and arrowheads on the left-hand side of each lane indicate the predicted immunogens and sequence-related polypeptides respectively. The open triangles on the right-hand side of each lane mark the physically associated proteins. Immunoprecipitation with anti-p105N (lane 3) and anti-p65 (lane 5) antisera detected similar complexes that contained p105, p80, p65, p50 and p36. However, when a p105C antiserum (reactive with only p105) was used, only p105, p80 (only apparent in Figure 3d, lane 7), p65 and p50, and not p36, were precipitated (lane 7). Conversely, the IκBα antiserum precipitated only p80, p65 and p36 but not p105 (lane 9, very faint for 80 and 65 kDa proteins in Figure 3a, but detectable in Figures 3c and 3d). All these reactivities were abolished by preincubating the antisera with the competing peptides, indicating the specificity of the immunoreactions (lanes 1, 3, 5, 7 compared with lanes 2, 4, 6, 8 respectively).

It is likely that the physically associated 105, 80, 65, 50 and 36 kDa proteins (triangles in Figure 3a) are the prototypical p105-NF-κB, p80-c-Rel, p65-NF-κB, p50-NF-κB and p36-IκBα molecules respectively. To confirm the identity of the labelled associated proteins in each immunocomplex detected in Figure 3a, the membrane was subjected to a series of Western-blot analyses using anti-p105C (Figure 3b), anti-p65 (Figure 3c), anti-c-Rel (Figure 3d) and anti-IκBα (Figure 3e) in order. The results show that the coprecipitated 105, 80, 65 and 36 kDa proteins are indeed the p105-NF-κB, p80-c-Rel, p65-NF-κB and p36-IκBα respectively. It is of interest that a 60 kDa protein was specifically detected in c-Rel, p105N and p65 immune complexes (Figure 3a, lanes 1, 3, 5). It probably represents a physically associated

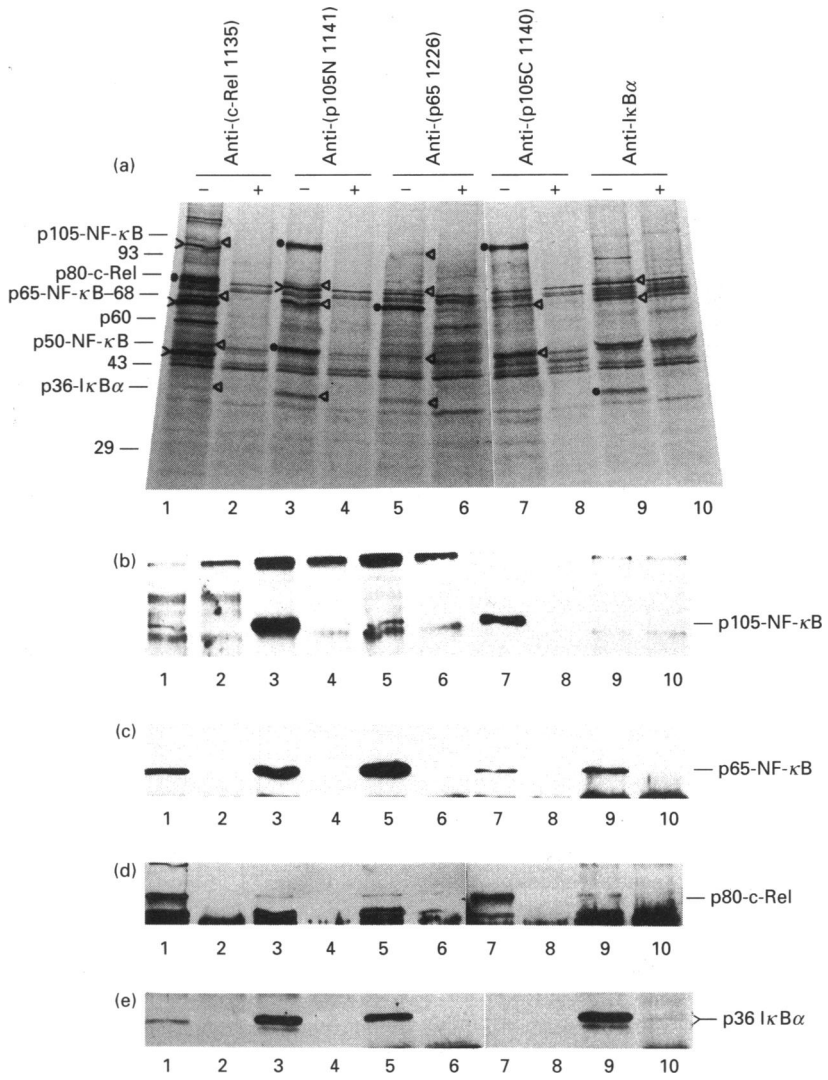

Figure 3 Physical association of NF-κB/Rel family members analysed by immunoprecipitation and subsequent Western blots

[^{35}S]Methionine-labelled Jurkat cells were lysed and the cleared lysate was immunoprecipitated with designated antisera with (lanes 2, 4, 6, 8, 10) or without (lanes 1, 3, 5, 7, 9) the presence of competing peptides (indicated by + or −). The washed immunoprecipitates were resolved by SDS/PAGE, transferred to membranes and autoradiography was performed for 5 days with double intensifying screens (**a**). The membrane was subsequently immunoblotted with anti-p105-NF-κB (**b**), anti-p65-NF-κB (**c**), anti-p80-c-Rel (**d**) and anti-p36-IκBα (**e**) in order, without removing the reactivity remaining from the previous analyses. Solid circles and arrowheads mean the same as in Figure 1; physically associated proteins are indicated by triangles on the right-hand side of lanes 1, 3, 5, 7 and 9. The light bands under p105-NF-κB in (**b**) are non-specific. The 65 kDa bands observed in (**d**) are reactivity remaining from (**c**); 93, 68, 43 and 29 kDa markers are indicated.

protein rather than a non-specific cellular protein detected as a result of cross-reactivity between the antisera (see the Discussion section).

The physically associated 50 kDa protein (Figure 3a, p50 triangles) identified in c-Rel (lane 1), p65 (lane 5) and p105C (lane 7) immunocomplexes migrated to the same position as the authentic p50-NF-κB in the p105N immunoprecipitate (p50 solid circle in lane 3). It is highly likely that this 50 kDa species is p50-NF-κB. However, this could not be confirmed in this specific experiment because the immunoreactivity of p50 was masked by the intense immunoglobulin band recognized by the secondary antibody used in the Western blot (results not shown). To confirm the identity of p50-NF-κB, we performed a c-Rel immunoprecipitation (as in Figure 3a, lane 1) from unlabelled cells and eluted the c-Rel complex with competing peptide. Residual unbound immunoglobulin molecules were then removed from the eluate with excess Protein A–Sepharose beads and the clarified eluate was boiled and analysed by SDS/PAGE and Western-blot analysis. The 50 kDa reactivity detected with the p105N antiserum (Figure 4, lane 2) confirms that the 50 kDa protein contained in the c-Rel complex is p50-NF-κB. This 50 kDa reactivity could not be the residual immunoglobulin background recognized by the anti-(rabbit immunoglobulin) serum (as seen in lane 3 control) because the same eluate analysed with anti-p65 did not detect the same 50 kDa reactivity (lane 4).

In summary, antisera against c-Rel, p105N and p65 all detected similar immunocomplexes containing p80-c-Rel, p105-NF-κB, p65-NF-κB, p50-NF-κB and p36-IκB-α. In other words, similar complexes were immunoprecipitated by different antisera directed to different components of the same complex. Although p105-NF-κB and p36-IκBα were each found associated with p80-c-Rel or p65-NF-κB, the two proteins were not found as members of the same complex (Figures 3a–3e, lanes 7–10).

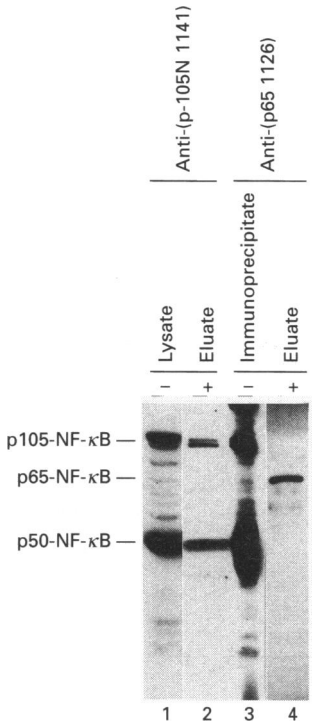

Figure 4 Identification of the c-Rel-associated 50 kDa protein as p50-NF-κB

Unlabelled Jurkat cell lysate was immunoprecipitated with anti-(c-Rel 1135), thoroughly washed, and the immunocomplex eluted by incubating with 20 μg of competing peptide for 15 h at 4 °C. The eluate was incubated with Protein A–Sepharose, in two cycles, to remove any residual unbound immunoglobulin. The clarified eluate was resolved by SDS/PAGE (10% gels) in two identical lanes, and analysed by Western blot with either anti-p105N (lane 2) or anti-p65 (lane 4) serum. Total Jurkat lysate (lane 1) and washed immunoprecipitate before elution (lane 3) were used as controls for anti-p105N blot.

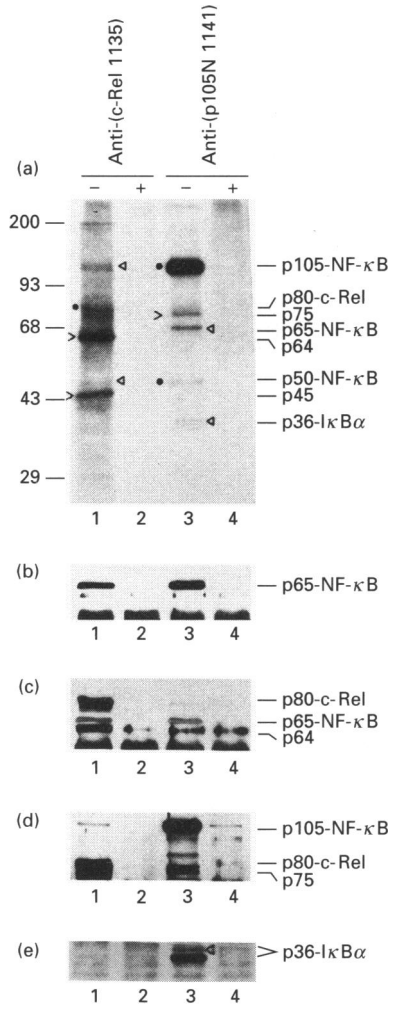

Figure 5 NF-κB/Rel family proteins are phosphoproteins – *in vivo* study

[^{32}P]P_i-labelled Jurkat cells were subjected to immunoprecipitation with anti-c-Rel (lanes 1 and 2) and anti-p105N (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of the competing peptides (indicated by + and −). The Western transferred membrane was exposed to X-ray film for 3 days (**a**), and subsequently immunoblotted with anti-p65-NF-κB (**b**), anti-c-Rel (**c**), anti-p105N (**d**) and anti-p36-IκBα (**e**) in order. The p36-IκBα detected in lane 3 of (**a**) corresponds to the upper band in (**e**), both indicated by triangles. All symbols are the same as in Figure 3. The p65-NF-κB in (**c**) and p80-c-Rel in (**d**) are residual reactivities from the previous blots shown in (**b**) and (**c**) respectively. Size markers are shown on the left hand side of (a).

Phosphorylation of the NF-κB/Rel family proteins

To examine whether the physically associated NF-κB/Rel family members are also phosphoproteins, experiments were carried out as described in Figure 3 except that the Jurkat cells were labelled with [^{32}P]P_i for 3 h before lysis. The autoradiograph (Figure 5a) showed that several proteins present in the immunocomplexes were phosphorylated *in vivo*. Subsequent serial Western-blot analyses using anti-p65, -c-Rel, -p105N and -IκBα sera (Figures 5b, 5c, 5d and 5e in order) specifically identified components present in the complex. The c-Rel immunocomplex contained proteins similar to those detected in Figure 3 (Figure 5, lanes 1). Multiple complex members, including p80-c-Rel (solid circle), p64 and p45 Rel-related proteins (arrowheads) and associated NF-κB proteins (triangles) are all phosphoproteins. Although p65-NF-κB is present in c-Rel complex (Figures 3 and 5b, lanes 1), this Rel-associated p65 was not detected as a phosphoprotein in this experiment (Figure 5a, lane 1). The highly phosphorylated 64 kDa protein present in the c-Rel complex (Figure 5a, lane 1, arrowhead) is not p65-NF-κB but the p64 Rel sequence-related protein (arrowheads in Figures 1, 2, 3a, 5a and 5c, lanes 1). Virtually no p36-IκBα was detected in the c-Rel complex in either the ^{32}P autoradiograph (Figure 5a, lane 1) or the subsequent immunoblot (Figure 5e, lane 1) (see the Discussion section). The immunocomplex precipitated by anti-p105N contained the prototypical NF-κB p105, p65, p50, p36-IκBα and a 75 kDa p105-related species (also seen in Figure 1, lane 3), all of which appeared to be phosphoproteins (Figures 5a–5e, lanes 3). It also contained low levels of p80-c-Rel (Figure 5c, lane 3) which was barely detectable as a phosphoprotein (Figure 5a, lane 3). Both phosphorylated p36-IκBα (Figures 5a and 5e, lanes 3, triangles) and unphosphorylated p36-IκBα (Figure 5e, lane 3) were detected in the complex; however, the unphosphorylated form predominated in the unstimulated Jurkat cells.

To characterize these phosphoproteins further, we carried out *in vitro* phosphorylation reactions on NF-κB/Rel immunoprecipitates isolated from unlabelled Jurkat cells. The results were very similar to those obtained from *in vivo* metabolically ^{32}P-labelled cells (Figure 6 compared with Figure 5). In order to characterize further the nature of the phosphoproteins, we performed phosphoamino acid analysis on NF-κB/Rel and IκBα

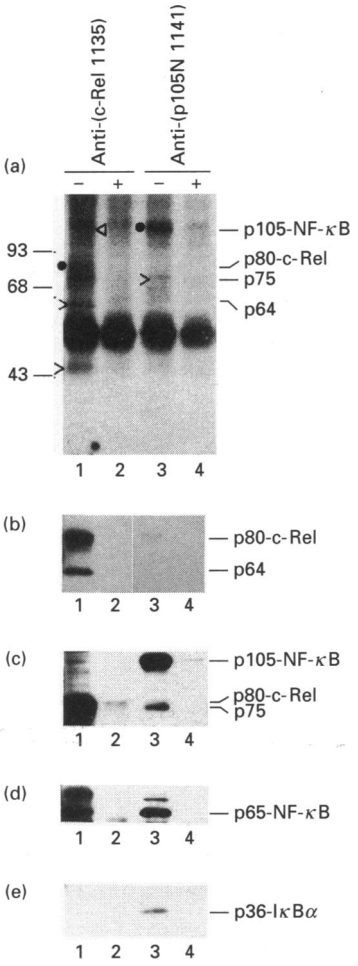

Figure 6 *In vitro* phosphorylation of anti-c-Rel and anti-p105N immunoprecipitates from unstimulated Jurkat cells

Unlabelled Jurkat cells were subjected to immunoprecipitation as described in Figure 5. *In vitro* phosphorylation was performed on washed precipitates and the results were analysed by SDS/PAGE (10% gel), Western transfer, autoradiography (**a**), and subsequent immunoblots using anti-c-Rel (**b**), anti-p105N (**c**), anti-p65 (**d**) and anti-IκBα (**e**) in order. All the symbols are the same as in Figure 3. The p80-c-Rel detected in (**c**) is residual reactivity from the previous anti-c-Rel blot. The heavy bands between 68 and 43 kDa size markers in (**a**) are immunoglobulins.

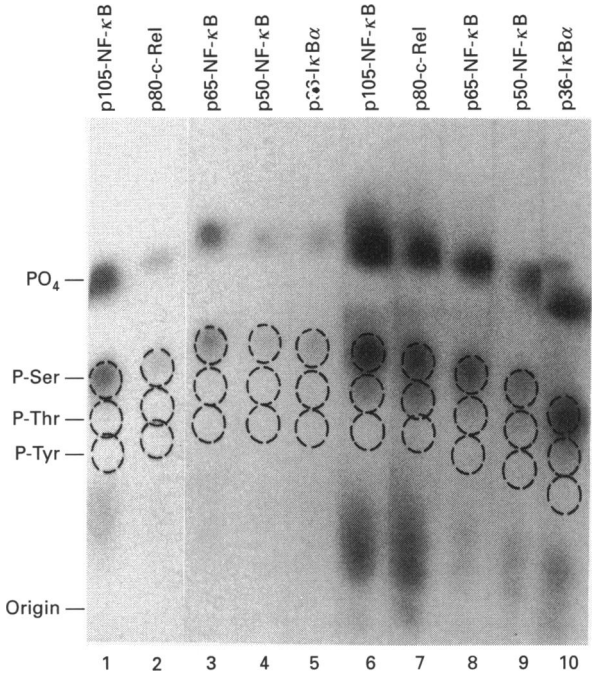

Figure 7 NF-κB/Rel family proteins and p36-IκBα from unstimulated cells contain phosphoserine and phosphothreonine residues

Appropriate protein bands detected from *in vivo* experiments (lanes 1–5) and *in vitro* phosphorylation reactions (lanes 6–10) were excised and the membrane slices were subjected to phosphoamino acid analysis. The origin, the positions of PO_4, and the stained phosphorylated residues are marked. The positions of phosphoserine, phosphothreonine and phosphotyrosine in each lane are marked with broken circles.

proteins phosphorylated *in vivo* (as in Figure 5a) or *in vitro* (as in Figure 6a) to determine whether the same residues were phosphorylated in both cases. As shown in Figure 7, both *in vivo* (lanes 1–5) and *in vitro* (lanes 6–10) labelled NF-κBRel proteins and p36-IκBα contain phosphoserine and phosphothreonine (phosphothreonine detected only with a longer exposure). None of the proteins appeared to be phosphorylated on tyrosine.

Phosphorylation of NF-κB/Rel family proteins in activated cells

Phosphorylation and subsequent degradation of IκB has been proposed as a required step in NF-κB activation. Although it has been shown that IκBα is rapidly proteolytically degraded 30 min after NF-κB stimulation (Sun et al., 1993; Brown et al., 1993; C.-C. H. Li, unpublished work), *in vivo* evidence for the phosphorylation of IκB has not been clearly demonstrated. To determine if NF-κB activation changes the physical association and phosphorylation patterns of NF-κB/Rel family proteins, we performed immunoprecipitations on [^{32}P]P_i-labelled cells with or without the stimulation of PMA and PHA (Figure 8a). A 10 min stimulation was used to study the events occurring before IκB degradation. As the c-Rel level in Jurkat cells is intrinsically low (Figures 1, 2 and 3), c-Rel immunoprecipitation was not performed. The results show that, after a 10 min stimulation with PMA and PHA, significantly more phosphorylation was detected in p105 and p50 (Figure 8a, lane 2 compared with lane 1, and lane 4 compared with lane 3) but not in p65 (lane 4 compared with lane 3). Surprisingly, p36-IκBα in PMA/PHA-stimulated cells was not more extensively phosphorylated than in the control cells (Figure 8a, lane 6 compared with lane 5). As equal numbers of cells were used and the subsequent Western blots also showed the presence of comparable amounts of protein (results not shown) in each immunoprecipitation, the different intensities observed in these bands represent the actual differences in the extent of phosphorylation. Moreover, this experiment also showed that phosphorylated IκBα is still capable of physically complexing with NF-κB family members. This observation was also demonstrated in earlier experiments (Figures 3a and 3e, lanes 3 and 9; Figures 5a and 5e, lanes 3). p105-NF-κB proteins from unstimulated (Figure 8a, lane 1) and stimulated (lane 2) cells were further subjected to partial proteolytic mapping. Similar but not identical patterns (Figure 8b) suggested that, in addition to the overall increased phosphorylation of p105, the phosphorylation profile of p105 is also changed in response to PMA/PHA stimulation.

DISCUSSION

Physically associated proteins are conventionally studied and identified by immunoprecipitation followed by proteolytic map-

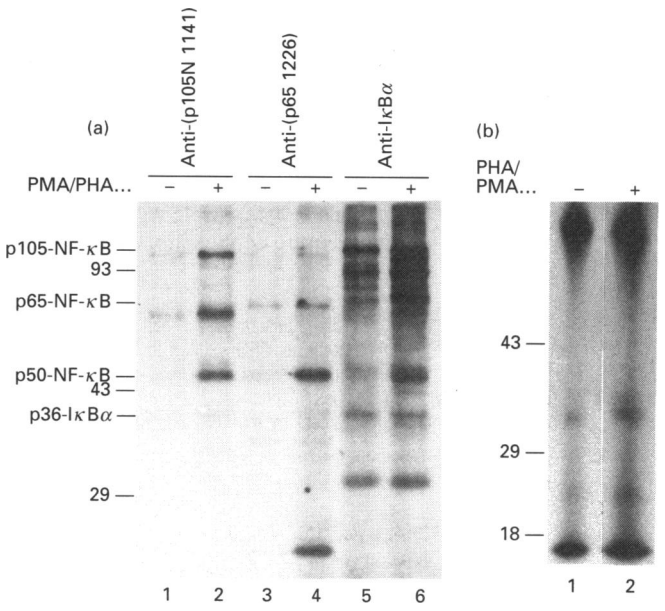

Figure 8 Increased phosphorylation of NF-κB proteins in PMA/PHA-stimulated cells and peptide mapping of p105 from untreated and stimulated cells

Jurkat cells were labelled with [^{32}P]P_i for 2 h either without stimulation (lanes 1, 3, 5) or with PMA/PHA stimulation for the final 10 min. The lysates were immunoprecipitated with the designated antisera. Washed precipitates were resolved by SDS/PAGE, dried on to filter paper and visualized by autoradiography for 20 h (**a**). p105 bands from lanes 1 and 2 were excised and compared by partial proteolytic mapping (**b**) (Cleveland et al., 1977). Each sample was digested with 0.5 μg of *Staphylococcus aureus* V8 protease per lane. The digested proteins were resolved on a 15% polyacrylamide gel and visualized by autoradiography for 3 weeks; 93, 43, 29 and 18 kDa markers are indicated.

ping or elution and reprecipitation. Both methods require large amounts of reagents and are rather time-consuming. Combined radioimmunoprecipitation and subsequent serial immunoblotting offers a rapid and easy way to analyse physically associated complexes and to identify unambiguously individual components of the complexes. In this report, we used this combined method to show that, in the unstimulated human T-cell line Jurkat, the c-Rel immunocomplex contains the prototype NF-κB proteins, p105, p65, p50 and p36-IκBα. The identical complex components were detected when immunoprecipitations using anti-p105N and anti-p65 were performed. Similar results were also obtained from experiments performed in malignant human B-cell lines (results not shown). Although physical associations between c-Rel, p105 and p36-IκBα have been extensively described (Simek and Rice, 1988; Morrison et al., 1989; Davis et al., 1990a,b, 1991; Kochel et al., 1991; Mosialos et al., 1991; Capobianco et al., 1992; Neumann et al., 1992; Mercurio et al., 1993), it is not clear whether p50 and p65 are normally contained in the c-Rel complex in unstimulated cells. Our report offers a more complete *in vivo* study and provides the first evidence that p50 and p65 can be found in association with c-Rel in unstimulated cells. Previous researchers may not have detected the p50 and p65 in their radioimmunoprecipitation assays because they only labelled the cells for 0.5–5 h. As p105 and p65 are rather stable proteins (C.-C. H. Li, unpublished work), a longer labelling time, e.g. 15 h, is needed to detect them.

The specificity of each antiserum was established by both Western blot (Figure 1) and immunoprecipitation from lysates diluted after boiling in SDS-containing buffer (Figure 2). The c-Rel antiserum unexpectedly detected a number of proteins of which p80-c-Rel was only a minor species. The low level of c-Rel is not surprising, as c-Rel is not an abundant cellular protein in unstimulated T-cells (Li et al., 1993). The other proteins (arrowheads on lanes 1 of Figures 1, 2, 3a, 5a and 6a) could be synthetic peptide sequence-related or c-Rel-related species. Interestingly, proteins of similar size were also detected using antisera raised against different Rel immunogens (C.-C. H. Li, unpublished work), suggesting that these proteins are probably Rel-related polypeptides. The physically associated proteins detected in the immunoprecipitation (triangles on lanes 1 of Figures 3a, 5a and 6a) could theoretically be complexing to these sequence-related proteins rather than p80-c-Rel itself. However, this is not likely because the complexes obtained from immunoprecipitations using p105N, p65, p105C and IκBα antisera all contained p80-c-Rel but not always the other proteins (Figure 3), and different c-Rel and NF-κB antisera consistently detected complexes that contained p80-c-Rel and NF-κB family members (results not shown). The doublet p80-c-Rel detected in both Western-blot (Figure 1a, lane 1) and immunoprecipitation (Figures 2a and 3a, lanes 1) analyses suggested that the slower-migrating p80 might be a phosphorylated form of p80-c-Rel. This notion was further supported by immunoprecipitation performed with ^{32}P-labelled cells (Figures 5a and 6a, lanes 1). The 100 kDa protein co-precipitated with p80-c-Rel (Figure 3a, lane 1, top arrowhead) is not the precursor of p50B-NF-κB (results not shown) but a c-Rel sequence-related protein (see lanes 1 in Figures 1 and 2). A 60 kDa protein was coimmunoprecipitated by anti-c-Rel (Figure 3a, lane 1), anti-p105N (Figure 3a, lane 3) and, to a lesser degree, anti-p65 (Figure 3a, lane 5), suggesting that this p60 is also a component of the NF-κB/Rel complex. It could be an unidentified new member of the family or a relative that did not show the cross-reactivity in western blots.

Our data suggest that the majority, if not all, of IκBα molecules are present in complex form (Figures 3a and 3e, compare the p36 reactivity in lane 9 with the summation of lanes 1 and 3). Most of the p36-IκBα is complexed with the prototypical NF-κB complex p50–p65 (Figures 3a and 3e, compare lanes 3, 5 and 9). The detection of low levels of p36-IκBα in the c-Rel complex in Jurkat cells was not always reproducible (compare Figures 3, 5 and 6, lanes 1). This could be explained, at least partially, by the low basal level of c-Rel in Jurkat cells. In addition the low affinity between c-Rel and p36-IκBα could be a Jurkat-specific phenomenon, as association between c-Rel and p36-IκBα was readily detectable in B-cells (Rice et al., 1992; C.-C. H. Li, unpublished work). Another interesting observation is that, although significant amounts of p65-NF-κB and p80-c-Rel are complexed with p36-IκBα (Figures 3c and 3d, lanes 9), they were barely detectable as ^{35}S-labelled species (Figure 3a, lane 9). This suggests that p36-IκBα has a high turnover rate (Sun et al., 1993) and that newly synthesized ^{35}S-labelled p36-IκBα will quickly associate with the pre-existing unlabelled p65-NF-κB and p80-c-Rel molecules which are probably much more stable than IκBα. This is also supported by our finding of a much longer half-life of NF-κB/Rel family proteins than p36-IκBα (C.-C. H. Li, unpublished work).

Both p36-IκBα and p105-NF-κB contain the ankyrin repeats which are postulated to be involved in protein–protein interactions. p105-NF-κB has been shown to function as an IκB molecule that sequesters other members of the NF-κB/Rel family in the cytoplasm. Our inability to detect p105 in IκB immunoprecipitates and vice versa (Figure 3, lanes 7–10) suggests and confirms the previous observation (Rice et al., 1992) that p105 and p36-IκBα do not coexist in one physical complex, i.e. they do

not directly associate with each other or indirectly through a third molecule. Therefore the detection of both p105 and p36 in c-Rel, p65 and p105N immunoprecipitates indicates that there are at least two kinds of complex present in each precipitate, one containing p105 and the other p36.

In summary, our physical-association data are consistent with, but do not conclusively prove, the following model. In addition to homodimerization, the NF-κB/Rel family members, specifically p50, p65 and p80-c-Rel, can heterodimerize with one another to form DNA-binding complexes of p50/p65, p50/p80 and p65/p80. Each of the family members and homo- or heterodimers may also associate with the p105 precursor which serves as an inhibitor (Naumann et al., 1993). In addition, they can associate with p36-IκBα, in that p65 seems to be a preferred associate (Urban and Baeuerle, 1990; Zabel and Baeuerle 1990; Davis et al., 1991; Kerr et al., 1991; Liou et al., 1992; Wulczyn et al., 1992). p105 does not associate with p36-IκBα, either directly or indirectly through a third molecule.

Both *in vivo* and *in vitro* phosphorylation experiments demonstrated that the NF-κB/Rel family members and p36-IκBα are indeed phosphoproteins (Figures 5–8). Although it has been shown in the literature that p105 (Neumann et al., 1992), c-Rel (Mosialos et al., 1991; Neumann et al., 1992) and IκB (Link et al., 1992) are phosphoproteins, this is the first report showing that p50 and p65 are also phosphoproteins. Phosphoamino acid analysis of proteins isolated from both *in vivo* and *in vitro* assays showed phosphorylated serine and threonine, but not tyrosine, residues. This result, obtained with unstimulated cells, does not necessarily conflict with that reported by Neumann and coworkers (1992) who found phosphorylated serine, threonine and tyrosine in p105-NF-κB and p80-c-Rel in stimulated cells. Although our method may not have been sufficiently sensitive to detect phosphotyrosine, phosphorylation of specific tyrosine residues could be a stimulation-responsive reaction.

An interesting and surprising result obtained in this study is that, 10 min after PMA/PHA stimulation, increased phosphorylation was detected in p105 and p50 but not IκBα (Figure 8a). After the completion of this work, Beg et al. (1993) also reported a lack of IκBα phosphorylation after PMA/PHA stimulation, consistent with our result. Both quantitative (Figure 8a) and qualitative (Figure 8b) changes were observed in p105 phosphorylation. The increased phosphorylation of p50 after stimulation was even more dramatic. As shown in both Figure 5a and Figure 8a, p50 was much less extensively phosphorylated than p105 in unstimulated cells, but was phosphorylated to at least the same extent as p105 after stimulation. This result is also consistent with our observation (C.-C. H. Li, unpublished work) that, after stimulation, a significant amount of phosphorylated p50 can be detected in the nucleus, presumably the activated DNA-binding form.

Our data show two forms of p36-IκBα with different electrophoretic mobility (Figures 3e and 5e). The phosphorylated p36 detected in ^{32}P-labelled cells (Figure 5a, lane 3, p36 triangle) corresponded to the slower-migrating form of p36 (Figure 5e, lane 3, triangle), indicating that the two forms represent hyper- and un-phosphorylated p36-IκBα. Unexpectedly, both forms of p36-IκBα are found associated with the NF-κB complex, presumably preventing it from translocating to the nucleus. This finding, together with the lack of increased IκB phosphorylation in response to PMA/PHA stimulation (Beg et al., 1993 and this study), seems to contradict the dogma that stimulation leads to phosphorylation of IκB and its dissociation from the NF-κB complex. As a recent report (Brown et al., 1993) showed IκB phosphorylation after tumour necrosis factor α stimulation, further investigation is required into whether this exception is a PMA/PHA-specific phenomenon. On the other hand, it is possible that IκB undergoes differential phosphorylation in stimulated cells. Although the overall level of IκB phosphorylation does not increase after PMA/PHA treatment, certain critical sites may be phosphorylated (or dephosphorylated) to activate the NF-κB activity. This is compatible with the finding that activation of NF-κB may require dephosphorylation of IκB (Link et al., 1992).

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