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RelA/p65 is a molecular target for the immunosuppressive action of protein kinase A

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Stimulation of the protein kinase A (PKA) signalling pathway exerts an inhibitory effect on the proliferation of numerous cells, including T lymphocytes. In CD4$^+$ T helper cells, stimulation of PKA leads to suppression of interleukin 2 (IL-2) induction, while induction of the genes coding for the lymphokines IL-4 and IL-5 is enhanced. We show that the differential effect of PKA activity on induction of the IL-2 and IL-4 genes is mediated through their promoters. One major target of the suppressive effect of PKA is the κB site in the IL-2 promoter. A κB site is missing in the IL-4 promoter. Mutations preventing factor binding to the IL-2 κB site result in a loss of PKA-mediated suppression of IL-2 promoter activity. Furthermore, activation of the PKA signalling pathway impairs the inducible activity of multiple κB sites of the IL-2 promoter, but not of other factor binding sites. The reduction in activity of κB sites in activated and PKA-stimulated T cells is accompanied by changes in the concentration and DNA binding of Rel/NF-κB factors. Stimulation of the PKA pathway in Jurkat T cells with the PKA activator forskolin leads to an increase in synthesis of c-Rel and p105/p50, while synthesis of p65/RelA remains unchanged. However, nuclear translocation and DNA binding of p65 is distinctly impaired, probably due to a retarded degradation of IκB-α. In a similar way, stimulation of the PKA signalling pathway inhibits nuclear translocation of p65 and generation of nuclear κB complexes in peripheral T lymphocytes from murine lymph nodes. These results indicate that PKA-mediated suppression of NF-κB activity plays an important role in the control of activation of peripheral T lymphocytes.
Key words: immunosuppression/NF-κB/protein kinase A/p65/RelA

Introduction

One key event in the immune response is activation of resting peripheral T lymphocytes. Processed foreign antigens presented by major histocompatibility complex molecules at the surface of macrophages, dendritic cells and B lymphocytes activate T cells through interaction with components of the T cell receptor (TCR) complexes. Further necessary stimuli are provided through the CD4/CD8 'co-receptors' and CD28 (see Linsley and Ledbetter, 1993; June *et al.*, 1994; Rudd *et al.*, 1994, for recent reviews). One of the earliest events is activation of the protein tyrosine kinases ZAP-70, p56^{lck} and p59$^{fyn(T)}$, which are members of the syk and src kinase families. As a result, numerous cytosolic targets, including the ζ subunit of TCR, phospholipase γ1 and CD 5 are phosphorylated (see Fraser *et al.*, 1993; Rudd *et al.*, 1994). Another early event following TCR triggering is the generation of GTP-bound p21^{ras}, the active form of Ras (Downward *et al.*, 1990). Active Ras is able to bind and activate Raf-1, a serine/threonine protein kinase, mediating its translocation to cellular membranes (see Daum *et al.*, 1994). In Jurkat T cells it has been shown that over-expression of an activated version of Raf-1 results in an increase in IL-2 production (Owaki *et al.*, 1993). Since MAP/Erk kinases which are downstream targets of Raf-1 also become activated during T cell activation (Whitehurst *et al.*, 1992; Izquierdo *et al.*, 1993), it is very likely that signalling transfer through the Ras−Raf−Erk pathway(s), described for other cells, also plays an important role in T cell activation.

In concert with early tyrosine protein phosphorylation and generation of active Ras, TCR stimulation also leads to an increase in the influx of extracellular Ca^{2+} and mobilization of Ca^{2+} from intracellular stores (see Rao, 1991). In T cells, a key enzyme of Ca^{2+} signalling pathways is calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase that is involved in the steps leading to proper induction of the IL-2 promoter (Clipstone and Crabtree, 1992; O'Keefe *et al.*, 1992). Potent inhibitors of calcineurin are the immunosuppressants cyclosporin A (CsA) and FK506 (see Liu, 1993). The binding of either of these compounds to low molecular weight cytosolic proteins results in complexes which are able to interact with calcineurin, thereby inhibiting this enzyme (Liu *et al.*, 1991). Calcineurin stimulates, directly or indirectly, the activity of inducible transcription factors, such as NF-AT. In resting T lymphocytes NF-AT is located in the cytoplasm. It is translocated into the nucleus in response to activation of calcineurin-mediated Ca^{2+} pathways (see Liu, 1993). CsA and FK506 suppress the nuclear translocation and DNA binding of NF-AT (Emmel *et al.*, 1989; Randak *et al.*, 1990).

Another class of immunosuppressants are glucocorticoid hormones. Whereas glucocorticoid hormones are known to induce gene expression and differentiation in several tissues, such as the mammary gland and liver, they play an inhibitory role in several other cell types. Glucocorticoid

hormones block tumour promotion in skin carcinogenesis (Scribner and Slaga, 1973), impair the proliferative response of cells in wound healing (Baxter and Forsham, 1972) and the growth of normal and transformed lymphoid cells (see Cupps and Fauci, 1988). Positive and negative effects of glucocorticoid hormones are mediated through their receptor, a transcription factor which translocates from the cytosol into the nucleus as a consequence of hormone binding. Within the nucleus, the glucocorticoid receptor binds to and transactivates promoters containing glucocorticoid responsive elements (GREs). The glucocorticoid receptor exerts a negative effect on many other promoters by interfering with other transcription factors, such as AP-1 (see Heck *et al.*, 1994, and references therein). It is very likely that the inhibitory effect glucocorticoid hormones exert on induction of the IL-2 promoter (see, for example, Vacca *et al.*, 1992) is mediated through suppression of AP-1, which binds, often in concert with other transcription factors, to several sites of the IL-2 promoter.

Similar to glucocorticoid hormones, the second messenger cyclic adenosine 3′,5′-monophosphate (cAMP) has a dual effect on numerous eukaryotic cells. Whereas cAMP acts as a mitogen in thyrocytes and 3T3 cells, it suppresses the proliferation of many other cells, including T lymphocytes (see Kammer, 1988, for a review). Elevated concentrations of intracellular cAMP have long been known to suppress the immune reponse and, at the molecular level, transcription of IL-2 and γ-interferon (γ-IFN) genes (Rappaport and Dodge, 1982; Hasler *et al.*, 1983). Prostaglandins of the E series, such as PGE2, are potent activators of the protein kinase A pathway(s) within the haematopoeitic compartment. They are synthesized by macrophages, follicular dendritic cells and mononuclear phagocytes in response to interleukin-1, tumour necrosis factor α, lipopolysaccharides and cross-linking of Fc receptors (see Phipps *et al.*, 1991). Suppression of T cell activation by PGE2 might be due to a block of the Ras−Raf−Erk signalling pathway, since it has been shown for several other cell types that activation of PKA interferes with intracellular signal transfer through this signal cascade (Burgering *et al.*, 1993; Graves *et al.*, 1993; Sevetson *et al.*, 1993; Wu *et al.*, 1993; Hordijk *et al.*, 1994; Russell *et al.*, 1994). Raf-1 is a substrate of PKA and it has been postulated that hyperphosphorylation of the N-terminal regulatory domain of Raf-1 leads to weakening of the interaction between Raf-1 and Ras (Wu *et al.*, 1993) and/or to an inhibition of Raf-1 activation by lck and protein kinase C α (Häfner *et al.*, 1994).

In T cells, PGE2 and other activators of PKA exert an opposite, stimulatory effect on expression of the lymphokines IL-4 and IL-5 (Phipps *et al.*, 1991). The observation that activators of PKA inhibit expression of IL-2 and γ-IFN, i.e. lymphokines specific for TH1 cells, and stimulate IL-4 and IL-5 expression, characteristic for TH2 cells, led to the hypothesis that activators of PKA might play an important role in differentiation of TH cells to TH1 and TH2 cells (see Betz and Fox, 1991). This assumption prompted us to investigate the effect of activators of PKA on the activity of the IL-2 and IL-4 promoters in T cells.

In this study we show that stimulation of PKA activity suppresses the IL-2 promoter in T lymphocytes through a κB binding site which is missing in the IL-4 promoter. Elevated PKA activities led to drastic changes in the synthesis, nuclear translocation and DNA binding of members of the Rel/NF-κB family of factors in human Jurkat T leukaemic cells and murine El4 T lymphoma cells. While PKA activation enhances *de novo* synthesis of c-Rel, p105/p50 and IκB, no increase in synthesis of p65/RelA was observed. The enhanced or unaltered synthesis was contrasted by impaired nuclear translocation and DNA binding of p65, while DNA binding of c-Rel was stimulated following PKA activation. Since suppressed nuclear translocation of p65 is correlated with retarded degradation of cytosolic IκB-α, it is likely that increased PKA activities interfere with release of p65 from its cytosolic inhibitor in T cells. A strong suppression of the generation of κB complexes and the cytosolic retardation of p65 were also observed in stimulated peripheral T lymphocytes after PKA activation, while no measurable suppressive effect on κB factors was detected in several TH1 and TH2 cell lines. These observations indicate that in peripheral T cells stimulation of the PKA signalling pathway leads to reduced binding of specific κB factors to the IL-2 promoter, a reduction in IL-2 gene expression and, finally, to suppression of the IL-2 dependent steps of the immune response.

Results

Stimulation of protein kinase A impairs induction of the IL-2 promoter through its κB site

The differential effects exerted by the PKA signalling pathway on expression of the IL-2 and IL-4 genes (Munoz *et al.*, 1990; Novak and Rothenberg, 1990; Betz and Fox, 1991; Lee,H.J. *et al.*, 1993) prompted us to investigate differences in the transcriptional control of both lymphokine genes. Plasmid constructs bearing chloramphenicol acetyltransferase (CAT) or luciferase (luc) reporter genes under the control of the IL-2 and IL-4 promoters (Figure 1A) were transfected into murine El4 T lymphoma cells and human Jurkat T leukaemic cells. As shown in Figure 1B and C for El4 cells, the activities of both the IL-2 and IL-4 promoters were stimulated upon activation of these cells by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the Ca^{2+} ionophore ionomycin. In the presence of 5 μM forskolin, which directly activates the catalytic subunit of adenylate cyclase (see Kammer, 1988), induction of the IL-4 promoter was enhanced ~3-fold, while activity of the IL-2 promoter was suppressed to at least 50% of the value observed after TPA/ionomycin induction. This suppressive effect was observed in transient CAT transfection experiments in which the cells were stimulated for 20 h (Figure 1B), as well as in luciferase experiments in which the cells were stimulated for 8 h (Figure 1C).

Suppression of TPA/ionomycin-mediated induction of the IL-2 promoter was also detected in the presence of 5 μM PGE2 or 100 μM Sp-5,6-dichloro-1-β-D-ribofuranosyl benzimidazole-3′,5′-monophosphorothioate (cBiMPS) (Figure 2A). PGE2 binds to surface receptors and stimulates the production of cAMP (see Phipps *et al.*, 1991). cBiMPS is a lipophilic and hydrolysis-resistant cAMP analogue and a selective activator of PKA in cell extracts and intact cells (Sandberg *et al.*, 1991). Thus all three substances which stimulate activity of the PKA

signalling pathway by different mechanisms impair induction of the IL-2 promoter in T cells (Figure 2A).

In order to determine which sequence elements of the IL-2 promoter might be negatively affected by the increase in PKA activity, IL-2–CAT constructs containing IL-2 promoter segments of various lengths were transfected into El4 cells. Forskolin-mediated inhibition of inducible CAT activity similar to that of the wild-type promoter was found in induced cells transfected with a construct carrying three copies of the distal half of the IL-2 promoter spanning nucleotide positions −180 to −293 (designated pILCAT 2/1-3×L; Figure 3A). In contrast, the activity of a construct containing three copies of a shorter segment (spanning nucleotides −200 to −293, pILCAT2/1-3×M) was not impaired, but stimulated by forskolin. Only the former construct has an intact κB/TCEd site. This was confirmed in electrophoretic mobility shift assays (EMSAs) in which two prominent complexes could be detected when nuclear extracts of induced El4 cells were incubated with a probe spanning nucleotides −180 to −223. No such complexes were detectable with a probe spanning nucleotides −200 to −223 (Figure 3B, compare lanes 2 and 3 with lane 9). Incubation with a 50-fold molar excess of the IL-2 κB/TCEd site (Figure 3B, lanes 6 and 7) or a κB consensus site (not shown) led to suppression of factor binding. In accordance with published results (Briegel *et al.*, 1991), these data indicate that κB-like factors are able to bind to the −180 to −293 IL-2 promoter DNA segment, but not to the shorter segment spanning nucleotides −200 to −293. These findings suggest that κB factors are the target of PKA-mediated suppression of the IL-2 promoter.

To substantiate this hypothesis we investigated the effect of forskolin on IL-2 promoter constructs containing mutated factor binding sites. In striking contrast to suppression of the wild-type IL-2 promoter, forskolin treatment led to a 3-fold increase in induction of a promoter bearing a defective κB/TCEd site. No such effect was observed using promoter constructs with mutated octamer or AP-1 binding sites (Figure 3C). Similar results were obtained when the effect of forskolin was tested on the activity of multiple copies of the IL-2 factor binding site. Only the activity of multiple κB/TCEd sites was markedly suppressed, whereas the activity of constructs with multiple NFAT-1 binding sites (4×Pu-b_d), octamer/AP-1 binding sites (5×UPS) or AP-1 consensus sites (5×TREcoll) was found to be enhanced by forskolin (Figure 3D).

The sequence of the κB/TCEd site differs from a κB consensus site. In experiments using T cell proteins in addition to NF-κB (Lenardo *et al.*, 1988; Hoyos *et al.*, 1989; Shibuya *et al.*, 1989; Kang *et al.*, 1992), other factors were also found to bind to this site (Briegel *et al.*, 1991). However, the suppressive effect of PKA appears

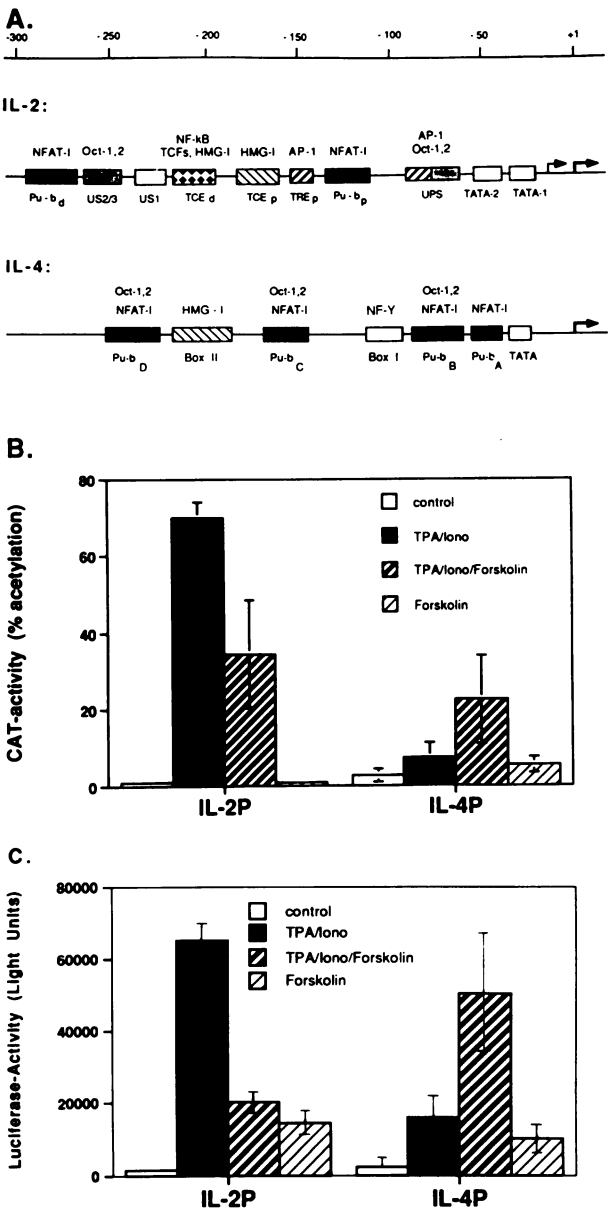


Fig. 1. Induction of the PKA signalling pathway by forskolin suppresses IL-2 promoter and stimulates IL-4 promoter activity. (**A**) Schematic structures of the murine IL-2 and IL-4 promoters. The κB-like TCEd site of the IL-2 promoter is shown as a box filled with black squares. The black boxes indicate the binding sites of NF-AT, i.e. the proximal and distal purine boxes (Pu-b_p and Pu-b_d) of the IL-2 promoter (Randak *et al.*, 1990) and the purine boxes A, B, C and D of the IL-4 promoter (Chuvpilo *et al.*, 1993). The grey, dotted boxes indicate TATA boxes and binding sites of octamer factors (UPS and upstream sites US 2/3) in the IL-2 promoter and the hatched boxes those for AP-1 (UPS and TREp) in the IL-2 promoter and the high mobility group protein HMG I(Y) in both promoters (TCEp and Box II) (Chuvpilo *et al.*, 1993). Box I of the IL-4 promoter is a binding site for NF-Y (Szabo *et al.*, 1993). (**B**) Effect of forskolin treatment on induction of the IL-2 and IL-4 promoters in CAT experiments. El4 cells were transfected with the CAT constructs pIlCAT 2/1+ (IL-2P), containing the murine IL-2 promoter/enhancer (from postions −7 to −293) in front of a tk–CAT indicator gene (Serfling *et al.*, 1989), and pmIL4CAT 5/1+, containing one copy of the murine IL-4 promoter (−12 to −307) in front of the tk–CAT indicator gene of the vector pBLCAT5 (Chuvpilo *et al.*, 1993). Twenty hours after transfection, the cells were divided. One part of the cells was left as an uninduced control, one part was induced with TPA/ionomycin (10 ng/ml and 0.5 μM respectively) in the absence or presence of forskolin (5 μM) and one part was treated with forskolin alone. Twenty hours later, the cells were sonicated and the cell lysates were used in CAT assays. (**C**) Effect of forskolin treatment on induction of the IL-2 and IL-4 promoters in luciferase transfection experiments. El4 cells were transfected with tk–luc constructs containing the murine IL-2 promoter (−7 to −293; IL-2P) or IL-4 promoter (−12 to −270; IL-4P) in front of the tk promoter in pBLCAT5 (Boshart *et al.*, 1992) in which the CAT gene was replaced by the luciferase reporter gene. The cells were stimulated as in (B) for 8 h. They were harvested and luciferase assays were performed according to a standard protocol (de Wet *et al.*, 1987).

to be mediated through NF-κB, since the inducible activity of multimers of κB consensus sites derived from the class II-associated invariant chain promoter (Pessara and Koch, 1990) (Figure 2B) and the HIV-1 promoter (data not shown) were also negatively affected by forskolin. This suppressive effect is likely due to an increase in cellular PKA activity, since co-transfections of κB−CAT constructs with a vector over-expressing the catalytic subunit of PKA impaired κB-mediated induction of CAT activity (Figure 2C, construct 7×κB). In the same assay, induction of the IL-2 promoter−CAT vector was also impaired, whereas that of a vector containing four copies of a cAMP responsive element (4×CRE) was strongly enhanced. Such a suppressive effect of PKA on NF-κB-mediated induction was not detected in murine A 20J B lymphoma cells and in human HeLa cells (unpublished results).

Activation of PKA stimulates DNA binding of c-Rel, but inhibits binding of p65

In order to investigate the suppressive effect of PKA stimulation on κB proteins, we first analysed binding of pulse-labelled Rel proteins to the κB sites of IL-2 and HIV-1 LTR promoters in DNAP assays, followed by high resolution, two-dimensional gel electrophoresis (Franza *et al.*, 1987). Jurkat cells were induced with TPA/ionomycin for 2 h in the absence or presence of 5 μM forskolin, followed by pulse-labelling with [^{35}S]methionine for 30 min. Whole cell extracts were incubated with biotinylated wild-type and mutant κB oligonucleotide probes of the IL-2 and HIV-1 promoters, the binding proteins were precipitated and fractionated by two-dimensional gel electrophoresis. As shown in Figures 4 and 5, a variety of labelled proteins were recovered by this technique. Several of them were specifically precipitated with the wild-type IL-2 κB/TCEd or HIV-1 κB sites. In accordance with earlier observations (Lee,J.H. *et al.*, 1991), the most prominent protein among the κB site-specific DNA binding proteins was c-Rel. DNA binding of c-Rel increases upon induction, presumably due to elevated *de novo* synthesis (see also Figure 7A). This increase is accompanied by the appearance of several new c-Rel isoforms. Forskolin treatment led to an increase in c-Rel induction, but did not change the isoform pattern (Figures 4C and E and 5C and E). Proteins designated A were precipitated with the IL-2 κB/TCEd probe more efficiently than with the HIV-1 κB site, while those designated B and C bound more efficiently to the HIV-1 κB site. The A proteins might be components of factor complexes TCF2 and TCF3, which we have described previously as binding to the IL-2 κB/TCEd site, but not to a κB consensus site in El4 cells (Briegel *et al.*, 1991).

Only minor amounts of ^{35}S pulse-labelled p50 and p65 were recovered in these DNAP assays. This has been shown by blotting of two-dimensional gels and subsequent identification of p65 and p50 proteins with specific antibodies (unpublished results). In order to investigate the effect of forskolin on these members of the Rel family, Rel proteins from unlabelled Jurkat cells were precipitated with biotinylated κB oligonucleotide probes, fractionated on one-dimensional gels and electrophoretically trans-

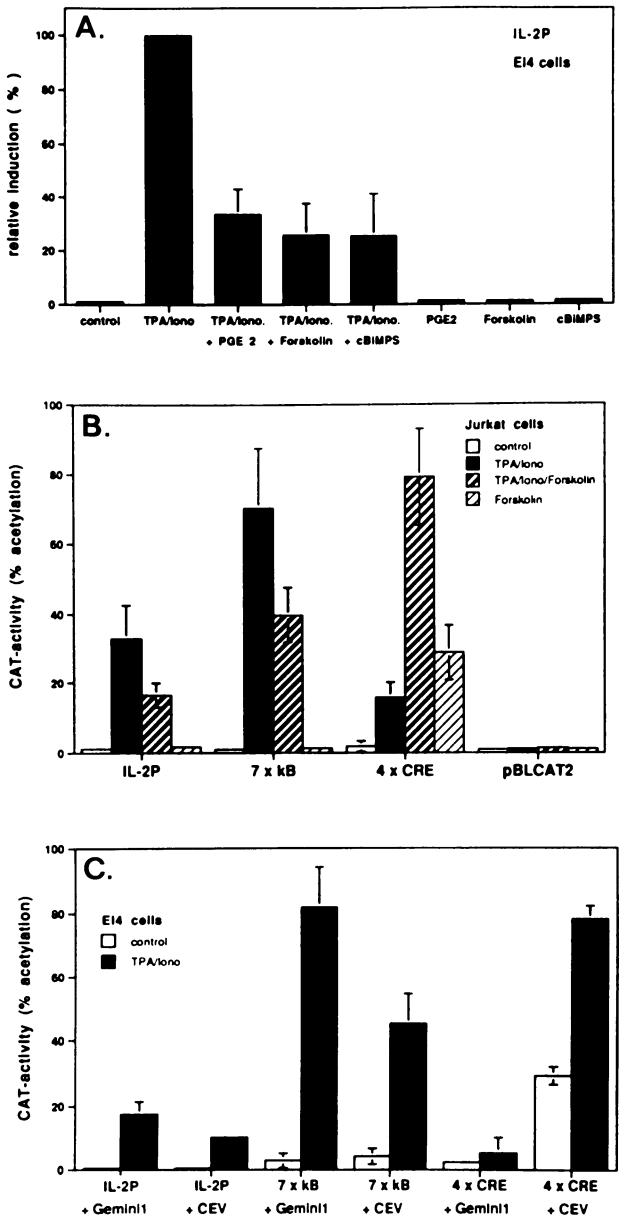
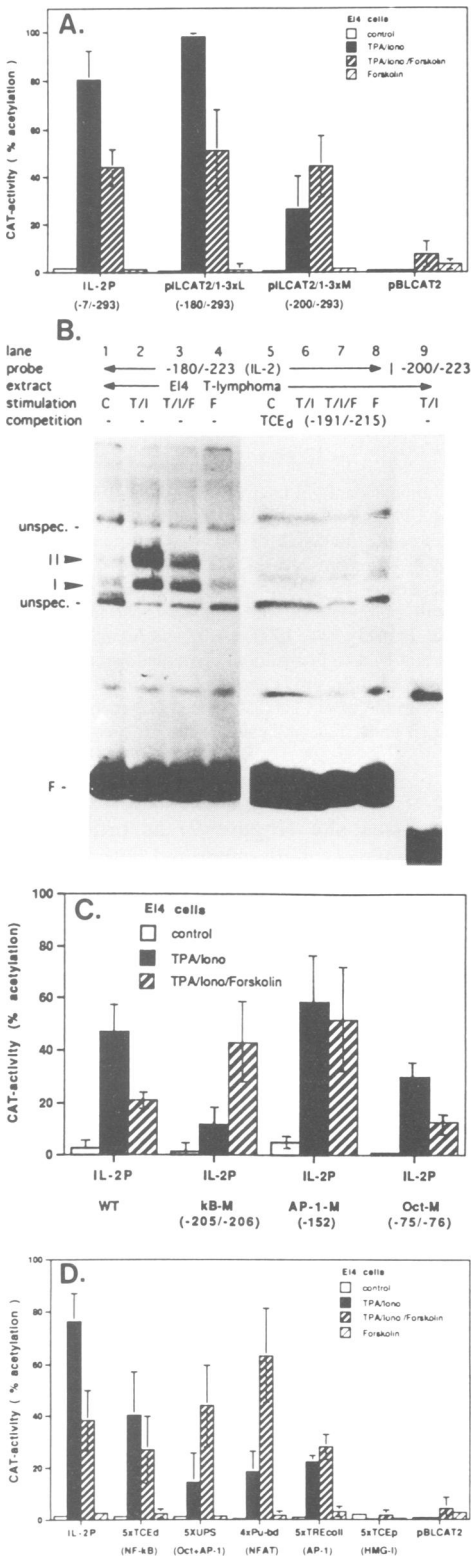


Fig. 2. Stimulation of the PKA pathway interferes with inducible activity of the IL-2 promoter and of κB consensus sites.
(**A**) Activators of the PKA pathway inhibit induction of the IL-2 promoter in El4 T lymphoma cells. El4 cells were transfected with the construct pIl2CAT5 containing the IL-2 promoter in front of the tk promoter and the CAT reporter gene in pBLCAT5. Twenty hours later, the cells were divided. One part of the cells was left uninduced (control), one part was induced with TPA/ionomycin in the absence or presence of PGE2 (5 μM), forskolin (5 μM) or cBiMPS (0.1 mM) or with PGE2, forskolin or cBiMPS alone. After incubation for 20 h the cells were sonicated and the cell lysates were used in CAT assays. (**B**) Activation of PKA activity reduces NF-κB-directed promoter activity in T cells. The IL-2 promoter construct pIlCAT2/1+ (IL-2P), the construct 7×κB, containing seven copies of the NF-κB binding site from the MHC class II invariant chain promoter (Pessara and Koch, 1990), the construct 4×CRE, containing four copies of a CREB binding site from the human cytomegalovirus enhancer (Niller and Hennighausen, 1990), and pBLCAT2 were transfected into human Jurkat T cells. The cells were treated as in Figure 1B. (**C**) Over-expression of the catalytic subunit of PKA leads to a reduction in NF-κB-directed promoter activity. DNA (2.5 μg) of the IL-2 promoter construct pIL2CAT5 (IL-2P) or the constructs 7×κB or 4×CRE were transfected into El4 cells, together with 5 μg expression vector pCEV coding for the catalytic subunit of PKA (Uhler and McKnight, 1987) or, to keep the DNA concentration constant, with the Gemini 1 vector as a control. Twenty hours later, the cells were divided. One half of the cells was left uninduced, the other half was induced with TPA/ionomycin for 20 h.

ferred to nitrocellulose. The immunoblots were probed with peptide antibodies raised against specific epitopes of c-Rel, p65 and p50. Results of such experiments using a c-Rel-specific antibody raised against a peptide from the unique C-terminus of c-Rel show that forskolin treatment led to an increase in κB site-specific DNA binding of c-Rel (Figure 6A, lanes 2 and 3 and 5 and 6). In contrast, forskolin strongly suppressed TPA/ionomycin-induced DNA binding of p65. This is shown in Figure 6B and C using a p65-specific antibody raised against a p65-specific oligopeptide (Figure 6B) and an antibody that cross-reacted with p65 and p50 (Figure 6C). Again, only minor amounts of p50 were found to bind to the κB sites, as detected in the DNAP assays using antibodies raised against whole p50 protein or a p50-specific oligopeptide (Figure 6C and D). Forskolin treatment had a weak suppressive effect on the poor κB site-specific DNA binding of p50 (see lanes 2 and 3 in Figure 6D).



Elevated PKA levels inhibit generation of nuclear κB complexes

Nuclear translocation of NF-κB is controlled by the association of Rel/NF-κB proteins with inhibitory cytosolic proteins, designated IκBs (see Beg and Baldwin, 1993, for a recent review). It is thought that phosphorylation of IκBs leads to release of NF-κB from cytosolic, inactive complexes and consequently to translocation of active NF-κB into the nucleus (Beg *et al.*, 1993; Brown *et al.*, 1993; Henkel *et al.*, 1993; Sun *et al.*, 1993). In order to investigate whether stimulation of the PKA signalling pathway interferes with this post-translational activation of NF-κB, immunoprecipitations were performed incubating Rel-specific antibodies with ^{35}S-labelled proteins derived from Jurkat cells induced in the presence or absence of forskolin. Figure 7 shows that

Fig. 3. Localization of the PKA-sensitive site within the IL-2 promoter. (**A**) Effect of PKA activation on the activity of distal DNA segments of the IL-2 promoter. The constructs pIlCAT2/1+, containing one copy of the entire IL-2 promoter from positions −7 to −293 (IL-2P), pILCAT2/1-3×L, containing three copies of the promoter region from −180 to −293, pILCAT2/1-3×M, containing three copies of the sequence from −200 to −293 (Serfling *et al.*, 1989), and the vector pBLCAT2 were transfected into El4 cells. Twenty hours after transfection, the cells were divided and induced as indicated for 20 h. (**B**) Inducible κB-like factors bind to the IL-2 promoter segment from −180 to −223, but not to the shorter segment from −200 to −223. Nuclear protein (4 μg) from uninduced El4 cells (lanes 1 and 5) or from cells induced with TPA/ionomycin (lanes 2, 6 and 9), TPA/ionomycin/forskolin (lanes 3 and 7) or forskolin alone (lanes 4 and 8) was incubated with DNA probes from the IL-2 promoter spanning nucleotides −180 to −223 (lanes 1–8) or −200 to −223 (lane 9). In lanes 5–8 a 100-fold molar excess of the κB/TCEd site of the IL-2 promoter (Briegel *et al.*, 1991) was added for specific competition. Note the generation of inducible complexes I and II after incubation with an oligonucleotide spanning nucleotides −180 to −223, but not −200 to −223, with nuclear proteins from TPA/ionomycin-induced cells (compare lanes 1, 2 and 9) and the reduction in the intensity of complex II in incubations with proteins from TPA/ionomycin/forskolin-induced cells (compare lanes 2 and 3). (**C**) Mutations within the κB/TCEd-site of the IL-2 promoter abolish the suppressive effect of PKA activation on the IL-2 promoter. CAT constructs containing the wild-type IL-2 promoter (WT) or IL-2 promoters bearing mutations within their κB/TCEd sites (κB-M, −205 to −206), AP-1/TREp site (AP-1-M, −152) and Oct/UPS site (Oct-M, −75 to −76) were transfected into El4 cells. After 20 h, the cells were divided. One third of the cells were left uninduced, one part was induced with TPA/ionomycin and one with TPA/ionomycin/forskolin for 20 h. (**D**) The effect of PKA stimulation on the activity of several factor binding sites of the IL-2 promoter. The IL-2 wild-type promoter construct pIlCAT2/1+ (IL-2P), the constructs 5×TCEd, containing five copies of the κB-like site TCEd, 5×UPS, containing five copies of the octamer and AP-1 binding site UPS, 4×Pu-b_d, containing four copies of the NFAT-1 binding site Pu-b_d, 5×TCEp, containing five copies of the HMG I(Y) binding site of the IL-2 promoter, 5×TREcoll, containing five copies of an AP-1 consensus site from the human collagenase promoter, and the vector pBLCAT2 were transfected into El4 cells. The cells were treated as in Figure 1B.

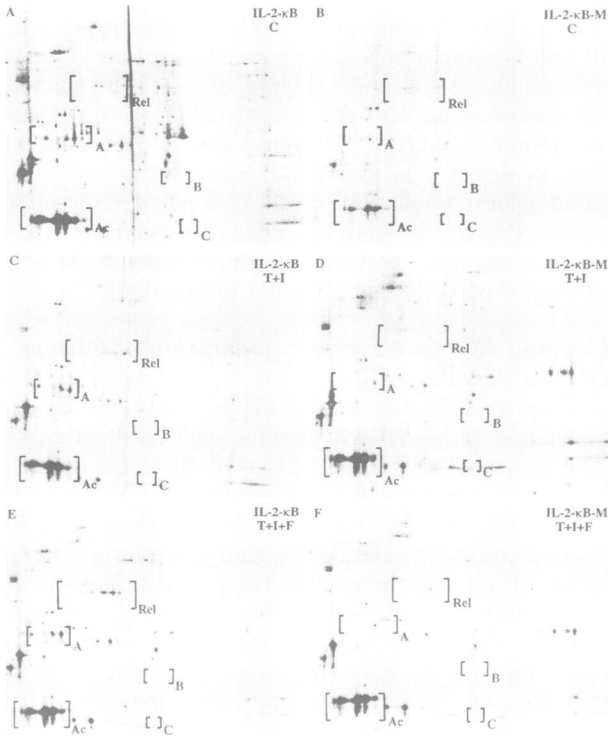


Fig. 4. Two-dimensional gel analysis of proteins binding to the κB/TCEd site of the IL-2 promoter in Jurkat T cells. Unstimulated Jurkat cells (C) (**A** and **B**) and Jurkat cells stimulated with TPA/ionomycin without (T+I) (**C** and **D**) or with forskolin (T+I+F) (**E** and **F**) for 2 h were labelled with [^{35}S]methionine *in vivo* for 30 min (2.5 mCi/100 mm dish). Whole cellular protein extracts were prepared and incubated with a biotinylated wild-type κB/TCEd probe of the IL-2 promoter (A, C and E) or a mutant κB/TCEd probe as a control (B, D and F). Protein–DNA complexes were precipitated with streptavidin–agarose, followed by two-dimensional gel electrophoresis as described earlier (Franza *et al.*, 1987; Neumann *et al.*, 1992). The isoforms of c-Rel (Rel) and actin (Ac) are indicated with brackets. The proteins binding more strongly to the κB/TCEd site of the IL-2 promoter than to the κB site of the HIV-1 LTR (see Figure 5) are also indicated and designated A; those binding more strongly to the HIV-1 κB site are designated B and C.

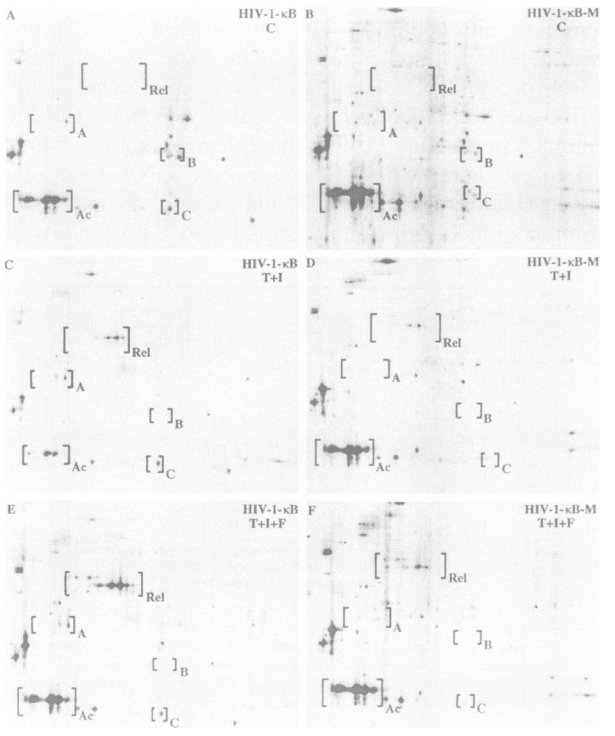


Fig. 5. Two-dimensional gel analysis of proteins binding to the κB site of the HIV-1 LTR in Jurkat T cells. Unstimulated Jurkat cells (**A** and **B**) and Jurkat cells stimulated for 2 h with TPA/ionomycin in the absence (**C** and **D**) or presence of forskolin (**E** and **F**) were labelled with [^{35}S]methionine for 30 min. Whole cellular protein extracts were incubated with a biotinylated wild-type κB-probe from the HIV LTR promoter (see Franza *et al.*, 1987) (A, C and E) or with a mutated κB probe (B, D and F) as a control and processed as described in Figure 4. The isoforms of c-Rel (Rel) and actin (Ac) and the proteins binding more strongly to the κB/TCEd site of the IL-2 promoter (A) or to the HIV κB site (B and C) are indicated with brackets.

forskolin treatment led to a distinct increase in *de novo* synthesis of c-Rel and p105, the precursor of p50. A slight stimulatory effect of forskolin on the generation of p50 was also observed, although only trace amounts of labelled p50 were synthesized in these pulse-labelling experiments (see the arrowhead between lanes 3 and 4 in Figure 7). In contrast, constitutive synthesis of p65 was neither enhanced by TPA/ionomycin nor affected by forskolin treatment (Figure 7C). This indicates that stimulation of PKA does not interfere with *de novo* synthesis of p65, but with its DNA binding and, possibly, its nuclear transport.

In order to test the hypothesis that increased PKA activity impairs nuclear transport of p65, immunoblots of nuclear and cytosolic proteins derived from Jurkat cells were probed with p65-, p50- and c-Rel-specific antibodies. The results of these experiments show that forskolin treatment led to a reduction in the amount of nuclear p65, whereas nuclear translocation of c-Rel and p50 remained unchanged. Thus, the PKA-mediated increase in *de novo* synthesis of c-Rel and p105 did not result in a measurable increase in steady-state levels of c-Rel and p50 (Figure 8A).

In EMSAs using the IL-2 κB/TCEd site (Figure 3B) or a κB consensus site (Figure 9) as probes, two major inducible complexes were generated with nuclear proteins from activated El4 cells or Jurkat cells. Forskolin treatment led to a distinct suppression of formation of the slower migrating complex II, while formation of complex I remained largely unaffected. EMSA 'supershift' assays using antibodies specific for Rel proteins showed that complex I consists mainly of p50 (Figure 9B, lane 5) and, therefore, represents p50–p50 homodimers. Complex II consists of several κB proteins. The most prominent component of complex II is p65, because incubation with a p65-specific antibody resulted in the supershift of most, albeit not all, protein of complex II (Figure 9B, lane 2). In addition, p50 and c-Rel are also components of this complex, because incubations with p50- and c-Rel-specific antibodies led to a slight decrease in formation of complex II (Figure 9B, lanes 3 and 5). Neither complex was recognized in supershift assays using antibodies directed against RelB and p52. RelB was detected in EMSAs performed with protein extracts from B lymphocytes using the same antibody (unpublished results). Taken together, these results and the data presented above indicate that stimulation of PKA impairs nuclear translocation of p65.

In order to test whether activation of PKA also impairs transcriptional transactivation by p65 in T cells, we co-transfected a CAT reporter construct containing five

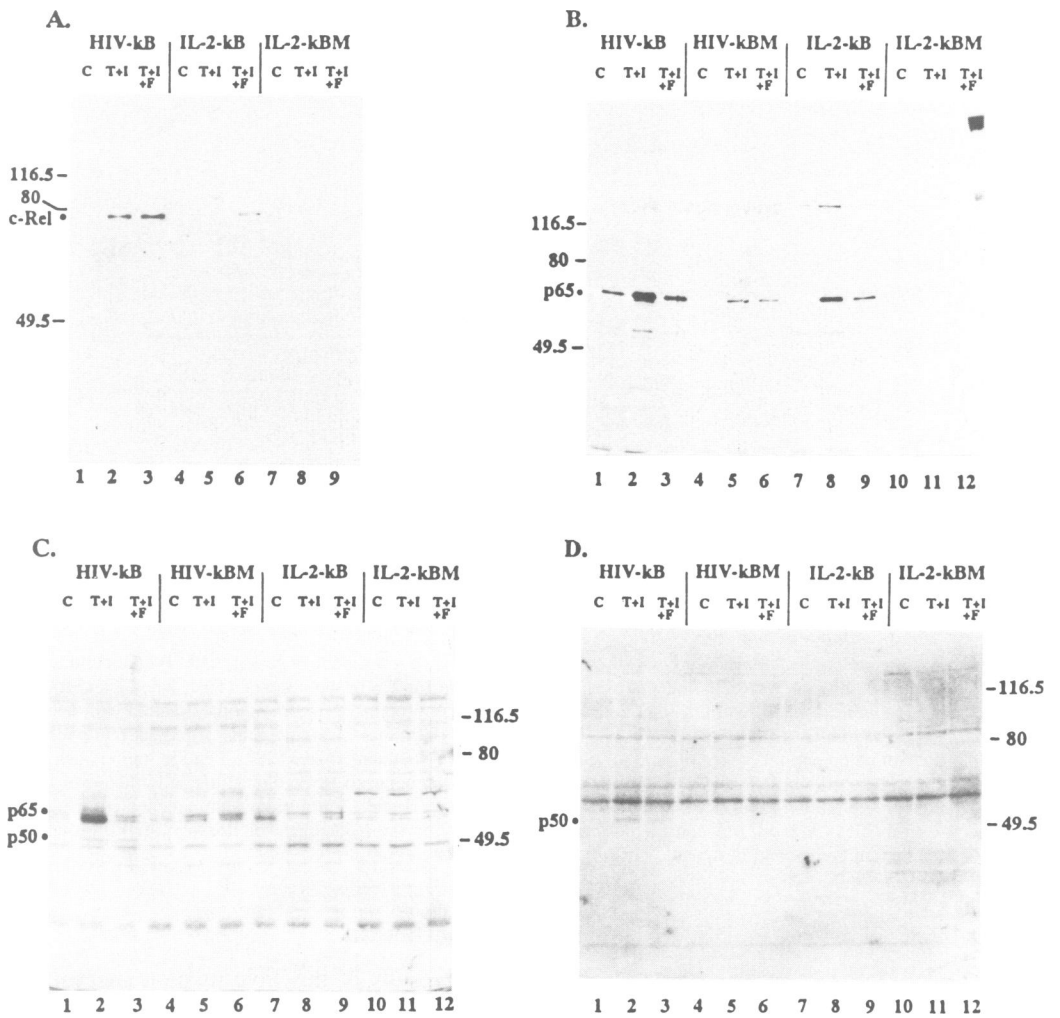


Fig. 6. PKA activation selectively affects the DNA binding of Rel proteins in Jurkat cells. Whole cellular protein extracts from uninduced Jurkat cells (C) or Jurkat cells induced with TPA/ionomycin for 2 h in the absence (T+I) or presence of 5 μM forskolin (T+I+F) were incubated with biotinylated wild-type κB probes and mutated probes from the HIV-1 LTR (HIV-κB and HIV-κB-M; Franza *et al.*, 1987) and the IL-2 promoter (IL-2-κB and IL-2-κB-M; Briegel *et al.*, 1991) respectively. After precipitation of protein–DNA complexes with streptavidin–agarose, the proteins were released by boiling, fractionated on 10% SDS–polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes. Immunodetections were performed using antibodies raised against: (**A**) a unique C-terminal peptide of human c-Rel (Neumann *et al.*, 1992); (**B**) a p65-specific antibody raised against the amino terminal domain of human p65 (sc-109; Santa Cruz); (**C**) an antibody raised against bacterially expressed human p50 which also cross-reacts with p65; (**D**) a p50-specific antibody raised against the NLS region of p50 (a kind gift of A.Tsimanis, Riga).

binding sites for the yeast factor Gal4 together with an expression vector coding for a chimeric Gal4–p65 protein into El4 cells. As shown in Figure 8B, expression of Gal4p65ΔN containing the DNA binding domain of Gal4 and the transactivation domains of p65 (Schmitz and Baeuerle, 1991) resulted in a strong increase in activity of the CAT reporter construct, which was further enhanced after stimulation of cells. Forskolin did not reduce, but further increased, p65-mediated transactivation, indicating that the transactivating potency of p65 is not impaired by activation of PKA.

Elevated PKA activity retards degradation of IκB-α

Nuclear translocation of NF-κB upon induction by phorbol esters, lipopolysaccharides, IL-1 and TNF-α is closely correlated with degradation of IKB-α (see Beg and Baldwin, 1993). Because of the PKA-mediated inhibition of nuclear translocation of NF-κB, one may speculate that increased PKA activity prevents degradation of IκB-α, which appears to be linked with its phosphorylation (Ghosh,S. and Baltimore, 1990; Link *et al.*, 1992). To test this hypothesis, we performed immunoblots using IκB-α-specific antibodies. We observed a strong IκB-α signal with cytosolic proteins derived from uninduced Jurkat cells. Upon TPA/ionomycin induction, the concentration of IκB-α decreased, while the amounts of nuclear p65 and nuclear p50–p65 complexes increased. These findings are in accordance with results obtained by other authors (Beg *et al.*, 1993; Brown *et al.*, 1993; Henkel *et al.*, 1993; Sun *et al.*, 1993). Forskolin treatment of TPA/ionomycin-stimulated Jurkat cells led to a smaller decrease in IκB-α, but also to a reduction in p65 protein concentration and p50–p65 complexes in the nuclei of T cells (Figure 9). This indicates that activation of PKA leads to stabilization of IκB-α by retarding degradation of IκB-α and, as a consequence, release and nuclear translocation of p65. The discrepancy between the concentration and DNA binding of nuclear p65, as notable in lane 4 of Figure 9A,

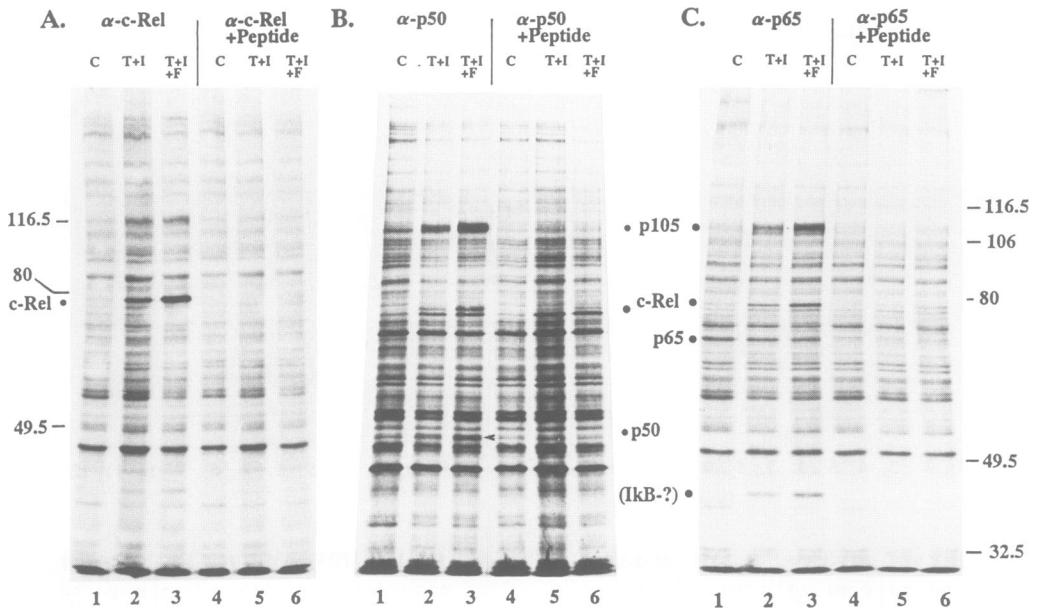


Fig. 7. PKA activation selectively increases *de novo* synthesis of c-Rel and p105/p50, but not p65, in Jurkat cells. Whole cellular proteins from uninduced Jurkat cells (C, lanes 1 and 4) or from cells induced with TPA/ionomycin for 2 h in the absence (T+I, lanes 2 and 5) or presence of forskolin (T+I+F, lanes 3 and 6) were labelled with [^{35}S]methionine for 30 min. Rel proteins were immunoprecipitated with antibodies raised against: (**A**) a C-terminal peptide of human c-Rel (Neumann *et al.*, 1992); (**B**) the N-terminal 21 amino acids of human p50/p105 (Kieran *et al.*, 1990); (**C**) the N-terminal domain of human p65 (Santa Cruz, sc-109), as described (Neumann *et al.*, 1992). Peptide immunogen (1 μg) was added to each precipitation assay in control experiments (lanes 4–6). Due to complex formation between Rel proteins, a small amount of labelled c-Rel was also precipitated in the immunoprecipitations using p105- and p65-specific antibodies (B and C) and vice versa; labelled p105 was also precipitated with c-Rel- and p65-specific antibodies (A and C). A considerable amount of protein in the molecular weight range of IκB proteins was precipitated only with c-Rel- and p65-specific antibodies and designated (IκB-?) (A and C).

might indicate that p65 not bound to DNA is rapidly degraded.

Activation of the PKA pathway prevents generation of nuclear κB complexes in peripheral T lymphocytes

In order to investigate the consequences of the elevated PKA activity in peripheral T cells and TH1/TH2 cells, we analysed the effect of forskolin on κB factors in these cells. Stimulation of murine lymph node T cells for 8 h with TPA/ionomycin gave rise to one prominent, inducible NF-κB complex (Figure 10A). Supershift experiments with antibodies specific for Rel proteins showed that this complex mainly consists of p50–p65 heterodimers (Figure 10B). Forskolin almost completely suppressed formation of the p50–p65 complexes (Figure 10A) and impaired nuclear translocation of p65 (Figure 10C). Similar to the situation in Jurkat cells, this might be due to stabilization of IκB-α, since a stronger IκB-α signal was observed after induction of peripheral T cells in the presence of forskolin (Figure 10C).

In contrast to the strong inhibitory effect of forskolin on induction of κB complexes in peripheral T cells, no suppressive effect of PKA activation was observed for NF-κB in TH1 and TH2 cells (M.Neumann and M.Lohoff, unpublished results). We investigated the TH1 cell line LNC-2, which synthesizes large amounts of IL-2 but no IL-4, upon stimulation with anti-CD3, anti-CD28 and anti-CD44 antibodies (M.Lohoff *et al.*, in preparation), and the TH2 cells clones L 1/1 and D10G4.1, which synthesize IL-4 but no IL-2 upon anti-CD3, anti-CD28 and anti-CD44 stimulation.

Discussion

The results of this investigation suggest the existence of a novel molecular control mechanism modulating the immune response *in vivo*. Activators of the PKA signalling pathway, such as PGE, are able to inhibit activation of T lymphocytes by suppressing formation of active nuclear κB factors. Elevated PKA activity impairs nuclear translocation of NF-κB, in particular nuclear transport of the strong transactivator p65/RelA. This seems to be due to stabilization of IκB-α, a cytosolic inhibitor of κB factors. As a consequence, translocation of active NF-κB into the nuclei of T cells is attenuated.

The role of NF-κB in activation of T lymphocytes

NF-κB was originally described as a B lymphocyte-specific transcription factor controlling transcriptional activity of the Ig light chain κ enhancer (Sen and Baltimore, 1986). Although NF-κB turned out to be a widely distributed, ubiquitous transcription factor, κB factors play a pivotal role in the immune response (see Baeuerle and Henkel, 1994, for a recent review). The rapid activation of NF-κB by post-transcriptional mechanisms upon inflammation and infection and the control of numerous lymphoid-specific genes, including those of lymphotropic viruses, by NF-κB predetermines this factor as a key regulator of the mammalian immune system (Grilli *et al.*, 1993; Baeuerle and Henkel, 1994).

NF-κB appears to be crucially involved in activation of T lymphocytes, since most, if not all, stimuli which activate T cells also activate NF-κB. However, activation of NF-κB is necessary, but not sufficient, for IL-2 synthesis

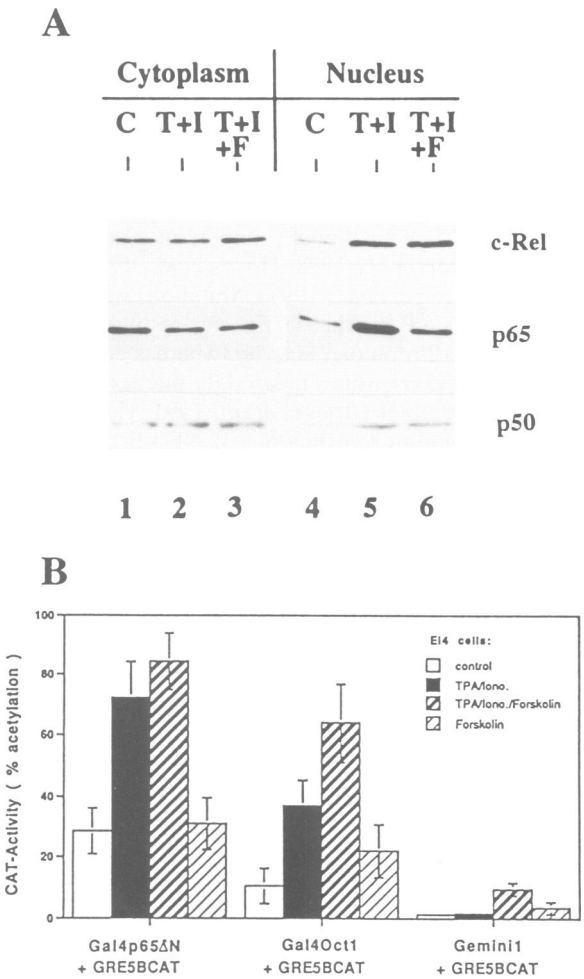


Fig. 8. PKA activation impairs the nuclear appearance but not transactivation of p65. (**A**) The effect of forskolin on nuclear translocation of Rel proteins in Jurkat cells. Cytoplasmic and nuclear protein extracts derived from uninduced Jurkat cells (C, lanes 1 and 4) or from cells induced with TPA/ionomycin for 2 h in the absence (T+I, lanes 2 and 5) or presence of forskolin (T+I+F, lanes 3 and 6) were prepared as described (Neumann *et al.*, 1992). Immunoprecipitations were performed and the proteins were fractionated on 10% SDS–polyacrylamide gels and electrophoretically transferred onto nitrocellulose filters. Immunodetections were performed with c-Rel-, p65- and p50-specific antibodies (see Figure 7). Note the considerable reduction in nuclear p65 in cells activated with TPA/ionomycin and forskolin. (**B**) Forskolin treatment does not reduce transactivation of p65 in El4 cells. A CAT reporter plasmid containing five binding sites of the yeast factor Gal4 (GRE5BCAT) was co-transfected into El4 cells with expression vectors coding for chimeric Gal4–p65 protein (Gal4p65ΔN; Schmitz and Baeuerle, 1991) or Gal4–Oct1 protein (Seipel *et al.*, 1992) and with Gemini 1 as a control. Twenty hours after transfection, the cells were divided and induced for 20 h as indicated.

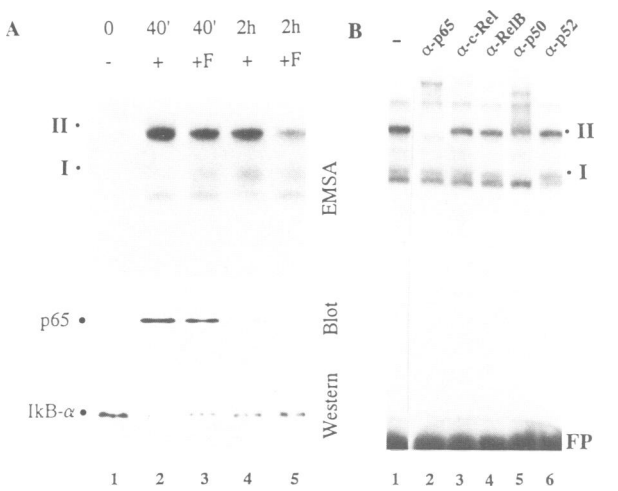


Fig. 9. Forskolin treatment impairs nuclear translocation of κB complexes and degradation of IκB-α in Jurkat cells. (**A**) EMSA and Western blot experiments with nuclear proteins (EMSA and p65 blot) and cytosolic proteins (IκB-α blot) from Jurkat cells. Nuclear and cytoplasmic proteins derived from uninduced Jurkat cells (0, lane 1) or from cells induced for 40 min or 2 h with TPA/ionomycin in the absence (40′+ and 2h+, lanes 2 and 4) or presence of forskolin (40′+F and 2h+F, lanes 3 and 5) were used in EMSAs and immunoblots as indicated. In EMSAs, 4 μg nuclear proteins were incubated with an IL-2 TCEd (A/C) κB probe (Briegel *et al.*, 1991). Note the rapid induction of nuclear complexes I and II after TPA/ionomycin stimulation and the reduction of formation of complex II by forskolin treatment. The band below complex I corresponds to a non-specific complex. The free probe was cut off. Immunoblots were performed using a p65-specific antibody (sc-109; Santa Cruz) to detect the concentration of p65 in the nuclei and a MAD-3-specific antibody (sc-203; Santa Cruz) was used to determine the concentration of IκB-α in the cytoplasm of Jurkat cells. The ECL system (Amersham, UK) was used for detection, according to the manufacturer's instructions. Note the reduced degradation of IκB-α in forskolin-treated cells (lanes 3 and 5). (**B**) Analysis of composition of inducible NF-κB complexes in supershift EMSAs. Nuclear proteins from Jurkat cells induced with TPA/ionomycin for 2 h (lane 1) were incubated with a κB probe and 1 μl of the following antibodies: lane 2, α-p65 (sc-109; Santa Cruz); lane 3, α-c-Rel raised against a C-terminal peptide of human c-Rel (Neumann *et al.*, 1992); lane 4, α-RelB (sc-226; Santa Cruz); lane 5, α-p50 raised against an N-terminal peptide of human p50 (Kieran *et al.* 1990); lane 6, α-p52 (sc 298; Santa Cruz). FP, free probe.

and T cell activation. Low concentrations of the NF-κB inhibitor PDTC are able to suppress CD2/CD28-mediated activation of peripheral human T lymphocytes (Costello *et al.*, 1993). Removal of the lectin concanavalin A, an efficient activator of T cells, impairs both nuclear translocation of NF-κB and IL-2 synthesis in murine lymph node T cells (E.Bohn *et al.*, in preparation). Phorbol esters, on the other hand, are efficient activators of NF-κB in many cells, including T cells, but are incapable of activating primary T lymphocytes without stimulation of the Ca^{2+} signalling pathway(s). Such an additional stimulus also enhances activation of NF-κB in many T cell lines. However, the moderate effects of the calcineurin inhibitors CsA and FK506 on NF-κB activity (Emmel *et al.*, 1989; Randak *et al.*, 1990; but see Frantz *et al.*, 1994) demonstrate that calcium plays a less prominent role in activation of NF-κB compared with other stimuli. This conclusion is supported by the observation that triggering of T cells by CD28 results in efficient stimulation of Rel proteins, in particular of c-Rel. This stimulation was shown to be insensitive to CsA (Verweij *et al.*, 1991; Ghosh,P. *et al.*, 1993; Bryan *et al.*, 1994).

Our results show that p65, p50 and c-Rel are the most prominent members of the NF-κB family involved in T cell activation (Molitor *et al.*, 1990). The high amount of DNA-bound c-Rel we observed in pulse-labelling experiments (Figures 4 and 5) might be due to very efficient labelling of c-Rel with [^{35}S]methionine. In all EMSAs performed with nuclear proteins from Jurkat cells and peripheral lymph node T cells, the κB-specific binding of c-Rel was lower compared with p65 and p50. It is known that in Jurkat cells nuclear translocation of c-Rel

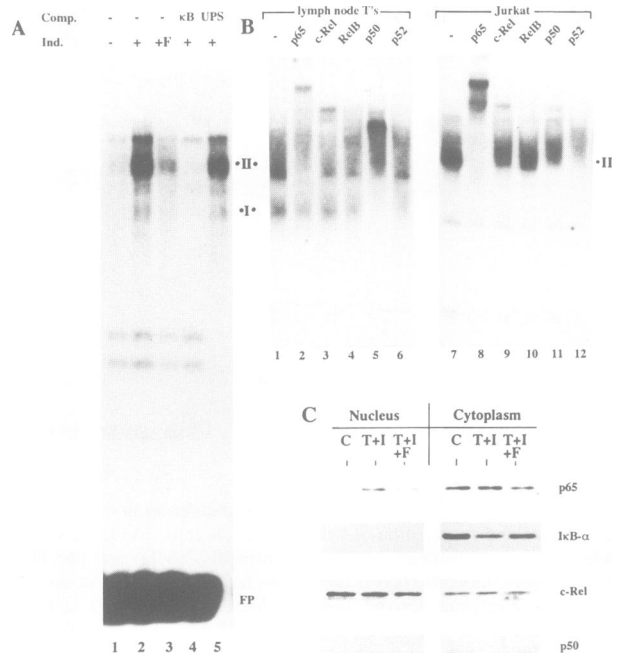


Fig. 10. Forskolin treatment prevents inducible generation of nuclear NF-κB complexes in T lymphocytes from murine lymph nodes. (**A**) EMSAs with nuclear proteins from T lymphocytes of murine lymph nodes. Nuclear proteins (4 μg) from uninduced T lymphocytes (lane 1) or lymphocytes induced for 8 h with TPA/ionomycin in the absence (lanes 2, 4 and 5) or presence of forskolin (lane 3) were incubated with an IL-2 TCEd (A/C) κB probe (Briegel *et al.*, 1991). For specific competition, a 100-fold molar excess of unlabelled κB oligonucleotide was added in lane 4. In lane 5, a 100-fold molar excess of an unlabelled IL-2 UPS oligonucleotide (i.e. an AP-1/octamer site; Pfeuffer *et al.*, 1994) was added. Note the reduced formation of κB complexes I and II in cells stimulated in the presence of forskolin (lane 4). (**B**) Detection of p65, p50 and c-Rel in the κB complexes generated with nuclear proteins from lymph node T cells. Supershift EMSAs of nuclear proteins from lymph node T cells induced for 8 h (lanes 1–6) and Jurkat cells induced for 2 h with TPA/ionomycin (lanes 7–12) using antibodies specific for Rel proteins (see Figure 9 for their specificity). (**C**) PKA activation reduces the nuclear appearance of p65 in lymph node T cells. Immunodetection of Rel/κB proteins in nuclear and cytosolic protein extracts from uninduced lymph node T cells (C) or cells induced with TPA/ionomycin for 8 h in the absence (T+I) or presence (T+I+F) of forskolin.

is induced with delayed kinetics relative to p65. Since c-Rel was found to interfere with p65-mediated transcriptional activation in Jurkat cells (Doerre *et al.*, 1993), it is possible that a PKA-mediated increase in c-Rel concentration might suppress the activity of κB sites in T cells. Although we have no proof for such a mechanism, this could contribute to PKA-mediated suppression of NF-κB activity in T cells.

Peripheral T cells contain higher concentrations of nuclear p50–p50 complexes than Jurkat T cells (see Figure 10B). It has been proposed that the predominance of p50–p50 complexes in resting, non-transformed T cells and their antigen-dependent intranuclear sequestration by a hypothetical inhibitory protein is important for induction of the IL-2 promoter (Kang *et al.*, 1992; see Grilli *et al.*, 1993). According to our data, such a mechanism can be excluded for PKA-mediated suppression of the IL-2 promoter. In EMSAs using proteins from peripheral T cells we never observed any increase in DNA binding of p50, but a decrease in p65 binding after PKA induction (Figure 10).

The majority of κB binding sites involved in control of lymphokine and lymphokine receptor promoters in T cells differ in their sequences from κB consensus motifs. Such subtle sequence differences often have profound effects on the binding affinity of NF-κB factors, the composition of κB complexes and, consequently, transactivating properties. We and others have shown that the IL-2 κB/TCEd site is a weak but functionally important binding site for NF-κB (Lenardo *et al.*, 1988; Hoyos *et al.*, 1989; Shibuya *et al.*, 1989; Briegel *et al.*, 1991). Mutations which prevent binding of NF-κB to the TCEd site result in a drastic decrease in IL-2 promoter activity, whereas conversion of TCEd to a κB consensus site strongly increases induction of the IL-2 promoter (Briegel *et al.*, 1991; Hentsch *et al.*, 1992). This implies that binding of NF-κB to the TCEd is of importance for IL-2 promoter activity. Low affinity NF-κB sites might be very sensitive to changes in the composition of κB complexes caused by PKA activation and other stimuli.

Elevated cAMP levels exert a dual effect in T cells. Expression of IL-2 and γ-IFN is enhanced, whereas expression of IL-4 and IL-5 is reduced. IL-2 and γ-IFN are characteristic for the TH1 subset of T helper cells, while IL-4 and IL-5 are typical for the TH2 subset of T helper cells. Based on these observations, several authors have suggested that differentiation of T helper cells into TH1 and TH2 cells is, at least in part, controlled by cAMP (Munoz *et al.*, 1990; Novak and Rothenberg, 1990; Betz and Fox, 1991; Lee,H.J. *et al.*, 1993). Furthermore, high cAMP concentrations were found in TH2 cells (Novak and Rothenberg, 1990). All these findings prompted us to investigate whether distinct differences exist in activation of κB factors between TH1 versus TH2 cells. One indication in support of this model was the description of reduced NF-κB complex formation in some TH2 cells (Lederer *et al.*, 1994). We compared the TH2 cell clones D10G4.1 (Kaye *et al.*, 1983) and L 1/1 (Lohoff *et al.*, 1988) with the TH1 cell line LNC-2 (Schmitt *et al.*, 1989). We were unable to detect any significant differences in the composition of Rel proteins between these cell lines. Although many parameters (e.g. the cell lines used, the induction of cells) could be responsible for these negative results, we conclude that the primary effect of elevated PKA activity on T cells is inhibition of T cell activation.

IκB-α is a molecular target of the suppressive effect of elevated PKA activity in T lymphocytes

Nuclear translocation of NF-κB is accompanied by rapid degradation of IκB-α. It has been shown that activation of Jurkat T cells and other cells by TNF-α and many other stimuli leads to rapid decay of IκB-α and the nuclear appearance of p65 (see Beg and Baldwin, 1993). IκB proteins are usually located in the cytoplasm. They are characterized by so-called ankyrin repeats. Ankyrin repeats are structural motifs of ~33 amino acids which are supposed to mediate protein complex formation. Through these motifs IκB-α seems to retain NF-κB in the cytosol by masking the nuclear localization signal (NLS) of Rel proteins (see Beg and Baldwin, 1993). All Rel proteins (except p52/NF-κB2) carry the PKA recogniton motif Arg–Arg–Pro–Ser near their NLS sequence. Disruption of the PKA recognition sequence of c-Rel results in the predominant nuclear appearance of this protein (Mosialos

et al., 1991). Nevertheless, a function for this sequence motif remains to be established for other Rel proteins. Both p105 and p65 were efficiently phosphorylated by PKA *in vitro*. Furthermore, p50–p65 heterodimers displayed increased DNA binding upon phosphorylation (Naumann and Scheidereit, 1994). Members of the IκB protein family are phosphorylated *in vitro* by a variety of kinases, including PKA (Ghosh,S. and Baltimore, 1990; Kerr *et al.*, 1991; Link *et al.*, 1992). However, it is not yet clear whether the same enzymes also phosphorylate IκB proteins *in vivo*. Different IκB proteins appear to have different binding affinities for the Rel proteins. Whereas IκB-α predominantly interacts with heterodimeric p50–p65 complexes and interferes with their nuclear translocation, DNA binding (and nuclear transport) of c-Rel was found to be less affected by IκB-α than by IκB-β (Kerr *et al.*, 1991). These functional differences of IκB proteins might explain why elevated PKA activity, which, as our results indicate, leads to accumulation of IκB-α, causes a selective inhibition of κB site-specific DNA binding of p65/NF-κB, but not of c-Rel.

We observed rather small changes in the amounts of IκB-α, especially in murine lymph node T-cells. However, in a recent study it was convincingly demonstrated that even small changes in cytoplasmic IκB result in profound changes in nuclear NF-κB activity (Miyamoto *et al.*, 1994). This conclusion is based on the fact that only 10% of NF-κB is translocated to the nucleus at steady-state. Therefore, even a 2-fold increase in IκB-α concentration might be sufficient to retain the entire NF-κB activity in the cytoplasm.

Elevated cAMP levels inhibit hormonal induction of the Ras–Raf–MAP kinase signalling pathway in non-lymphoid cells (Burgering *et al.*, 1993; Cook and McCormick, 1993; Sevetson *et al.*, 1993; Wu *et al.*, 1993). Since the Ras–Raf–MAP kinase cascade appears to play an important role in induction of the IL-2 promoter (Owaki *et al.*, 1993; Park and Levitt, 1993) and the HIV LTR promoter through their κB sites (Bruder *et al.*, 1993; Finco and Baldwin, 1993), one may conclude that PKA-mediated suppression of NF-κB in T lymphocytes is mediated through this pathway. However, other signalling pathways controlled by PKA which can also modulate the phosphorylation status of IκB, p65 and other members of the Rel family could also result in reduced nuclear translocation of p65 (see Naumann and Scheidereit, 1994, and discussion therein). Experiments are in progress to investigate whether PKA stimulation modifies *in vivo* phosporylation of Rel proteins in T cells.

PKA activation and T cell proliferation

The formation of catalytically active complexes between cyclins and cyclin-dependent kinases, designated cdks, is a prerequisite for cells to progress through the cell cycle. It has recently been shown that elevated PKA activities in macrophages increase the concentration of $p27^{Kip1}$, an inhibitor of phosphorylation of cdks (Kato *et al.*, 1994). In peripheral T lymphocytes IL-2 stimulates generation of cyclin–cdk2 complexes, which are necessary for entry into S phase (Firpo *et al.*, 1994). Thus elevated PKA activities could block T cell proliferation at at least two levels, i.e. by suppression of expression of the growth factor IL-2 and by stimulating the growth inhibitor $p27^{Kip1}$. This indicates the close interdependence of control of expression of growth factors, like IL-2, and components of the cell cycle, like the cdks and cell cycle inhibitors.

Materials and methods

Cells, DNA transfections and CAT assays

Human Jurkat T leukaemic cells and murine El4 T lymphoma cells were grown in RPMI medium containing 5% fetal calf serum. Usually, 4×10^7 cells were transfected with 20 μg DNA. The cells were cultured for 20 h after transfection and then divided. One quarter of the cells were left as an uninduced control, one quarter were induced with 10 ng/ml TPA and 0.5 μM ionomycin, one quarter with TPA + ionomycin and 5 μM forskolin (or, if indicated, with 5 μM PGE2 or 0.1 mM cBiMPS as PKA activators) and one quarter with 5 μM forskolin (or PGE2 or cBiMPS) alone. After a further 20 h incubation, cells were harvested and CAT activities determined as described. In co-transfection experiments, 2.5 μg of CAT indicator plasmid DNA were transfected together with 5 μg of expression plasmid. The amount of DNA was kept constant at 7.5 μg.

Murine lymph node cells from Balb/c mice were prepared according to a standard protocol. Red blood cells were removed by ammonium chloride (0.83%) lysis. T cells were purified by panning on plastic dishes coated with a rabbit anti-mouse Ig monoclonal antibody (DAKO A/S, Glostrup, Denmark) as described (Kincade *et al.*, 1981). Non-adherent cells were further purified by depletion of cells having bound J11D monoclonal antibody (anti-heat stable antigen; Bruce *et al.*, 1981) on plastic dishes coated with a rabbit anti-rat Ig monoclonal antibody. The purity of T cells was controlled by immunofluorescence and flow cytometry using anti-B220 and anti-CD3 monoclonal antibodies. The purity of T cells prepared in this way was always >95%.

The TH1 cell line LNC-2 (Schmitt *et al.*, 1989) and the TH2 cell clones L1/1 (Lohoff *et al.*, 1988) and D10G4.1 (Kaye *et al.*, 1983) were propagated as described previously (Lohoff *et al.*, 1990). Three weeks after the last antigenic stimulation and 10 days after IL-2 addition, 10^6 cells were stimulated in a volume of 1.5 ml in Costar culture plates coated with anti-CD3 antibodies (2 μg/ml; Leo *et al.*, 1987) and anti-CD44 antibodies (0.5 μg/ml; 01221D; Dianova, Hamburg, Germany). The cells were also stimulated with soluble anti-CD28 antibodies (0.1 μg/ml; 01671D; Dianova). The combination of these three antibodies has been shown to induce maximal production of IL-2 by TH1 cells. After 5 h stimulation, the cells were harvested and the nuclear proteins were prepared.

Preparation of protein extracts and electrophoretic mobility shift assays (EMSAs)

Nuclear and cytosolic proteins from Jurkat cells, El4 cells and murine lymph node T lymphocytes were prepared according to Schreiber *et al.* (1989), with the exception that NP40 was omitted and nuclei were prepared by passing the cells 10 times through an injection needle (26G3/8). Jurkat cells and El4 cells were induced with TPA/ionomycin for 45 min or 2–3 h as indicated in the figure legends. Lymph node T cells were induced with TPA/ionomycin for 8 h.

In EMSAs, 4 μg nuclear proteins were incubated with 5000 c.p.m. (equivalent to ~0.2 ng) of a ^{32}P-labelled oligonucleotide probe and 2 μg poly(dI–dC) as non-specific competitor. After incubation for 30 min on ice, the samples were fractionated on non-denaturing 5% polyacrylamide gels at 200 V/15 cm at room temperature followed by autoradiography. In supershift experiments, 1 μl of the following antibodies were added to the EMSA incubation mixtures: α-c-Rel (Neumann *et al.*, 1992), α-p65 (Santa Cruz, sc-109), α-p50 (Santa Cruz, sc-114), α-RelB (Santa Cruz, sc-226) and α-p52 (Santa Cruz, sc-218).

DNA affinity precipitation (DNAP) assays

Oligonucleotides corresponding to the HIV-1 LTR κB site and the same site containing point mutations which suppress NF-κB binding (Franza *et al.*, 1987) and the IL-2 κB/TCEd and a mutant site $TCEd_M$ (Briegel *et al.*, 1991) were synthesized by ISIS Pharmaceuticals (San Diego, CA). They contained a biotin group at the 3′-end and a phosphothiorate backbone, which renders them nuclease-resistant. Cell pellets were lysed in 600 μl DNAP buffer as described previously (Franza *et al.*, 1987). Dithiothreitol (1 mM), sodium fluoride (50 mM) and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) (1 mM) and leupeptin (10 μM) were added immediately before extraction. The final KCl concentration was 250 mM for the extraction and 50 mM for the binding

assay. In the binding reaction, 100 pmol biotinylated oligonucleotide and a 40-fold excess of poly(dI−dC) were used. The oligonucleotide−protein complexes were recovered by binding to streptavidin−agarose beads (GIBCO BRL, Gaithersburg, MD). The beads were washed four times with 500 µl DNAP buffer containing 50 mM NaF and 75 mM KCl. A DNase/RNase digest was performed for 5 min in ice. Finally, the protein−DNA complexes were heated at 95°C for 5 min in 50 µl Laemmli sample buffer.

Immunoprecipitations

Pellets of 5×10^7–10^8 Jurkat cells were extracted at 4°C for 30 min with 800 µl lysis buffer containing 1 mM sodium vanadate, 100 µM ammonium molybdate, 50 mM NaF, 250 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM EDTA, 0.001% sodium azide, 0.1% Triton X-100, 20% glycerol and 50 mM Tris, pH 8; 1 mM PMSF and 10 µM leupeptin were added just before lysis. Cell debris was spun down and the supernatant was transferred to a fresh tube to which 5 µl rabbit pre-immune serum were added. After 20 min in ice, the solution was transferred to a new tube containing 0.5 ml Zysorbin slurry (Zymed) previously washed in lysis buffer. After 30 min, the Zysorbin was spun down for 5 min. The extract was then divided. The antibody incubation was performed for 1–2 h on ice. One µg of antigenic oligopeptide was included in control reactions. After addition of 70 µl protein A−agarose beads (Pierce), the incubation was performed at 4°C under rotation. The beads were then pelleted and the supernatant was discarded. The beads were washed four times with lysis buffer and the proteins were recovered by heating at 95°C for 5 min in 50 µl Laemmli sample buffer.

Gel electrophoresis and immunoblotting

The samples for DNAP assays and immunoprecipitations were filtered through Millex Eppendorf tube filters (Ultrafree-MC; Millipore). The samples were resolved on one-dimensional 8% polyacrylamide Laemmli gels. For the two-dimensional gel analysis an additional wash of the beads with 50 µl DB buffer containing 20% glycerol, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 100 µM sodium vanadate, 0.001% sodium azide, 0.5 mM EDTA, 0.5 mM EGTA and 50 mM Tris, pH 8, was performed. The beads were then resuspended in 50 µl 0.3% SDS, 1% β-mercapto-ethanol and 50 mM Tris, pH 8, and heated for 5 min at 95°C. The beads were pelleted and the supernatant filtered through Millex tube filters. The samples were frozen in dry ice and lyophilized with a Speedvac. After resuspension in 50 µl sample buffer containing 9.95 M urea, 4% NP-40, 2% ampholytes (6–8) and 0.1 M DTT, the samples were resolved on two-dimensional gels consisting of a pH 3.5–10 isoelectric focusing first dimension gel and a 10% polyacrylamide second dimension gel as described (Garrels and Franza, 1989). After resolution, the gels were dried and exposed for autoradiography or phosphorimaging. For immuno-detection, the proteins were electrophoretically transferred to nitro-cellulose membranes (Schleicher & Schuell) for 2 h at 500 mA. Immunodetections were performed using the ECL detection system (Amersham, UK) according to the instructions of the manufacturer.

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Note added in proof

In a recent study Chen and Rothenberg [(1994) *J. Exp. Med.*, **179**, 931–942] also observed a suppressive effect of PKA stimulation on NF-κB factors in E14 T lymphoma cells. Our data confirm and extend these findings.