I κ B α is a target for the mitogen-activated 90 kDa ribosomal S6 kinase

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The activity of transcription factor NF κ B is regulated by its subcellular localization. In most cell types, NF κ B is sequestered in the cytoplasm due to binding of the inhibitory protein I κ B α . Stimulation of cells with a wide variety of agents results in degradation of I κ B α , which allows translocation of NF κ B to the nucleus. Degradation of I κ B α is triggered by phosphorylation of two serine residues, i.e. Ser32 and Ser36, by as yet unknown kinases. Here we report that the mitogen-activated 90 kDa ribosomal S6 kinase (p90^{rsk1}) is an I κ B α kinase. p90^{rsk1} phosphorylates I κ B α at Ser32 and it physically associates with I κ B α *in vivo*. Moreover, when the function of p90^{rsk1} is impaired by expression of a dominant-negative mutant, I κ B α degradation in response to mitogenic stimuli, e.g. 12-*O*-tetradecanoylphorbol 13-acetate (TPA), is inhibited. Finally, NF κ B cannot be activated by TPA in cell lines that have low levels of p90^{rsk1}. We conclude that p90^{rsk1} is an essential kinase required for phosphorylation and subsequent degradation of I κ B α in response to mitogens.

Keywords: adenovirus E1A/I κ B α /NF κ B/
phosphorylation/RSK

Introduction

Mammalian transcription factor NF κ B is a key regulator of a broad range of genes involved in inflammatory processes, growth control and apoptosis (reviewed in Grilli *et al.*, 1993; Baeuerle and Henkel, 1994; Beg and Baltimore, 1996; Liu *et al.*, 1996; Wu *et al.*, 1996). NF κ B is a heterodimer that is composed of a 50 and a 65 kDa subunit. These subunits belong to a larger group of proteins, generally referred to as the family of Rel-related proteins. The members of the family of Rel-related proteins share sequence homology with the product of the *v-rel* oncogene from the avian reticuloendotheliosis virus strain T (Stephens *et al.*, 1983). On the basis of their structure, they can be divided into two subclasses. The first consists

of p65-RelA, relB and c-Rel which are synthesized as mature proteins and have sequence homology within the N-terminal 300 amino acids, the so-called Rel homology domain (RHD) (reviewed in Verma *et al.*, 1995). The RHD is responsible for DNA binding, dimerization and nuclear localization of the dimers (Logéat *et al.*, 1991; Bressler *et al.*, 1993). In addition, these proteins contain non-conserved transactivation domains. The second class of Rel-related proteins is composed of p50-NF κ B1 and p52-NF κ B2. These proteins are produced as precursor proteins p105-NF κ B1 and p100-NF κ B2, respectively (reviewed in Verma *et al.*, 1995). In addition to a highly homologous RHD, these precursor proteins have a C-terminal ankyrin repeat domain (ARD). The ARD is removed by ubiquitin-dependent proteolytic processing in the 26S proteasome, giving rise to mature p50-NF κ B1 and p52-NF κ B2 (Fan and Maniatis, 1991; Palombella *et al.*, 1994; Orian *et al.*, 1995). With the exception of relB (which only forms heterodimers with p50-NF κ B1 or p52-NF κ B2), the members of both subclasses can form all possible homo- and heterodimers, generally referred to as Rel-NF κ B.

Two types of inactive Rel-NF κ B complexes are found in the cytoplasm of unstimulated cells. The first is formed by Rel dimers that are bound to the inhibitory proteins I κ B α , I κ B β , I κ B γ or Bcl-3 (reviewed in Verma *et al.*, 1995). These inhibitory proteins have ARDs consisting of 5–7 ankyrin repeats, which are required for binding to the RHD of Rel-NF κ B. Binding of I κ B to Rel-NF κ B masks the nuclear localization signal (NLS) of the latter and, thus, causes cytoplasmic retention of the ternary complex (Kerr *et al.*, 1991; Beg *et al.*, 1992; Franzoso *et al.*, 1992; Hatada *et al.*, 1992; Inoue *et al.*, 1992; Wulckzyn *et al.*, 1992; Naumann *et al.*, 1993). A second type of inactive complex is formed by the unprocessed precursor proteins p105-NF κ B1 and p100-NF κ B2 (Capobianco *et al.*, 1992; Liou *et al.*, 1992; Rice *et al.*, 1992; Hatada *et al.*, 1993; Mercurio *et al.*, 1993; Naumann *et al.*, 1993; Scheinmann *et al.*, 1993; Dobrzanski *et al.*, 1994). These proteins dimerize with mature Rel-related proteins, giving rise to inactive heterodimers. The ARD in the C-terminus of p105-NF κ B1 and p100-NF κ B2 is functionally homologous to I κ B. It masks the NLS of the dimer and retains it in the cytoplasm.

Inactive cytoplasmic Rel-NF κ B can be activated by stimulation of cells with a broad range of NF κ B-inducing agents (reviewed in Grilli *et al.*, 1993; Baeuerle and Henkel, 1994; Israël, 1995), including mitogens (e.g. the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate; TPA) and stress factors (e.g. tumour necrosis factor- α ; TNF- α). Stimulation of cells with NF κ B-activating agents leads to phosphorylation of I κ B, which is followed rapidly by its ubiquitin-dependent degradation in the 26S proteasome (Brown *et al.*, 1993; Henkel *et al.*, 1993; Mellits *et al.*,

1993; Rice and Ernst, 1993; Finco *et al.*, 1994; Miyamoto *et al.*, 1994; Traenckner *et al.*, 1994; Chen *et al.*, 1995; DiDonato *et al.*, 1995; Lin *et al.*, 1995). In addition, p105-NF κ B1 and p100-NF κ B2 are phosphorylated and processed (Mellits *et al.*, 1993; Mercurio *et al.*, 1993; Naumann and Scheidereit, 1994; MacKichan *et al.*, 1996). Both the degradation of I κ B α and the processing of p105-NF κ B1 result in unmasking of the NLS, which allows nuclear translocation of Rel-NF κ B and activation of specific genetic programs. Much research has focused on the activation of the NF κ B (the p50-NF κ B1-p65-RelA dimer) bound to the inhibitory protein I κ B α . Ligand-induced phosphorylation of I κ B α occurs at Ser32 and Ser36, giving rise to a phosphorylated form of I κ B α which has a reduced mobility in SDS-PAGE as compared with non-induced I κ B α (Brockman *et al.*, 1995; Brown *et al.*, 1995; Traenckner *et al.*, 1995; Whiteside *et al.*, 1995; DiDonato *et al.*, 1996). Mutation of both Ser32 and Ser36 completely inhibits ligand-induced phosphorylation of I κ B α , whereas mutation of one of these residues inhibits ligand-induced I κ B α phosphorylation only partially, yielding a phosphorylated form of I κ B α with an electrophoretic mobility intermediate between those of non-induced and ligand-induced phosphorylated wild-type I κ B α (Traenckner *et al.*, 1995; Whiteside *et al.*, 1995). Furthermore, mutation of one or both of these serines renders I κ B α resistant to ubiquitination and subsequent degradation in the 26S proteasome (Chen *et al.*, 1995; DiDonato *et al.*, 1996; Roff *et al.*, 1996), showing that phosphorylation of both serines is an absolute requirement for the induction of I κ B α degradation. However, the identity of the kinase or kinases that phosphorylate I κ B α at these serine residues is not known.

The mitogen-activated 90 kDa ribosomal S6 kinase (p90^{rsk1}) is a serine/threonine kinase involved in the transduction of signals which are induced by stimuli that activate the Ras/mitogen-activated protein (MAP) kinase cascade (Blenis, 1993). It is phosphorylated and subsequently activated by the extracellularly regulated kinases (ERKs) p44^{erk1} and p42^{erk2} but is also capable of autophosphorylation (Sturgill *et al.*, 1988; Chung *et al.*, 1991; Grove *et al.*, 1993). To date, several isoforms of p90^{rsk1} have been described in mouse and human (Alcorta *et al.*, 1989; Moller *et al.*, 1994; Zhao *et al.*, 1995). Originally, p90^{rsk1} was identified as a kinase that phosphorylates the S6 protein of the ribosomal 40S subunit *in vitro* (Erikson and Maller, 1986). However, later it became apparent that, instead of p90^{rsk1}, the non-related 70 kDa ribosomal S6 kinase (p70^{s6k}) is responsible for mitogen-induced phosphorylation of S6 *in vivo* (Chung *et al.*, 1992; Price *et al.*, 1992; Thomas, 1993). Interestingly, mitogen-induced activation of p90^{rsk1} parallels the induction of immediate early genes, suggesting that p90^{rsk1} is involved in the regulation of some of these early events. Thus far, it has been shown that p90^{rsk1} phosphorylates the early transcription factors c-Fos (Chen *et al.*, 1993), serum response factor (Rivera *et al.*, 1993) and Nur77 (Fisher and Blenis, 1996) *in vitro*, but direct evidence for the involvement of p90^{rsk1} in the phosphorylation of these transcription factors *in vivo* has not been provided yet.

In the present study, we report that p90^{rsk1} is a kinase that phosphorylates I κ B α both *in vitro* and *in vivo*. p90^{rsk1} phosphorylates I κ B α at Ser32, an event that has been

shown to be pivotal for the induction of polyubiquitination and degradation of I κ B α and subsequent activation of NF κ B. Moreover, we show that p90^{rsk1} physically associates with I κ B α *in vivo*. Ectopic expression of a dominant-negative mutant of p90^{rsk1} in cells that are normally responsive to TPA, i.e. adenovirus type 5 early region 1 (Ad5E1)-transformed baby rat kidney (BRK) cells, inhibits degradation of I κ B α after TPA stimulation. Overexpression of dominant-negative p90^{rsk1} does not interfere with I κ B α degradation in response to TNF- α . Consistently, in cells that have low levels of p90^{rsk1}, i.e. adenovirus type 12 early region 1 (Ad12E1)-transformed BRK cells, NF κ B cannot be activated by TPA. These data prove that p90^{rsk1} is an essential kinase required for TPA-induced phosphorylation and subsequent degradation of I κ B α *in vivo*. Furthermore, these data suggest that multiple independent NF κ B activation pathways exist, each requiring distinct I κ B α kinases.

Results

NF κ B is not activated in response to TPA in Ad12E1-transformed BRK cells

Identification of kinases involved in the phosphorylation of I κ B α may come from studying cell types in which the regulation of NF κ B is disturbed. Previously, we have shown that Ad12E1-transformed BRK cells have reduced levels of NF κ B and hardly any KBF1 (the homodimer of p50-NF κ B1) due to inefficient processing of p105-NF κ B1 (Schouten *et al.*, 1995). The reduced processing of p105-NF κ B1 can be caused either by Ad12E1A-mediated interference with the initiation of p105-NF κ B1 processing or by inhibition of the activity of the 26S proteasomes, in which p105-NF κ B1 processing takes place. The processing of p105-NF κ B1 and ligand-induced degradation of I κ B α are thought to be coupled processes, both occurring in the 26S proteasome (reviewed in Thanos and Maniatis, 1995). Therefore, we used Ad12E1-transformed BRK cells to study their responsiveness to NF κ B-inducing agents and compared them with Ad5E1-transformed BRK cells, which have high levels of NF κ B. Ad5E1- and Ad12E1-transformed BRK cells were treated with TNF- α or TPA and tested for the induction of the DNA-binding activity of NF κ B to the H2TF1 element of the mouse MHC class I promoter in an electrophoretic mobility shift assay (EMSA). Figure 1A shows that treatment with TNF- α caused rapid induction of NF κ B, both in Ad5E1- (lanes 1–5) and Ad12E1-transformed (lanes 6–10) BRK cells. The kinetics of NF κ B induction in each cell type was not significantly different, although the absolute level of DNA-bound NF κ B was significantly lower in Ad12E1-transformed cells as compared with Ad5E1- or non-transformed BRK cells. This is in agreement with the finding that Ad12E1-transformed cells contain reduced amounts of NF κ B due to inefficient p105-NF κ B1 processing (Schouten *et al.*, 1995). In contrast, TPA treatment activated NF κ B in Ad5E1-transformed BRK cells (lanes 11–15), but not in Ad12E1-transformed BRK cells (lanes 16–20). In this particular experiment, the cells were treated for up to 1 h with TPA. Treatment of Ad12E1-transformed cells for longer periods of time (up to 4 h) also did not cause NF κ B activation (data not shown). This excludes

Fig. 1. (A) Ligand-induced DNA-binding activity of NF κ B in AdE1-transformed BRK cells. Exponentially growing Ad5E1- and Ad12E1-transformed BRK cells were treated with TNF- α (lanes 1–10) or TPA (lanes 11–20) for the indicated periods of time. A total of 5 μ g of whole cell extract was incubated with a radiolabelled double-stranded oligonucleotide representing the H2TF1 element from a mouse MHC class I promoter. Two retarded complexes were formed in extracts from Ad5E1-transformed BRK cells (lanes 1 and 11), representing NF κ B and KBF1 (indicated by arrows). No retarded complexes were formed in extracts of untreated Ad12E1-transformed BRK cells (lanes 6 and 16). Treatment with TNF- α rapidly induced the DNA-binding activity of NF κ B, both in Ad5E1- (lanes 1–5) and Ad12E1-transformed cells (lanes 6–10). TPA treatment induced NF κ B only in Ad5E1-transformed BRK cells (lanes 11–15), whereas no NF κ B activation occurred in Ad12E1-transformed BRK cells (lanes 16–20). (B) Ligand-induced degradation of I κ B α in AdE1-transformed BRK cells. Exponentially growing cells were treated with TNF- α (lanes 1–10) or TPA (lanes 11–20) for the indicated periods of time. Whole cell extract (30 μ g) was fractionated on a 10% polyacrylamide gel and tested for I κ B α levels on a Western blot. TNF- α treatment caused rapid degradation of I κ B α , both in Ad5E1- (lanes 1–5) and Ad12E1-transformed BRK cells (lanes 6–10), and newly synthesized I κ B α appeared during the course of the chase period (lanes 5 and 10). Treatment with TPA caused I κ B α degradation only in Ad5E1-transformed cells (lanes 11–15), but not in Ad12E1-transformed cells (lanes 16–20).

the possibility that TPA-induced activation of NF κ B is delayed in Ad12E1-transformed cells.

Next, we examined whether the observed TNF- α - and TPA-induced activation of NF κ B was caused by enhanced degradation of its inhibitor I κ B α . Therefore, AdE1-transformed cells were treated with these agents for various time periods and extracts from the treated cells were assayed on a Western blot. Figure 1B shows that TNF- α treatment of both Ad5E1- (lanes 1–5) and Ad12E1-transformed BRK cells (lanes 6–10) caused rapid degradation of I κ B α . In the course of the experiment, newly synthesized I κ B α appeared (lanes 5 and 10). Since the expression of I κ B α is controlled by NF κ B (Arenzana-Seisdedos *et al.*, 1995, and references therein), this experiment showed that TNF- α -induced NF κ B is transcriptionally active in both Ad5E1- and Ad12E1-transformed cells. In contrast, TPA treatment only induced degradation of I κ B α in Ad5E1-transformed cells (lanes 11–15), whereas in Ad12E1-transformed cells (lanes 16–20) the levels of I κ B α remained unaffected. These data show that the TPA-induced degradation of I κ B α and subsequent activation of NF κ B is inhibited in Ad12E1-transformed cells. This is not due to inactivation of the 26S proteasome, since the induction of I κ B α degradation and NF κ B activation by TNF- α was not abolished in these cells. Therefore, it is likely that earlier events in the pathway, e.g. TPA-induced phosphorylation of I κ B α , are affected in Ad12E1-transformed cells.

Ad12E1-transformed cells have reduced levels of p90^{rsk1}

Ligand-induced degradation of I κ B α is triggered by its phosphorylation. Since it is the TPA-induced degradation

of I κ B α that is blocked in Ad12E1-transformed cells, we screened AdE1-transformed cells for kinases that are responsive to TPA. The Western blot of Figure 2A (upper panel) shows that the levels of mitogen-inducible p90^{rsk1} are much lower in Ad12E1-transformed BRK cells (lanes 3 and 4) than in Ad5E1-transformed BRK cells (lanes 1 and 2). The antibody recognized three distinct forms of p90^{rsk1}, which represent differentially phosphorylated forms of p90^{rsk1} (see below). The transformed cells were also tested for the presence of the mitogen-inducible 'extracellularly regulated kinases' p44^{erk1} and p42^{erk2}. Neither p44^{erk1} nor p42^{erk2} protein levels were affected in AdE1-transformed BRK cells (Figure 2A, lower panel). Furthermore, the p90^{rsk} isoforms p90^{rsk2} and p90^{rsk3} (Moller *et al.*, 1994; Zhao *et al.*, 1995; Xing *et al.*, 1996) were not down-regulated in Ad12E1-transformed BRK cells (Figure 2B). This shows that specifically the amount of p90^{rsk1} was reduced in Ad12E1-transformed cells. Down-regulation of p90^{rsk1} in Ad12E1-transformed cells occurred at the level of RNA (data not shown).

The correlation between low levels of p90^{rsk1} and lack of TPA inducibility of NF κ B in Ad12E1-transformed cells suggests that p90^{rsk1} might be required for this process. However, based on these data, we cannot exclude that Ad12E1-transformed cells are not responsive to TPA at all, for example due to lack of expression of certain protein kinase C isoforms. Therefore, we treated AdE1-transformed BRK cells with TPA and determined the phosphorylation state of p90^{rsk1} by measuring the formation of hyperphosphorylated p-p90^{rsk1} on a Western blot. Inactive hypophosphorylated p90^{rsk1} has a higher mobility in SDS-PAGE than TPA-activated hyperphosphorylated p-p90^{rsk1} (Grove *et al.*, 1993). Figure 2C shows that TPA

Fig. 2. (A) Reduced levels of p90^{rsk1} in Ad12E1-transformed cells. Whole cell extracts (30 µg) from exponentially growing AdE1-transformed BRK cells were fractionated on a 10% polyacrylamide gel and tested for the amount of p90^{rsk1} (upper panel), p44^{erk1} and p42^{erk2} (lower panel) on a Western blot. The amount of p90^{rsk1} was much lower in Ad12E1-transformed BRK cells (lanes 3 and 4, upper panel) than in Ad5E1-transformed BRK cells (lanes 1 and 2). All cell types have comparable amounts of p44^{erk1} and p42^{erk2} (lower panel). (B) p90^{rsk} isoforms p90^{rsk2} and p90^{rsk3} are not down-regulated in Ad12E1-transformed cells. Whole cell extracts (30 µg) from exponentially growing AdE1-transformed BRK cells (Ad5E1-c22 and Ad12-c33) were fractionated on a 10% polyacrylamide gel and tested for the amount of p90^{rsk2} (lanes 1 and 2) and p90^{rsk3} (lanes 3 and 4) with the antibodies SC1430 α-p90^{rsk2} and SC1431 α-p90^{rsk3}. Expression of both p90^{rsk} isoforms is higher in Ad12E1-transformed cells. (C) TPA activates p90^{rsk1} both in Ad5E1- and Ad12E1-transformed cells. Exponentially growing AdE1-transformed cells were treated with TPA (lanes 1–10) for the indicated periods of time. Whole cell extracts (30 µg) were fractionated on a 10% polyacrylamide gel and tested for p90^{rsk1} expression on a Western blot. TPA activates p90^{rsk1} both in Ad5E1 (lanes 1–5) and Ad12E1-transformed cells (lanes 6–10), as measured by the formation of a hyperphosphorylated form of p90^{rsk1} (p-p90^{rsk1}). (D) TNF-α does not activate p90^{rsk1} in AdE1-transformed BRK cells (lanes 1–5).

treatment induced the formation of hyperphosphorylated p-p90^{rsk1} in Ad5E1-transformed cells (lanes 1–5). Moreover, TPA treatment caused hyperphosphorylation of the low level of p90^{rsk1} in Ad12E1-transformed cells (lanes 6–10). All three distinct forms of p90^{rsk1} were converted into one discrete form of p-p90^{rsk1}, suggesting that the three forms of p90^{rsk1} in unstimulated cells represent differentially phosphorylated forms of p90^{rsk1}. In contrast, treatment of Ad5E1-transformed cells with TNF-α did not cause formation of p-p90^{rsk1} (Figure 2D, lanes 1–5). Apparently, p90^{rsk1} is not part of the signal transduction route that is activated by TNF-α in AdE1-transformed cells. These data demonstrate that both Ad5E1- and Ad12E1-transformed cells can be responsive to TPA.

p90^{rsk1} phosphorylates IκBα *in vivo* at Ser32

To investigate whether p90^{rsk1} is involved directly in the TPA-induced phosphorylation of IκBα, we transiently transfected COS1 cells with a vector encoding haemagglutinin (HA)-tagged p90^{rsk1} or, as a control, with a vector encoding an HA-tagged kinase that acts directly upstream of p90^{rsk1}, i.e. p44^{erk1}. Both kinases were activated by treating the transfected cells with TPA, and the kinases were purified by immunoprecipitation. Subsequently, the activity of these kinases towards human GST-IκBα was determined *in vitro*. Figure 3 shows that p90^{rsk1} phosphorylated GST-IκBα (lane 2) whereas p44^{erk1} failed to do so (lane 3) under

identical reaction conditions. Precipitation of an endogenous IκBα kinase with the 12CA5 α-HA antibody was excluded, since no IκBα kinase activity was precipitated from mock-transfected cells (lane 1). Neither p90^{rsk1} nor p44^{erk1} were found to phosphorylate GST alone (data not shown). As a control for the activity of the kinases, the kinase activity towards known substrates was assayed. p90^{rsk1} phosphorylated the ribosomal protein S6 (lane 5) and p44^{erk1} phosphorylated c-Jun (lane 6), showing that both kinases were active. These data clearly show that p90^{rsk1} phosphorylates IκBα *in vitro*.

We next examined whether p90^{rsk1} is able to phosphorylate IκBα *in vivo*. COS1 cells were transiently transfected with a vector encoding wild-type human IκBα with or without a vector encoding HA-tagged p90^{rsk1} or HA-tagged p44^{erk1}, or an empty vector. Subsequently the cells were treated with TPA for either 10 or 20 min and assayed for phosphorylation of IκBα, as measured by a decreased mobility of IκBα in SDS-PAGE. Since the transfected IκBα is strongly overexpressed due to the presence of an SV40 origin of replication, IκBα phosphorylation by endogenous kinases was circumvented. As shown in Figure 4A, IκBα was not phosphorylated when an empty vector (lanes 1–3) or a p44^{erk1}-encoding vector (lanes 4–6) was co-transfected, showing that phosphorylation of IκBα by putative endogenous IκBα kinases is limited under these assay conditions. In contrast, co-expression of p90^{rsk1} did

Fig. 3. p90^{rsk1} phosphorylates GST-IκBα *in vitro*. COS1 cells were transfected with a vector expressing HA-tagged p90^{rsk1} or HA-tagged p44^{erk1} and the transfected cells were treated with TPA (100 ng/ml) for 15 min. The activated kinases were purified by immunoprecipitation with 12CA5 α-HA after which kinase assays were performed with the indicated substrates (0.5 μg of GST-IκBα, 0.5 μg of c-Jun or 25 μg of 40S ribosomes). HA-tagged p90^{rsk1} (lane 2) phosphorylated GST-IκBα, whereas HA-tagged p44^{erk1} (lane 3) did not. As a control for the p90^{rsk1} and p44^{erk1} kinase activity, the p90^{rsk1}-mediated phosphorylation of the S6 protein of the ribosomal 40S subunit (lane 5) and p44^{erk1}-mediated phosphorylation of c-Jun (lane 6) were measured. Neither p90^{rsk1} nor p44^{erk1} was found to phosphorylate GST (not shown). Autophosphorylated p90^{rsk1} is indicated. No kinase activity was precipitated from mock-treated cells (lanes 1 and 4).

result in phosphorylation of IκBα (lanes 7–9), giving rise to the slower migrating form of IκBα, p-IκBα. Phosphorylation of IκBα occurred even in untreated cells (lane 7), most likely due to the high levels of p90^{rsk1} in the transiently transfected cells. IκBα phosphorylation was enhanced further by TPA treatment (lanes 8 and 9). Both HA-tagged p44^{erk1} and HA-tagged p90^{rsk1} were highly expressed (data not shown).

In the experiments discussed above, both IκBα and p90^{rsk1} were strongly overexpressed. In order to test whether p90^{rsk1} can phosphorylate IκBα under more physiological circumstances, we transfected Ad12E1-transformed cells (which express low levels of p90^{rsk1}) with human IκBα and HA-tagged p90^{rsk1}. The transfected human IκBα and the endogenously expressed rat IκBα ran with different mobilities in SDS-PAGE, which allowed us to study the TPA-induced phosphorylation of the two proteins separately. Figure 4B shows that neither the endogenous rat IκBα (rIκBα) nor the transfected human IκBα (hIκBα) was phosphorylated by endogenous IκBα kinases following TPA treatment (lanes 1–4). In contrast, co-expression of p90^{rsk1} caused rapid phosphorylation of hIκBα in response to TPA (lanes 5–8). On the other hand, the phosphorylation of endogenous rIκBα was low in

Fig. 4. (A) p90^{rsk1} phosphorylates IκBα *in vivo*. COS1 cells were transfected with a vector expressing human IκBα together with an empty vector or a vector encoding HA-tagged p90^{rsk1} or HA-tagged p44^{erk1}. The transfected cells were treated either with TPA (100 ng/ml) for 10 or 20 min, or left untreated. Extracts were prepared and Western blotting was performed with the antibody SC371 α-IκBα. Human IκBα (IκBα) and the more slowly migrating hyperphosphorylated form of IκBα (p-IκBα) are indicated. IκBα was phosphorylated when HA-tagged p90^{rsk1} was co-expressed (lane 7), and IκBα-phosphorylation increased dramatically upon TPA treatment (lanes 8 and 9). IκBα phosphorylation was caused specifically by co-expressed p90^{rsk1}, since no phosphorylation was observed when no kinase (lanes 1–3) or p44^{erk1} (lanes 4–6) was co-expressed. This rules out the possibility that the highly overexpressed IκBα is phosphorylated by endogenous IκBα-kinases. (B) p90^{rsk1}-mediated phosphorylation of IκBα in Ad12E1-transformed BRK cells. Ad12E1-transformed BRK cells were transfected with a vector encoding human IκBα, together with a vector encoding HA-tagged p90^{rsk1} or an empty vector. The transfected cells were either treated with TPA (100 ng/ml) for 15, 30 or 60 min, or left untreated. Extracts were prepared and Western blotting was performed with the antibody SC371 α-IκBα. Human wt-IκBα (hIκBα), its hyperphosphorylated form (p-hIκBα) and endogenous rat IκBα (rIκBα) are indicated. TPA-induced phosphorylation of hIκBα required co-expression of p90^{rsk1} (lanes 5–8). No TPA-induced phosphorylation of IκBα was observed when an empty expression vector was co-transfected (lanes 1–4). (C) p90^{rsk1} phosphorylates IκBα at Ser32. COS1 cells were transfected with a vector expressing human wt-IκBα, 32A-IκBα, 36A-IκBα or 32A36A-IκBα, together with a vector encoding HA-tagged p90^{rsk1}. The transfected cells were treated either with TPA (100 ng/ml) for 10 or 20 min, or left untreated. Extracts were prepared and Western blotting was performed with antibody SC371 α-IκBα. p90^{rsk1} phosphorylated wt-IκBα (lane 1–3), but failed to phosphorylate 32A-IκBα (lanes 4–6) and 32A36A-IκBα (lanes 10–12). In contrast, p90^{rsk1} did phosphorylate 36A-IκBα in response to TPA (lanes 7–9). Thus p90^{rsk1} phosphorylates IκBα at Ser32.

these transfected cells (lanes 5–8). The Ad12E1-transformed cells had been transfected with an efficiency of <10%. Thus, <1 out of 10 cells co-expressed significant amounts of p90^{rsk1} (transfected HA-tagged p90^{rsk1}) and endogenous IκBα. In contrast, almost all transfected cells (<10% of the total culture) will co-express hIκBα and HA-tagged p90^{rsk1}, when HA-tagged p90^{rsk1} and hIκBα are co-transfected (lanes 5–8). This explains why p90^{rsk1}-mediated phosphorylation of IκBα was observed only with hIκBα (lanes 5–8). It should be noted that co-transfection of rIκBα and p90^{rsk1} into COS cells caused TPA-induced phosphorylation of rIκBα (data not shown). Thus p90^{rsk1} can phosphorylate IκBα *in vivo*.

Stimulation of cells with NFκB-inducing agents causes phosphorylation of IκBα at Ser32 and Ser36. Phosphorylation of these residues is a pivotal step in the activation of NFκB, since mutation of either one or both of these serines into alanines abolishes ligand-induced degradation of IκBα (Brockman *et al.*, 1995; Brown *et al.*, 1995; Traenckner *et al.*, 1995; Whiteside *et al.*, 1995; DiDonato *et al.*, 1996). In order to determine whether Ser32 and/or Ser36 was phosphorylated by p90^{rsk1}, we tested the p90^{rsk1} kinase activity towards human wild-type IκBα (wt-IκBα) and its mutant forms 32A-IκBα, 36A-IκBα and 32A36A-IκBα, in which Ser32, Ser36 or both were replaced by alanines (Whiteside *et al.*, 1995). Figure 4C shows that p90^{rsk1} phosphorylated wt-IκBα (lanes 1–3) in response to TPA, giving rise to p-IκBα. Mutation of both Ser32 and Ser36 (lanes 10–12) or mutation of Ser32 alone (lanes 4–6) abolished p90^{rsk1}-mediated phosphorylation of IκBα. In contrast, mutation of Ser36 did not inhibit the formation of p-IκBα (lanes 7–9). p90^{rsk1} phosphorylates serine residues in the consensus sequence RXXS, where X may be any amino acid (Erikson and Maller, 1988). In agreement with this, Ser32, but not Ser36, of IκBα lies in such a consensus sequence: RHDS. This specific sequence is conserved in IκBα of human, mouse, rat, pig and chicken.

IκBα and p90^{rsk1} associate *in vivo*

Next, we studied whether p90^{rsk1} physically associates with IκBα. IκBα was transiently expressed in COS1 cells with or without HA-tagged p90^{rsk1} or HA-tagged p44^{erk1}. Prior to cell lysis, the transfected cells were treated with TPA for 15 min, or left untreated. IκBα and p90^{rsk1} were immunoprecipitated under mild conditions and the immunoprecipitates were assayed on a Western blot. Figure 5A shows that p90^{rsk1} co-precipitated with IκBα (lane 3) and vice versa (lanes 7, 8 and 10). TPA treatment of the transfected cells caused dissociation of the IκBα–p90^{rsk1} complex (lanes 4, 9 and 11), indicating that this complex is disrupted upon p90^{rsk1}-mediated phosphorylation of IκBα. Co-precipitation with IκBα was specific for p90^{rsk1}, since p44^{erk1} did not co-precipitate (Figure 5B, lanes 1 and 2). Cross-reactivity of the antibodies was excluded, since the antibodies SC231 (α-p90^{rsk1}; Figure 5A, lane 5) and 12CA5 (α-HA; Figure 5B, lane 2) did not precipitate IκBα in the absence of HA-tagged p90^{rsk1}. Furthermore, the α-IκBα antibody SC371 did not cross-react with HA-tagged p90^{rsk1} (Figure 5A, lane 2). These data show that p90^{rsk1} and IκBα form a complex *in vivo*.

Fig. 5. IκBα associates with p90^{rsk1} *in vivo*. COS1 cells were transfected with a vector encoding rat IκBα together with a vector encoding HA-tagged p90^{rsk1} or HA-tagged p44^{erk1}, or an empty vector. The transfected cells were either treated with TPA (100 ng/ml) for 15 min, or left untreated. Extracts were prepared in mild buffer and immunoprecipitations were performed with specific antibodies, as indicated. The immunoprecipitates were analysed by Western blotting with the antibodies 12CA5 α-HA or SC371 α-IκBα. (A) HA-tagged p90^{rsk1} (p90^{rsk1}) co-precipitated with IκBα (lane 3) and vice versa (lanes 7, 8 and 10). Treatment with TPA caused hyperphosphorylation and activation of p90^{rsk1} (as measured by the formation of p-p90^{rsk1}), and dissociation of the IκBα–p90^{rsk1} complex (lanes 4, 9 and 11). Cross-reactivity of the antibodies was excluded since SC371 α-IκBα did not precipitate HA-tagged p90^{rsk1} (lane 2), and SC231 α-p90^{rsk1} and 12CA5 α-HA did not precipitate IκBα (A, lane 5; B, lane 2). The open circle indicates a background band that was precipitated with 12CA5 α-HA. (B) HA-tagged p44^{erk1} and IκBα do not associate (lanes 1 and 2).

Stable overexpression of dominant-negative p90^{rsk1} inhibits degradation of IκBα in response to TPA

To determine whether p90^{rsk1} is a physiological IκBα kinase, we have inactivated this kinase in Ad5E1 cells, by overexpressing a dominant-negative mutant. p90^{rsk1} contains two distinct kinase domains. The amino-terminal kinase domain is involved in substrate phosphorylation, whereas both domains contribute to autophosphorylation (Bjorbaek *et al.*, 1995). With site-directed mutagenesis, the ATP-binding site of the amino-terminal kinase domain was disrupted by replacing aspartic acid on position 205 in the DFG consensus sequence by asparagine (D205N)

Fig. 6. Overexpression of dominant-negative p90^{rsk1} inhibits degradation of I κ B α in response to TPA. Ad5E1-c22 cells were transfected with pcDNA3-Rsk1-D205N or pcDNA3. Monoclonal cell lines were established. **(A)** Western blot with extracts from cell lines BX-RSK-M13, BX-RSK-M15 and control cell line BX-D11. Whole cell extracts (30 μ g) were fractionated on a 10% polyacrylamide gel and tested for p90^{rsk1} and HA-p90^{rsk1}-D205N expression on a Western blot. Expression levels of HA-p90^{rsk1}-D205N were estimated to be 10- to 25-fold higher than endogenous p90^{rsk1} (lanes 2 and 3). **(B)** Ligand-induced degradation of I κ B α in BX-D11, BX-RSK-M13 and BX-RSK-M15. Exponentially growing cells were treated with TPA (lanes 1–6) or TNF- α (lanes 7–11) for the indicated periods of time. Whole cell extracts (30 μ g) were fractionated on a 10% polyacrylamide gel and tested for I κ B α expression on a Western blot. Degradation of I κ B α upon TPA stimulation was inhibited in cells overexpressing HA-p90^{rsk1}-D205N. In contrast, degradation of I κ B α in response to TNF- α was not inhibited in these cells. In this experiment, the appearance of newly synthesized I κ B α was slightly delayed for BX-D11 (compare also Figure 1B, lanes 1–5).

(van den Heuvel and Harlow, 1993). As expected, this mutant does not phosphorylate I κ B α *in vitro* and *in vivo* (data not shown). Subsequently, Ad5E1-transformed BRK cells were stably transfected with a vector encoding both HA-tagged p90^{rsk1}-D205N and the neomycin resistance gene. As a control, the same type of cells were transfected with a vector encoding only the neomycin resistance gene. Stable cell lines were established and screened for expression of p90^{rsk1}-D205N. The expression levels were estimated to be 10- to 25-fold higher than the endogenous p90^{rsk1} (Figure 6A). In cell lines expressing p90^{rsk1}-D205N, TPA-induced degradation of I κ B α was inhibited, whereas in the control cell lines BX-D11 (Figure 6B, lanes 1–6) and BX-D3 (data not shown) degradation was not affected. In contrast, degradation of I κ B α in response to TNF- α was not inhibited by the expression of p90^{rsk1}-D205N. These experiments show that p90^{rsk1} is an essential kinase that is required for TPA-inducible phosphorylation and subsequent degradation of I κ B α *in vivo*. Moreover, these data suggest that different NF κ B-inducing signals activate distinct I κ B α kinases.

p90^{rsk1} associates with p105-NF κ B1 and phosphorylates its C-terminus

Treatment of cells with NF κ B-activating agents not only causes phosphorylation and degradation of I κ B α , but also leads to phosphorylation and processing of p105-NF κ B1 (Mellits *et al.*, 1993; Mercurio *et al.*, 1993; Naumann and Scheidereit, 1994; MacKichan *et al.*, 1996). Therefore, we examined whether p90^{rsk1} is also involved in the phosphorylation of p105-NF κ B1. The human p105-NF κ B1 protein contains two putative p90^{rsk1} phosphorylation sites RXXS, i.e. Ser338 and Ser938. Ser338 is located in the RHD of the protein and Ser938 in the C-terminal part of the protein. In order to discriminate between these two serine residues, we tested two forms of p105-NF κ B1 as substrates in a p90^{rsk1} kinase assay, i.e. full-length Myc-tagged p105-NF κ B1 and Myc-tagged p97, a C-terminal deletion mutant of p105-NF κ B1 lacking Ser938 (Fan and Maniatis, 1991). COS1 cells were transfected with a vector expressing HA-tagged p90^{rsk1} or HA-tagged p44^{erk1}, and treated with TPA for 15 min. The kinases were purified by immunoprecipitation and their kinase activities were determined towards the different substrates. Figure 7A shows that full-length p105 was phosphorylated by p90^{rsk1} (lane 2). Most likely, phosphorylation of p105-NF κ B1 occurred at Ser938 and not at Ser338, since p97 was not phosphorylated by p90^{rsk1} (lane 5). p44^{erk1} failed to phosphorylate either p105 (lane 3) or p97 (lane 6). The 12CA5 α -HA antibody did not cross-react with an endogenous p105-NF κ B1 kinase, since no kinase activity was precipitated from the mock-treated cells (lanes 1 and 4). p90^{rsk1} and p44^{erk1} phosphorylated the S6 protein of the ribosomal 40S subunit (lane 7) and c-Jun (lane 8), respectively, indicating that both kinases were active.

As p90^{rsk1} specifically phosphorylates the C-terminus of p105-NF κ B1, we also determined whether p90^{rsk1} binds to p105-NF κ B1. COS1 cells were transfected with pCMV-105T or pCMV-97T (encoding Myc-tagged p105-NF κ B1 or Myc-tagged p97-NF κ B1) with or without a vector expressing HA-tagged p90^{rsk1} or HA-tagged p44^{erk1}. Co-immunoprecipitations were performed under mild conditions and the immunoprecipitates were analysed on a Western blot. Figure 7B shows that p105 and p97 co-precipitated with p90^{rsk1} (lanes 14 and 16) and vice versa (lanes 6 and 9). Only the inactive hypophosphorylated form of p90^{rsk1} associated with p105 (lane 9). Furthermore, TPA treatment of the transfected cells caused dissociation of the p90^{rsk1}-p105 complex (lanes 10 and 17). On the other hand, the p90^{rsk1}-p97 complex did not dissociate following TPA treatment (lanes 7 and 15). Instead, a hyperphosphorylated form of p90^{rsk1} (p-p90^{rsk1}) co-precipitated with p97 after TPA treatment (lane 7). This suggests that TPA-induced disruption of the p105-p90^{rsk1} complex is caused by p90^{rsk1}-mediated phosphorylation of the C-terminus of p105-NF κ B1 (the part of p105 that is missing in p97), rather than by phosphorylation of p90^{rsk1} itself. Thus, the p90^{rsk1}-p97 complex is more stable than the p90^{rsk1}-p105 complex, explaining why p90^{rsk1} co-precipitated less efficiently with p105 than with p97 (compare lanes 9 and 16 with lanes 6 and 14). Figure 7C shows that p97 specifically associates with p90^{rsk1} (lanes 4 and 9), whereas no complex formation can be detected with p44^{erk1} (lanes 5 and 10). Cross-reaction of the

Fig. 7. (A) p90^{rsk1} phosphorylates the C-terminus of p105-NFκB1. COS1 cells were transfected with a vector encoding HA-tagged p90^{rsk1} or HA-tagged p44^{erk1} and the transfected cells were treated with TPA (100 ng/ml) for 15 min. The activated kinases were purified by immunoprecipitation with 12CA5 α-HA, and kinase assays with the indicated substrates (Myc-tagged p105-NFκB1, Myc-tagged p97-NFκB1, c-Jun or 40S ribosomes) were performed. The full-length Myc-tagged p105-NFκB1 protein (lane 2), but not a C-terminal deletion mutant lacking Ser938, i.e. Myc-tagged p97-NFκB1 (lane 5), was phosphorylated by HA-tagged p90^{rsk1}. Under identical conditions HA-tagged p44^{erk1} failed to phosphorylate Myc-tagged p105-NFκB1 (lane 3) or Myc-tagged p97-NFκB1, stressing the specificity of p105 phosphorylation by p90^{rsk1}. The activity of precipitated p90^{rsk1} and p44^{erk1} was checked by performing kinase reactions with the S6 protein of the ribosomal 40S subunit (lane 7) and c-Jun (lane 8), respectively. Autophosphorylated HA-tagged p90^{rsk1} is indicated (lanes 2, 5 and 7). (B) Inactive p90^{rsk1} associates with p105 *in vivo*. COS1 cells were transfected with a vector encoding Myc-tagged p105-NFκB1 or Myc-tagged p97-NFκB1, together with a vector encoding HA-tagged p90^{rsk1} or HA-tagged p44^{erk1}. Cells were either treated with TPA (100 ng/ml) or left untreated. Extracts were prepared in mild buffer and immunoprecipitations were performed with the indicated antibodies. The immunoprecipitates were analysed by Western blotting with the antibodies 9E10 α-Myc and 12CA5 α-HA. HA-tagged p90^{rsk1} co-precipitated both with Myc-tagged p105 (Myc-p105) and Myc-tagged p97 (Myc-p97) and vice versa (lanes 6, 9, 14 and 16). TPA treatment abolished co-precipitation of p90^{rsk1} with Myc-p105 (lanes 10 and 17), but did not fully dissociate the complex between p90^{rsk1} and Myc-p97 (lanes 7 and 15). Instead, a slower migrating hyperphosphorylated form of p90^{rsk1} co-precipitated with Myc-p97 (p-p90^{rsk1}, lane 7). Addition of 0.1% SDS during the immunoprecipitation abolished association between p90^{rsk1} and Myc-p97 (lane 8). Cross-reactivity of the antibodies was excluded, since 9E10 α-Myc did not precipitate HA-tagged p90^{rsk1} (lane 1) and 12CA5 α-HA did not precipitate Myc-p97 or Myc-p105 (lanes 12 and 13). (C) p97 associates with p90^{rsk1} but not with p44^{erk1}. Myc-p97 co-precipitated specifically with p90^{rsk1} (lanes 4 and 9), whereas it did not co-precipitate with p44^{erk1} under identical conditions (lanes 5 and 10). Cross-reactivity of the antibodies can be excluded, since 9E10 α-Myc did not precipitate HA-tagged p90^{rsk1} or HA-tagged p44^{erk1} (lanes 1 and 2) and 12CA5 α-HA did not precipitate Myc-p97 (lane 8).

antibodies was excluded, since p90^{rsk1} could not be precipitated with the 9E10 α-Myc antibody in the absence of Myc-tagged p97 (lane 1) and p97 could not be precipitated with the 12CA5 α-HA antibody in the absence of HA-tagged p90^{rsk1} (lane 8). More importantly, co-immunoprecipitation of p90^{rsk1} with p97 was completely abolished by addition of 0.1% SDS (Figure 7B, lane 8). Thus, p90^{rsk1} physically associates with p105-NFκB *in vivo*.

Discussion

In the present study, we provide evidence that the mitogen-inducible kinase p90^{rsk1} is an IκBα kinase. It phosphoryl-

ates IκBα at Ser32 *in vivo*. Moreover, p90^{rsk1} physically associates with IκBα. Interestingly, the p90^{rsk1}-IκBα complex dissociates upon treatment of cells with TPA. Based on the finding that TPA-induced dissociation of the p90^{rsk1}-p105-NFκB1 complex occurred only when the p90^{rsk1} phosphorylation site in the C-terminus of p105-NFκB1 was present (see below), we suggest that dissociation of the p90^{rsk1}-IκBα complex is caused by p90^{rsk1}-induced phosphorylation of IκBα. Impairing the function of p90^{rsk1} *in vivo*, by expression of a dominant-negative mutant, inhibits the degradation of IκBα in response to TPA. Concomitant with the role of p90^{rsk1} as an IκBα kinase required for NFκB activation in response to mitogenic

agents, NF κ B cannot be activated by TPA in cells that have low levels of p90^{rsk1}.

Phosphorylation of Ser32 has been reported previously to be pivotal for the ubiquitination of I κ B α and subsequent degradation of I κ B α in response to NF κ B-activating agents (Brockman *et al.*, 1995; Brown *et al.*, 1995; Chen *et al.*, 1995; Traenckner *et al.*, 1995; Whiteside *et al.*, 1995; DiDonato *et al.*, 1996). However, p90^{rsk1}-mediated phosphorylation of I κ B α does not cause its degradation. This can be explained by the fact that I κ B α has to be phosphorylated not only at Ser32 but also at Ser36 before degradation occurs (Brockman *et al.*, 1995; Brown *et al.*, 1995; Traenckner *et al.*, 1995; Whiteside *et al.*, 1995; DiDonato *et al.*, 1996). Since p90^{rsk1} failed to phosphorylate Ser36, the p90^{rsk1}-phosphorylated I κ B α was not degraded. This also explains why co-transfection of a vector expressing p90^{rsk1} and an NF κ B-dependent luciferase reporter does not lead to increased luciferase activity (data not shown). These data thus suggest that multiple I κ B α kinases are required for the induction of I κ B α degradation. This assumption is attractive, since it allows fine-tuning of the regulation of I κ B α degradation and NF κ B activation. Furthermore, it explains why I κ B α is phosphorylated in response to a wide variety of stimuli that activate different subsets of kinases. Indeed dominant-negative p90^{rsk1} interferes with TPA-induced, but not with TNF- α -induced degradation of I κ B α *in vivo*. This underlines the existence of multiple activation pathways, that independently mediate I κ B α phosphorylation. Fine-tuning of NF κ B activation is enhanced further by the existence of multiple I κ B proteins, e.g. I κ B α and I κ B β . In contrast to I κ B α , the degradation of I κ B β is induced only by a limited number of stimuli, i.e. lipopolysaccharide and interleukin-1, whereas others, such as TPA, have no effect (Thompson *et al.*, 1995). I κ B β degradation is induced by phosphorylation of serine residues 19 and 23 (DiDonato *et al.*, 1996). Interestingly, these serine residues do not lie in the p90^{rsk1} consensus sequence RXXS, which might explain why I κ B β is not degraded in response to TPA.

Thus far, several kinases have been reported to be involved in ligand-induced phosphorylation of I κ B α and activation of NF κ B. The double-stranded (ds) RNA-activated p86 protein kinase (PKR) phosphorylates I κ B α in response to dsRNA *in vivo* (Kumar *et al.*, 1994; Maran *et al.*, 1994). Antisense PKR abolishes dsRNA-induced degradation of I κ B α , but it does not affect I κ B α degradation induced by other stimuli. This rules out the requirement of PKR for NF κ B activation in general (Maran *et al.*, 1994). Furthermore, p70^{s6k} has been reported to be involved in the activation of NF κ B (Lai and Tan, 1994), although this kinase has not been shown to phosphorylate I κ B α directly *in vivo*. More recently, it has been reported that I κ B α is phosphorylated by casein kinase II (CKII), both *in vitro* and *in vivo* (Barroga *et al.*, 1995; Lin *et al.*, 1996; McElhinny *et al.*, 1996; Schwarz *et al.*, 1996). Ser32 and Ser36 lie within the CKII consensus sequence S/TXXZ, in which Z must be an acidic amino acid or a phosphorylated serine or threonine (Pinna, 1990). The X residues preferentially should be acidic amino acids, but for Ser32 and Ser36 the residues represented by X are not acidic, suggesting that the consensus sequence is imperfect. In agreement with this, CKII-mediated phosphorylation

of I κ B α was found only at serine and threonine residues located in the C-terminus of I κ B α , whereas no phosphorylation occurred within the first 60 amino acids. Furthermore, CKII phosphorylated I κ B α constitutively (Barroga *et al.*, 1995; Lin *et al.*, 1996; McElhinny *et al.*, 1996; Schwarz *et al.*, 1996). Finally, a large multisubunit ubiquitination-dependent protein kinase that phosphorylates I κ B α has been isolated from HeLa cells. Interestingly, this kinase complex phosphorylated I κ B α at Ser32 and/or Ser36 (Chen *et al.*, 1996), the same residues that are phosphorylated upon stimulation of cells with NF κ B-inducing agents. However, the identity of the kinase(s) is not known yet. Recently, an alternative mechanism to inactivate I κ B α was reported. Stimulation of Jurkat T cells with pervanadate, or reoxygenation of hypoxic HeLa cells, led to inactivation of I κ B α due to its phosphorylation at Tyr42, although this inactivation was not linked to I κ B α degradation (Imbert *et al.*, 1996).

Several lines of evidence indicate that the Ras/MAP kinase cascade mediates ligand-induced activation of NF κ B. First, several protein kinase C (PKC) isoenzymes induce phosphorylation of I κ B α (Diaz-Meco *et al.*, 1993, 1994; Steffan *et al.*, 1995; Janosch *et al.*, 1996; Lindholm *et al.*, 1996), but PKC failed to phosphorylate I κ B α directly (Janosch *et al.*, 1996). Secondly, Ha-Ras and Raf-1 were shown to be required for ligand-induced NF κ B activation (Devary *et al.*, 1993; Finco and Baldwin, 1993; Bertrand *et al.*, 1995; Folgueira *et al.*, 1996; Janosch *et al.*, 1996). Contradicting results have been reported on the requirement for Raf-1. The Raf-1 kinase domain was shown to phosphorylate I κ B α *in vitro* and to associate with I κ B α in a yeast two-hybrid system (Li and Sedivy, 1993). However, full-length Raf-1 failed to associate with I κ B α or to phosphorylate it (Diaz-Meco *et al.*, 1994; Janosch *et al.*, 1996). Based on the data presented in this study, we propose that the involvement of the Ras/MAP kinase cascade is transduction of mitogen-induced signals to the I κ B α kinase p90^{rsk1}, which subsequently phosphorylates I κ B α . Finally, NF κ B is induced during the G₀-G₁ transition of the cell cycle (Baldwin *et al.*, 1991). This coincides with the activation of p90^{rsk1} (Chen *et al.*, 1991, 1992). Based on the data presented in this study, it is likely that p90^{rsk1} functions as an I κ B α kinase in this particular process.

Concomitant with I κ B α degradation, stimulation of cells with NF κ B-activating agents causes enhanced processing of p105-NF κ B1 (Mellits *et al.*, 1993; Mercurio *et al.*, 1993; Naumann and Scheidereit, 1994). This event is also preceded by phosphorylation of p105-NF κ B1 (Mellits *et al.*, 1993; Naumann and Scheidereit, 1994). Interestingly, p90^{rsk1} phosphorylates p105-NF κ B1 and physically associates with it. Since both I κ B α and p105-NF κ B1 contain an ARD, it is likely that this domain is responsible for association of these proteins with p90^{rsk1}. Accordingly, p90^{rsk1} does not associate with p50-NF κ B1, the processed form of p105-NF κ B1 that lacks the ARD (data not shown). TPA-induced activation of p90^{rsk1} causes dissociation of the p90^{rsk1}-p105-NF κ B1 complex, which requires the presence of the C-terminus of p105-NF κ B1, the part of p105-NF κ B1 that is phosphorylated by p90^{rsk1}. This suggests that phosphorylation of the C-terminus of p105 induces a change in its conformation, leading to dissociation of the p105-p90^{rsk1} complex. The assumed

p90^{rsk1}-induced conformational change in p105-NFκB1 might also commit its C-terminus to ubiquitination and degradation. Interestingly, Ad12E1-transformed cells that express low amounts of p90^{rsk1} process p105-NFκB inefficiently (Schouten *et al.*, 1995), which suggests that p90^{rsk1} indeed is required for the initiation of p105-NFκB1 processing.

Materials and methods

Cell culture and DNA transfection

Monolayers of COS1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum and antibiotics. The BRK cell lines transformed by Ad5E1 and Ad12E1 are described elsewhere (Schouten *et al.*, 1995). Monoclonal cell lines BX-RSK-M13 and BX-RSK-M15 were established by transfection of the Ad5E1-transformed BRK cell line BXC22 with pcDNA3-p90Rsk1-D205N and subsequent selection for G418 resistance. Control monoclonal cell lines BX-D3 and BX-D11 were established by transfection of BXC22 with parental vector pcDNA3 and subsequent selection for G418 resistance. Monolayers of AdE1-transformed cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% newborn calf serum and antibiotics. Tissue culture media and sera were purchased from Gibco (Grand Island, NY). All tissue culture plastics were obtained from Greiner (Nürtingen, Germany). Two hours prior to transfection, cells were plated at 30% density on 90 mm or 30 mm dishes. Two hours after plating, cells were incubated for 15 h with calcium phosphate-precipitated DNA as described previously (van der Eb and Graham, 1980). Subsequently, the transfected cells were cultured for 30 h, cells were harvested and extracts were prepared. Treatment with 100 ng/ml TPA (Sigma) was performed for the indicated periods of time.

Electrophoretic mobility shift assay

Preparation of whole cell extracts, binding reactions and electrophoresis were performed essentially as described elsewhere (Bernards, 1991). The double-stranded H2TF1 oligonucleotide dCCCAGGGCTGGGG-ATTCCCCATTGCA was labelled by filling in recessed 3' ends with [α -³²P]dCTP and the Klenow fragment of DNA polymerase I. After electrophoresis, the gels were dried and exposed to Kodak XAR-5 film at -80°C.

Plasmids, proteins and antibodies

The vectors expressing human wt-IκBα and the mutant IκBα proteins 32A-IκBα, 36A-IκBα and 32A36A-IκBα have been described elsewhere (Whiteside *et al.*, 1995). The pCMV-rIκBα expression vector was constructed as follows: the full-length IκBα cDNA from rat (RL/IF1) was isolated from Bluescript-IκBα (Tewari *et al.*, 1992) by digestion with *Hind*III and *Eco*RI. Subsequently, the full-length cDNA was ligated into a *Hind*III-*Eco*RI-digested pcDNA3 vector (Invitrogen), giving rise to pCMV-rIκBα. Expression vectors pCMV-p105T and pCMV-p97T (Fan and Maniatis, 1991), which encode Myc-tagged p105-NFκB1 and a C-terminally deleted form of p105-NFκB1, respectively, were kindly provided by Dr T.Maniatis. The plasmids pMT2-Rsk1-epi, encoding HA-tagged p90^{rsk1} (Grove *et al.*, 1993) and pcDNA-p44-tag, encoding HA-tagged p44^{erk1} (Meloche *et al.*, 1992), were kind gifts of Drs P.J.Coffer and J.Pouyssegur, respectively. pcDNA3-Rsk1-epi was constructed by cloning a 3.3 kb *Eco*RI fragment of pMT2-Rsk1-epi into the *Eco*RI site of pcDNA3 (Invitrogen). Site-directed *in vitro* mutagenesis of p90^{rsk1} was performed using the Altered Sites System, according to instructions of the manufacturer (Promega). A 2410 bp *Bam*HI-*Sal*I fragment of pMT2-Rsk1-epi was cloned into the multiple cloning site of the pSelect1 vector. The ATP-binding site of the amino-terminal kinase domain of p90^{rsk1} was mutated using oligonucleotide 5'-GCCAC-ATCAAACCTCACAAATTTGGCCTGAGCAAGG-3' (pSelect1-Rsk1-D205N). This mutation replaces aspartic acid on position 205 by asparagine and introduces a diagnostic *Apo*I restriction site. Full-length HA-Rsk1-D205N was created by replacing a 725 bp *Bst*EII-*Pf*MI fragment from pcDNA3-Rsk1-epi by the *Bst*EII-*Pf*MI fragment from pSelect1-Rsk1-D205N. Purified GST-IκBα and c-Jun proteins were purchased from Santa Cruz Biotechnology Inc. and Promega, respectively. The 40S ribosomes were isolated from rat liver and were a gift from Dr J.Dijk. The mouse monoclonal antibodies 12CA5 α-HA and 9E10 α-Myc are described elsewhere (Wilson *et al.*, 1984; Evan

et al., 1985). The antibodies SC231 α-RSK1, SC1430 α-RSK2, SC1431 α-RSK3 and SC371 α-IκBα were purchased from Santa Cruz Biotechnology Inc.

Immunoprecipitations

Cells were washed twice with phosphate-buffered saline (PBS), and lysed and scraped in mild buffer [50 mM Tris pH 7.4, 250 mM NaCl, 5 mM EDTA and 0.1% Triton X-100, supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml Na₄P₂O₇ and 0.1 mg/ml trypsin inhibitor, NaF, β-glycerophosphate and Na₃VO₄]. After 30 min incubation on ice, the lysates were cleared by centrifugation. Immunoprecipitations were performed by incubation of extracts derived from half a 90 mm dish with 1 μg of specific antibody and 30 μl of 10% protein A beads for 2 h. After extensive washing, the immunoprecipitates were subjected to Western blotting.

Western blotting

Cells were washed twice with PBS, and lysed and scraped in RIPA (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS, supplemented with 1 mM PMSF, 1 mg/ml Na₄P₂O₇ and 0.1 mg/ml trypsin inhibitor, NaF, β-glycerophosphate and Na₃VO₄). After 30 min incubation on ice, the lysates were cleared by centrifugation. Protein concentrations were determined by the Bio-Rad protein assay. Whole cell extracts or immunoprecipitates were fractionated by SDS-PAGE on 10% gels. Proteins were transferred onto Immobilon-P membranes and incubated with specific antibodies as indicated. The secondary antibodies were horseradish peroxidase-coupled goat anti-rabbit, goat anti-mouse and donkey anti-goat IgG (Santa Cruz Biotechnology Inc.). The antibody complexes were visualized with the ECL detection system according to the manufacturer's protocol (Amersham, UK).

Kinase assay

Kinase assays were performed essentially as described previously (Chen and Blenis, 1990), with some minor modifications. TPA-treated cells were lysed and scraped in lysis buffer (1% NP-40, 10 mM KH₂PO₄, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl₂ and 50 mM β-glycerophosphate pH 7.2, supplemented with 1 mM PMSF, 1 mg/ml Na₄P₂O₇ and 0.1 mg/ml trypsin inhibitor, NaF and Na₃VO₄). After 30 min incubation on ice, the lysates were cleared by centrifugation. Subsequently, HA-tagged p90^{rsk1} and HA-tagged p44^{erk1} were purified by immunoprecipitation with 12CA5 α-HA. Kinase reactions were performed with the indicated substrates (purified GST-IκBα, c-Jun, 40S ribosomes, purified Myc-tagged p105-NFκB1 or purified Myc-tagged p97-NFκB1) in kinase buffer [20 mM HEPES pH 7.2 and 10 mM MgCl₂, supplemented with 0.1 mg/ml trypsin inhibitor, 0.1 mg/ml Na₃VO₄, 50 μM ATP, 0.1 mg/ml bovine serum albumin (BSA) and 2 mM dithiothreitol (DTT)] in the presence of [γ -³²P]ATP for 30 min at 30°C. The phosphorylated proteins were fractionated by SDS-PAGE on 10% gels. The gels were dried and exposed to Kodak XAR-5 film at -80°C.

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References

- Alcorta,D.A., Crews,C.M., Sweet,L.J., Bankston,L., Jones,S.W. and Erikson,R.L. (1989) Sequence and expression of chicken and mouse rsk: homologs of *Xenopus laevis* ribosomal S6 kinase. *Mol. Cell. Biol.*, **9**, 3850-3859.
- Arenzana-Seisdedos,F., Thompson,J., Rodriguez,M.S., Bachelier,F., Thomas,D. and Hay,R. (1995) Inducible nuclear expression of newly synthesized IκBα negatively regulates DNA-binding and transcriptional activities of NFκB. *Mol. Cell. Biol.*, **15**, 2689-2696.
- Baeuerle,P.A. and Henkel,T. (1994) Function and activation of NFκB in the immune system. *Annu. Rev. Immunol.*, **12**, 141-179.

- Baldwin, A.S., Jr, Azizkhan, J.C., Jensen, D.E., Beg, A.A. and Coodly, L.R. (1991) Induction of NF κ B DNA-binding activity during the G₀ to G₁ transition in mouse fibroblasts. *Mol. Cell. Biol.*, **11**, 4943–4951.
- Barroga, C.F., Stevenson, J.K., Schwarz, E.M. and Verma, I.M. (1995) Constitutive phosphorylation of I κ B α by casein kinase II. *Proc. Natl Acad. Sci. USA*, **92**, 7637–7641.
- Beg, A.A. and Baltimore, D. (1996) An essential role for NF κ B in preventing TNF- α -induced cell death. *Science*, **274**, 782–784.
- Beg, A.A., Ruben, S.M., Scheinman, R.I., Haskill, S., Rosen, C.A. and Baldwin, A.S., Jr (1992) I κ B interacts with the nuclear localization sequences of the subunits of NF κ B: a mechanism for cytoplasmic retention. *Genes Dev.*, **6**, 1899–1913.
- Bernards, R. (1991) N-myc disrupts protein kinase C-mediated signal transduction in neuroblastoma. *EMBO J.*, **10**, 1119–1125.
- Bertrand, F., Philippe, C., Antoine, P.J., Baud, L., Groyer, A., Cepeau, J. and Cherqui, G. (1995) Insulin activates nuclear factor κ B in mammalian cells through a Raf-1-mediated pathway. *J. Biol. Chem.*, **270**, 24435–24441.
- Bjorbaek, C., Zhao, Y. and Moller, D.E. (1995) Divergent functional roles for p90^{rsk} kinase domains. *J. Biol. Chem.*, **270**, 18848–18852.
- Blenis, J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl Acad. Sci. USA*, **90**, 5889–5892.
- Bressler, P., Brown, K., Timmer, W., Bours, V., Siebenlist, U. and Fauci, A.S. (1993) Mutational analysis of the p50 subunit of NF κ B and inhibition of NF κ B activity by trans-dominant p50 mutants. *J. Virol.*, **67**, 288–293.
- Brockman, J.A., Scherer, D.C., McKinsey, T.A., Hall, S.M., Qi, X., Lee, W.Y. and Ballard, D.W. (1995) Coupling of a signal response domain in I κ B α to multiple pathways for NF κ B activation. *Mol. Cell. Biol.*, **15**, 2809–2818.
- Brown, K., Park, S., Kanno, T., Fransozo, G. and Siebenlist, U. (1993) Mutual regulation of the transcriptional activator NF κ B and its inhibitor, I κ B α . *Proc. Natl Acad. Sci. USA*, **90**, 2532–2536.
- Brown, K., Gerstberger, S., Carlson, L., Fransozo, G. and Siebenlist, U. (1995) Control of I κ B α proteolysis by site-specific, signal-induced phosphorylation. *Science*, **267**, 1485–1488.
- Capobianco, A.J., Chang, D., Mosialos, G. and Gilmore, T.D. (1992) p105: the NF κ B p50 precursor protein, is one of the cellular proteins complexed with the v-Rel oncoprotein in transformed chicken spleen cells. *J. Virol.*, **66**, 3758–3767.
- Chen, R.-H. and Blenis, J. (1990) Identification of *Xenopus* S6 protein kinase homologs (pp90^{rsk}) in somatic cells: phosphorylation and activation during initiation of cell proliferation. *Mol. Cell. Biol.*, **10**, 3204–3215.
- Chen, R.-H., Chung, J. and Blenis, J. (1991) Regulation of pp90^{rsk} phosphorylation and S6 phosphotransferase activity in Swiss 3T3 cells by growth factor-, phorbol ester-, and cyclic AMP-mediated signal transduction. *Mol. Cell. Biol.*, **11**, 1861–1867.
- Chen, R.-H., Sarnecki, C. and Blenis, J. (1992) Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol. Cell. Biol.*, **12**, 915–927.
- Chen, R.-H., Abate, C. and Blenis, J. (1993) Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. *Proc. Natl Acad. Sci. USA*, **90**, 10952–10956.
- Chen, Z.J., Hagler, J., Palombella, V.J., Melandri, F., Scherer, D., Ballard, D. and Maniatis, T. (1995) Signal-induced site-specific phosphorylation targets I κ B α to the ubiquitin-proteasome pathway. *Genes Dev.*, **9**, 1586–1597.
- Chen, Z.J., Parent, L. and Maniatis, T. (1996) Site specific phosphorylation of I κ B α by a novel ubiquitination-dependent protein kinase activity. *Cell*, **84**, 853–862.
- Chung, J., Pelech, S.L. and Blenis, J. (1991) Mitogen-activated Swiss mouse 3T3 RSK kinases I and II are related to pp44^{mkp} from sea star oocytes and participate in the regulation of pp90^{rsk} activity. *Proc. Natl Acad. Sci. USA*, **88**, 4981–4985.
- Chung, J., Kuo, C.J., Crabtree, G.R. and Blenis, J. (1992) Rapamycin-FKBP specifically blocks growth-dependent activation of and signalling by the 70kd S6 protein kinases. *Cell*, **69**, 1227–1236.
- Devary, Y., Rosette, C., DiDonato, J.A. and Karin, M. (1993) NF κ B activation by ultraviolet light not dependent on a nuclear signal. *Science*, **261**, 1442–1445.
- Diaz-Meco, M.T. et al. (1993) A dominant negative protein kinase C ζ subspecies blocks NF κ B activation. *Mol. Cell. Biol.*, **13**, 4770–4775.
- Diaz-Meco, M.T. et al. (1994) ζ PKC induces phosphorylation and inactivation of I κ B α in vitro. *EMBO J.*, **13**, 1842–1848.
- DiDonato, J., Mercurio, F. and Karin, M. (1995) Phosphorylation of I κ B α precedes but is not sufficient for its dissociation from NF κ B. *Mol. Cell. Biol.*, **15**, 1302–1311.
- DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S. and Karin, M. (1996) Mapping of the inducible I κ B phosphorylation sites that signal its ubiquitination and degradation. *Mol. Cell. Biol.*, **16**, 1295–1304.
- Dobrzanski, P., Ryseck, R.P. and Bravo, R. (1994) Differential interactions of Rel-NF κ B complexes with I κ B α determine pools of constitutive and inducible NF κ B activity. *EMBO J.*, **13**, 4608–4616.
- Erikson, E. and Maller, J.L. (1986) Purification and characterization of a protein kinase from *Xenopus* eggs highly specific for ribosomal protein S6. *J. Biol. Chem.*, **261**, 350–355.
- Erikson, E. and Maller, J.L. (1988) Substrate specificity of ribosomal protein S6 kinase II from *Xenopus* eggs. *Second Messenger Phosphoproteins*, **2**, 135–143.
- Evan, G.I., Lewis, G.K., Ramsay, G. and Bishop, J.M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.*, **5**, 3610–3616.
- Fan, C.M. and Maniatis, T. (1991) Generation of p50 subunit of NF κ B by processing of p105 through an ATP-dependent pathway. *Nature*, **354**, 395–398.
- Finco, T.S. and Baldwin, A.S., Jr (1993) κ B site-dependent induction of gene expression by diverse inducers of nuclear factor κ B requires Raf-1. *J. Biol. Chem.*, **268**, 17676–17679.
- Finco, T.S., Beg, A.A. and Baldwin, A.S., Jr (1994) Inducible phosphorylation of I κ B α is not sufficient for its dissociation from NF κ B and is inhibited by protease inhibitors. *Proc. Natl Acad. Sci. USA*, **91**, 11884–11888.
- Fisher, T.L. and Blenis, J. (1996) Evidence for two catalytically active kinase domains in pp90^{rsk}. *Mol. Cell. Biol.*, **16**, 1212–1219.
- Folgueira, L., Algeciras, A., MacMorran, W.S., Bren, G.D. and Paya, C.V. (1996) The ras-raf pathway is activated in human immunodeficiency virus-infected monocytes and participates in the activation of NF κ B. *J. Virol.*, **70**, 2332–2338.
- Fransozo, G., Bours, V., Park, S., Tomita-Yamaguchi, M., Kelly, K. and Siebenlist, U. (1992) The candidate oncoprotein Bcl-3 is an antagonist of p50/NF κ B-mediated inhibition. *Nature*, **359**, 339–342.
- Grilli, M., Chiu, J.J.S. and Lenardo, M.J. (1993) NF κ B and rel—participants in a multiform transcriptional regulatory system. *Int. Rev. Cytol.*, **143**, 1–62.
- Grove, J.R., Price, D.J., Banerjee, P., Balasubramanyam, A., Ahmad, M.F. and Avruch, J. (1993) Regulation of an epitope-tagged recombinant Rsk-1 S6 kinase by phorbol ester and erk/MAP kinase. *Biochemistry*, **32**, 7727–7738.
- Hatada, E.N., Nieters, A., Wulczyn, F.G., Naumann, M., Meyer, R., Nucifora, G., McKeithan, T.W. and Scheidereit, C. (1992) The ankyrin repeat domains of the NF κ B precursor p105 and the proto oncogene bcl-3 act as specific inhibitors of NF κ B DNA binding. *Proc. Natl Acad. Sci. USA*, **89**, 2489–2493.
- Hatada, E.N., Naumann, M. and Scheidereit, C. (1993) Common structural constituents confer I κ B activity to NF κ B p105 and I κ B/MAD-3. *EMBO J.*, **12**, 2781–2788.
- Henkel, T., Machleidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y. and Baeuerle, P.A. (1993) Rapid proteolysis of I κ B α is necessary for activation of transcription factor NF κ B. *Nature*, **365**, 182–185.
- Imbert, V. et al. (1996) Tyrosine phosphorylation of I κ B α activates NF- κ B without proteolytic degradation of I κ B α . *Cell*, **86**, 787–798.
- Inoue, J., Kerr, L.D., Kakizuka, A. and Verma, I.M. (1992) I κ B- γ , a 70kD protein identical to the C-terminal half of p110 NF κ B: a new member of the I κ B-family. *Cell*, **68**, 1109–1120.
- Israel, A. (1995) A role for phosphorylation and degradation in the control of NF κ B activity. *Trends Genet.*, **11**, 203–205.
- Janosch, P., Scheller, M., Seitz, T., Reim, P., Eulitz, M., Briellemeier, M., Kolch, W., Sedivy, J.M. and Mischak, H. (1996) Characterization of I κ B kinases. *J. Biol. Chem.*, **271**, 13868–13874.
- Kerr, L.D., Inoue, J.I., Davis, N., Link, E., Baeuerle, P.A., Bose, H.R., Jr and Verma, I.M. (1991) The Rel-associated pp40 protein prevents DNA binding of rel and NF κ B—relationship with I κ B β and regulation by phosphorylation. *Genes Dev.*, **5**, 1464–1476.
- Kumar, A., Haque, J., Lacoste, J., Hiscott, J. and Williams, B.R. (1994) Double-stranded RNA-dependent protein kinase activates transcription factor NF κ B by phosphorylating I κ B. *Proc. Natl Acad. Sci. USA*, **91**, 6288–6292.
- Lai, J.H. and Tan, T.H. (1994) CD28 signaling causes a sustained down-regulation of I κ B α which can be prevented by the immunosuppressant rapamycin. *J. Biol. Chem.*, **269**, 30077–30080.
- Li, S. and Sedivy, J.M. (1993) Raf-1 protein kinase activates the NF κ B transcription factor by dissociating the cytoplasmic NF κ B-I κ B complex. *Proc. Natl Acad. Sci. USA*, **90**, 9247–9251.

- Lin, R., Beauparlant, P., Makris, C., Meloche, S. and Hiscott, J. (1996) Phosphorylation of I κ B α in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. *Mol. Cell. Biol.*, **16**, 1401–1409.
- Lin, Y.C., Brown, K. and Siebenlist, U. (1995) Activation of NF κ B requires proteolysis of the inhibitor I κ B α : signal-induced phosphorylation of I κ B α alone does not release active NF κ B. *Proc. Natl Acad. Sci. USA*, **92**, 552–556.
- Lindholm, P.F., Tamami, M., Makowski, J. and Brady, J.N. (1996) Human T-cell lymphotropic virus type I Tax₁ activation of NF κ B: involvement of the protein kinase C pathway. *J. Virol.*, **70**, 2525–2532.
- Liou, H.C., Nolan, G.P., Ghosh, S., Fujita, T. and Baltimore, D. (1992) The NF κ B p50 precursor, p105, contains an internal I κ B like inhibitor that preferentially inhibits p50. *EMBO J.*, **11**, 3003–3009.
- Liu, Z.-G., Hsu, H., Goeddel, D.V. and Karin, M. (1996) Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF κ B activation prevents cell death. *Cell*, **87**, 565–576.
- Logeat, F., Israël, N., Ten, R., Blank, V., Le Bail, O., Kourilsky, P. and Israël, A. (1991) Inhibition of transcription factors belonging to the rel/NF κ B family by a transdominant negative mutant. *EMBO J.*, **10**, 1827–1832.
- MacKichan, M.L., Logeat, F. and Israël, A. (1996) Phosphorylation of p105 PEST sequence via a redox-insensitive pathway up-regulates processing of p50-NF κ B. *J. Biol. Chem.*, **271**, 6084–6091.
- Maran, A., Maitra, R.K., Kumar, A., Dong, B., Xiao, W., Li, G., Williams, B.R.G., Torrence, P. and Silverman, R.H. (1994) Blockage of NF κ B signaling by selective ablation of an mRNA target by 2-5A antisense chimeras. *Science*, **265**, 789–792.
- McElhinny, J.A., Trushin, S.A., Bren, G.D., Chester, N. and Paya, C.V. (1996) Casein kinase II phosphorylates I κ B α at S-283, S-289, S-293, and T-291 and is required for its degradation. *Mol. Cell. Biol.*, **16**, 899–906.
- Mellits, K.H., Hay, R.T. and Goodbourn, S. (1993) Proteolytic degradation of MAD3 (I κ B α) and enhanced processing of the NF κ B precursor p105 are obligatory steps in the activation of NF κ B. *Nucleic Acids Res.*, **21**, 5059–5066.
- Meloche, S., Pages, G. and Pouyssegur, J. (1992) Functional expression and growth factor activation of an epitope-tagged p44 mitogen-activated protein kinase, p44 mapk. *Mol. Biol. Cell*, **3**, 63–71.
- Mercurio, F., DiDonato, J.A., Rosette, C. and Karin, M. (1993) p105 and p98 proteins play an active role in NF κ B-mediated signal transduction. *Genes Dev.*, **7**, 705–718.
- Miyamoto, S., Maki, M., Schmitt, M.J., Hatanaka, M. and Verma, I.M. (1994) Qualitative changes in the subunit composition of κ B binding complexes during murine B cell differentiation. *Proc. Natl Acad. Sci. USA*, **91**, 12740–12744.
- Moller, D.E., Xia, C.-H., Tang, W., Zhu, A.X. and Jakubowski, M. (1994) Human rsk isoforms: cloning and characterization of tissue-specific expression. *Am. J. Physiol.*, **266**, 351–359.
- Naumann, M. and Scheidereit, C. (1994) Activation of NF κ B *in vivo* is regulated by multiple phosphorylations. *EMBO J.*, **13**, 4597–4607.
- Naumann, M., Wulczyn, F.G. and Scheidereit, C. (1993) The NF κ B precursor p105 and the proto-oncogene product Bcl-3 are I κ B molecules and control nuclear translocation of NF κ B. *EMBO J.*, **12**, 213–222.
- Orian, A., Whiteside, S., Israël, A., Stancovski, I., Schwartz, A.L. and Ciechanover, A. (1995) Ubiquitin-mediated processing of NF κ B transcriptional activator precursor p105. *J. Biol. Chem.*, **270**, 21707–21714.
- Palombella, V.J., Rando, O.J., Goldberg, A.L. and Maniatis, T. (1994) The ubiquitin–proteasome pathway is required for processing the NF κ B1 precursor protein and the activation of NF κ B. *Cell*, **78**, 773–785.
- Pinna, L.A. (1990) Casein kinase 2: an ‘eminence grise’ in cellular regulation? *Biochim. Biophys. Acta*, **1054**, 267–284.
- Price, D.J., Grove, J.R., Calvo, V., Avruch, J. and Bierer, B.E. (1992) Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. *Science*, **257**, 973–977.
- Rice, N.R. and Ernst, M.K. (1993) *In vivo* control of NF κ B activation by I κ B α . *EMBO J.*, **12**, 4685–4695.
- Rice, N.R., MacKichan, M.L. and Israël, A. (1992) The precursor of NF κ B p50 has I κ B α -like functions. *Cell*, **71**, 243–253.
- Rivera, V.M., Miranti, C.K., Misra, R.P., Ginty, D.D., Chen, R.-H., Blenis, J. and Greenberg, M.E. (1993) A growth factor-induced kinase phosphorylates the serum response factor at a site that regulates its DNA binding activity. *Mol. Cell. Biol.*, **13**, 6260–6273.
- Roff, M., Thompson, J., Rodriguez, M.S., Jacque, J.-M., Baleux, F., Arenzana-Seisdedos, F. and Hay, R.T. (1996) Role of I κ B α ubiquitination in signal-induced activation of NF κ B *in vivo*. *J. Biol. Chem.*, **271**, 7844–7850.
- Scheinman, R.I., Beg, A.A. and Baldwin, A.S., Jr (1993) NF κ B p100 (I κ B-10) is a component of H2TF1 and can function as an I κ B α -like molecule. *Mol. Cell. Biol.*, **13**, 6089–6101.
- Schouten, G.J., van der Eb, A.J. and Zanema, A. (1995) Downregulation of MHC class I expression due to interference with p105-NF κ B1 processing by Ad12E1A. *EMBO J.*, **14**, 1498–1507.
- Schwarz, E.M., van Antwerp, D. and Verma, I.M. (1996) Constitutive phosphorylation of I κ B α by casein kinase II occurs preferentially at serine 293: requirement for degradation of free I κ B α . *Mol. Cell. Biol.*, **16**, 3554–3559.
- Steffan, N.M., Bren, G.D., Frantz, B., Tocci, M.J., O’Neill, E.A. and Paya, C.V. (1995) Regulation of I κ B α phosphorylation by PKC- and Ca²⁺-dependent signal transduction pathways. *J. Immunol.*, **155**, 4685–4691.
- Stephens, R.M., Rice, N.R., Hiebsch, R.R., Bose, H.R., Jr and Gilden, R.V. (1983) Nucleotide sequence of *v-rel*: the oncogene of the reticuloendotheliosis virus. *Proc. Natl Acad. Sci. USA*, **80**, 6229–6233.
- Sturgill, T.W., Ray, L.B., Erikson, E. and Maller, J.L. (1988) Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature*, **334**, 715–718.
- Tewari, M., Dobrzanski, P., Mohn, K., Cressman, D.E., Hsu, J.C., Bravo, R. and Taub, R. (1992) Rapid induction in regenerating liver of RL/IF-1 (an I κ B that inhibits NF κ B, Rel B-p50, and c-Rel-p50) and PHF, a novel κ B site-binding complex. *Mol. Cell. Biol.*, **12**, 2898–2908.
- Thanos, D. and Maniatis, T. (1995) NF κ B: a lesson in family values. *Cell*, **80**, 529–532.
- Thomas, G. (1993) p70 S6K/p85 S6K: mechanism of activation, effects of rapamycin and role in mitogenesis. *Biochem. Soc. Trans.*, **21**, 901–904.
- Thompson, J.E., Phillips, R.J., Erdjument-Bromage, H., Tempst, P. and Ghosh, S. (1995) I κ B β regulates the persistent response in a biphasic activation of NF κ B. *Cell*, **80**, 573–582.
- Traenckner, E.B.M., Wilk, S. and Baeuerle, P.A. (1994) A proteasome inhibitor prevents activation of NF κ B and stabilizes a newly phosphorylated form of I κ B α that is still bound to NF κ B. *EMBO J.*, **13**, 5433–5441.
- Traenckner, E.B.M., Pahl, H.L., Henkel, T., Schmidt, K.N., Wilk, S. and Baeuerle, P.A. (1995) Phosphorylation of human I κ B α on serines 32 and 36 controls I κ B α proteolysis and NF κ B activation in response to diverse stimuli. *EMBO J.*, **14**, 2876–2883.
- van den Heuvel, S. and Harlow, E. (1993) Distinct roles for cyclin dependent kinases in cell cycle control. *Science*, **262**, 2050–2054.
- van der Eb, A.J. and Graham, F.L. (1980) Assay of transforming activity of tumor virus DNA. *Methods Enzymol.*, **65**, 826–839.
- Verma, I.M., Stevenson, J.K., Schwarz, E.M., van Antwerp, D. and Miyamoto, S. (1995) Rel/NF κ B/I κ B family: intimate tales of association and dissociation. *Genes Dev.*, **9**, 2723–2735.
- Whiteside, S.T., Ernst, M.K., LeBail, O., Laurent-Winter, C., Rice, N. and Israël, A. (1995) N- and C-terminal sequences control degradation of MAD3/I κ B α in response to inducers of NF κ B activity. *Mol. Cell. Biol.*, **15**, 5339–5345.
- Wilson, I.A., Niman, H.L., Hougston, R.A., Cherenson, A.R., Connolly, M.L. and Lerner, R.A. (1984) The structure of an antigenic determinant in a protein. *Cell*, **37**, 767–778.
- Wu, M. *et al.* (1996) Inhibitor of NF κ B/Rel induces apoptosis of murine B cells. *EMBO J.*, **15**, 4682–4690.
- Wulczyn, F.G., Naumann, M. and Scheidereit, C. (1992) Candidate proto-oncogene *bcl-3* encodes a subunit-specific inhibitor of transcription factor NF κ B. *Nature*, **358**, 597–599.
- Xing, J., Ginty, D.D. and Greenberg, M.E. (1996) Coupling of the RAS–MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science*, **273**, 959–963.
- Zhao, Y., Bjorbaek, C., Weremowicz, S., Morton, C. and Moller, D.E. (1995) RSK3 encodes a novel pp90rsk isoform with a unique N-terminal sequence: growth factor-stimulated kinase function and nuclear translocation. *Mol. Cell. Biol.*, **15**, 4353–4363.

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