Retroviral oncoprotein Tax deregulates NF- κ B by activating Tak1 and mediating the physical association of Tak1–IKK

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The Tax oncoprotein of human T-cell leukaemia virus type I (HTLV-I) persistently activates nuclear factor- κ B (NF- κ B), which is required for HTLV-I-mediated T-cell transformation. Tax activates NF- κ B by stimulating the activity of I κ B kinase (IKK), but the underlying mechanism remains elusive. Here, we show that Tax functions as an intracellular stimulator of an IKK-activating kinase, Tak1 (TGF- β -activating kinase 1). In addition, Tax physically interacts with Tak1 and mediates the recruitment of IKK to Tak1. In HTLV-I-infected T cells, Tak1 is constitutively activated and complexed with both Tax and IKK. We provide genetic evidence that Tak1 is essential for Tax-induced IKK activation. Furthermore, unlike cellular stimuli, the Tax-specific NF- κ B signalling does not require the ubiquitin-binding function of IKK γ . These findings show a pathological mechanism of IKK activation by Tax and provide an example for how IKK is persistently activated in cancer cells.

Keywords: HTLV-I; Tax; NF- κ B; Tak1; IKK

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INTRODUCTION

Human T-cell leukaemia virus type I (HTLV-I) is an oncogenic retrovirus aetiologically associated with an acute T-cell malignancy termed adult T-cell leukaemia (ATL; Yoshida, 2001). HTLV-I encodes a regulatory protein, Tax, which is important in HTLV-I-induced T-cell transformation (Grassmann *et al*, 2005). The oncogenic action of Tax involves aberrant induction of a large array of cellular genes regulating cell growth and survival (Sun & Ballard, 1999; Ng *et al*, 2001). Tax induces many of these genes by activating nuclear factor- κ B (NF- κ B; Sun & Yamaoka, 2005), a family of cellular transcription factors that are important for the control of cell proliferation and apoptosis inhibition (Karin & Lin,

2002). The activity of NF- κ B is tightly regulated through its cytoplasmic sequestration by the I κ B family of inhibitory proteins, predominantly I κ B α (Karin & Ben-Neriah, 2000). Activation of NF- κ B by diverse cellular stimuli involves stimulation of a multisubunit I κ B kinase (IKK), which is composed of two catalytic subunits, IKK α and IKK β , and a regulatory subunit termed IKK γ (Karin & Ben-Neriah, 2000). On activation, IKK phosphorylates I κ B α , triggering its ubiquitination and degradation, which allows NF- κ B to move to the nucleus and exert its transcription function.

The mechanism mediating IKK activation by different stimuli is not completely understood, although a number of upstream kinases have been implicated in the activation of IKK. However, recent genetic evidence suggests an essential role for TGF- β -activating kinase 1 (Tak1) in IKK activation by a number of cellular stimuli (Sato *et al*, 2005, 2006; Shim *et al*, 2005; Liu *et al*, 2006; Omori *et al*, 2006; Wan *et al*, 2006). Tak1 phosphorylates IKK β as well as MKK6, leading to activation of both NF- κ B and c-Jun amino-terminal kinase (JNK) pathways (Ninomiya-Tsuji *et al*, 1999). Recent evidence also suggests that IKK activation by immune stimuli involves lysine 63 (K63)-linked polyubiquitination of IKK γ and upstream regulators, including tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and receptor-interacting protein 1 (RIP1; Sun *et al*, 2004; Zhou *et al*, 2004; Chen *et al*, 2006). Ubiquitination of RIP1 and TRAF6 appears to act as a mechanism that recruits IKK and Tak1 (Ea *et al*, 2006; Li *et al*, 2006; Wu *et al*, 2006). Indeed, both IKK γ and a Tak1-associated protein, Tab2, are ubiquitin-binding proteins (Kanayama *et al*, 2004; Ea *et al*, 2006; Wu *et al*, 2006). Thus, the polyubiquitin chains might provide a platform for IKK activation by Tak1. As the signal-induced protein ubiquitination is quickly reversed by deubiquitinating enzymes (Wilkinson, 2000), this mechanism explains the rapid and transient nature of NF- κ B activation by cellular stimuli.

In contrast to the transient activation of NF- κ B by cellular stimuli, Tax stimulates persistent NF- κ B activation, which is essential for HTLV-I-induced T-cell transformation (Robek & Ratner, 1999; Sun & Ballard, 1999; Hiscott *et al*, 2001). Although precisely how Tax induces persistent NF- κ B activation is obscure, this viral-specific pathway does not require the upstream

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Fig 1 | TGF- β -activating kinase 1 is required for Tax-stimulated activation of I κ B kinase and nuclear factor- κ B. (A) Tak1^{-/-} and wild-type (Tak^{+/+}) MEFs were transfected with expression vectors encoding green fluorescent protein (pEGFP) or Tax (pCMV4-Tax). Around 36 h after transfection, nuclear extracts were isolated and subjected to electrophoretic mobility shift assay using NF- κ B (upper panel) or NF- Υ (lower panel) probes. (B) Cytoplasmic extracts (panels 1,2) and whole cell extracts (panels 3,4) from the cells used in (A) were subjected to immunoblotting (IB) using the indicated antibodies. (C) MEFs were transfected with expression vectors encoding either GFP or Tax. Around 36 h after transfection, RNA was prepared and subjected to semiquantitative reverse transcription-PCR to analyse the messenger RNA levels of tumour necrosis factor- α (TNF- α) and the housekeeping gene GAPDH. (D) The I κ B kinase (IKK) complex was isolated from the cells used in (A), by immunoprecipitation using anti-IKK γ followed by *in vitro* kinase assays (KA) using glutathione S-transferase (GST)-I κ B α (1-54) as substrate (panel 1). IB was carried out to monitor the expression level of IKK β , Tax and the loading control tubulin (panels 2-4). (E) Whole-cell lysates prepared from the cells used in (A) were subjected to IB to detect the indicated proteins. GAPDH, glyceraldehyde phosphate dehydrogenase; MEF, mouse embryonic fibroblasts; Tak1, TGF- β -activating kinase 1.

signalling factors, such as TRAFs and RIP1 (Geleziunas *et al*, 1998; Chu *et al*, 1999). Instead, Tax seems to use a mechanism that involves its physical interaction with IKK through the IKK γ subunit (Chu *et al*, 1999; Harhaj and Sun, 1999; Jin *et al*, 1999). However, it is presently unknown how the Tax-IKK association causes the activation of IKK. In this study we show that Tax stimulates the catalytic activity of the IKK-activating kinase Tak1 and also mediates the physical recruitment of IKK to Tak1. This viral-specific mechanism does not require the ubiquitin-binding function of IKK γ . These findings emphasize a pathological mechanism of IKK activation and provide an example for how IKK is persistently activated in cancer cells.

RESULTS AND DISCUSSION

Tak1 is required for activation of NF- κ B and IKK by Tax

Although Tax is known to physically interact with IKK, how Tax stimulates the catalytic activity of IKK remains unclear (Sun & Yamaoka, 2005). It is generally believed that Tax might activate IKK through either inducing IKK oligomerization or recruiting IKK to an upstream kinase (Sun & Ballard, 1999). However, neither of these hypotheses has been experimentally tested. To date, genetic evidence for the involvement of upstream kinases is lacking. To investigate further the mechanism of NF- κ B activation by Tax, we investigated the role of Tak1 by using a genetic approach. Mouse

embryonic fibroblasts (MEFs) derived from Tak1-deficient (Tak1^{-/-}) and wild-type control (Tak1^{+/+}) mice were transfected with the Tax expression vector followed by an analysis of the activation of NF- κ B by electrophoretic mobility shift assay (EMSA). As expected, expression of Tax in wild-type MEFs resulted in potent activation of NF- κ B DNA-binding activity (Fig 1A). Furthermore, this function of Tax was associated with a marked loss of the NF- κ B inhibitor I κ B α (Fig 1B). Remarkably, the Tak1 deficiency abrogated the Tax-stimulated activation of NF- κ B (Fig 1A) and also the loss of I κ B α (Fig 1B). Consistently, the loss of Tak1 also diminished Tax-stimulated expression of the TNF- α gene (Fig 1C).

We next examined whether Tak1 is required for Tax activation of IKK. When expressed in wild-type cells, Tax stimulated the catalytic activity of endogenous IKK (Fig 1D, lane 2). By contrast, Tax failed to enhance the IKK activity in Tak1^{-/-} cells (Fig 1D, lane 4). This signalling defect was not due to the alteration in the expression level of IKK subunits (Fig 1E). Together, these results show an essential role for Tak1 in mediating Tax-stimulated activation of IKK and NF- κ B.

Tax stimulates the catalytic activity of Tak1

The essential role of Tak1 in Tax-induced IKK activation prompted us to investigate whether Tax activates the catalytic activity of Tak1. We first analysed whether Tax promotes the IKK-activating

Fig 2 | Tax stimulates the catalytic activity of TGF- β -activating kinase 1. (A) 293 cells were either mock transfected (–) or transfected (+) with expression vectors encoding HA-tagged Tak1, Tax or both expression vectors. The IKK complex was isolated by immunoprecipitation (IP) using anti-IKK γ followed by *in vitro* kinase assay (KA) using glutathione S-transferase (GST)–I κ B α (1–54) as substrate (panel 1). The kinase assay membrane was blotted with anti-IKK β to detect the IKK protein in the immune complex (panel 2). The expression level of Tak1 and Tax in cell lysates was detected by immunoblotting (IB; panels 3,4). (B) 293 cells were transfected (in 24-well plates) with (+) or without (–) the indicated expression vectors along with a κ B-luciferase reporter (100 ng) and a control *Renilla* luciferase reporter driven by the constitutive thymidine kinase promoter (pRL-tk-luc, 40 ng). The κ B-specific luciferase activity was normalized on the basis of the control *Renilla* luciferase. Data are representative of three independent experiments. (C) 293 cells were transfected with (+) or without (–) HA-Tak1 or Tax as indicated. Tak1 was isolated by IP and subjected to *in vitro* kinase assays using MAP kinase kinase 6 (MKK6) as substrate (panel 1). The kinase assay membrane was subjected to IB to detect Tak1 protein (panel 2). IB was also carried out with the cell lysates to detect the expression levels of Tak1 and Tax (panels 3,4). (D) Tak1 was isolated by IP from the HTLV-negative SupT1 and the indicated Tax-expressing T cells transformed by either HTLV-I (C8166, SLB-1 and HUT102) or Tax (Tax1). Tak1 kinase assay (panel 1) and IB (panels 2–4) were carried out as in (B). (E) SupT1 cells were infected with retroviral vectors encoding either green fluorescent protein (GFP) or Tax followed by analysis of the Tak1 kinase activity and protein expression levels as in (B). HA, haemagglutinin; HTLV-I, human T-cell leukaemia virus type I; IKK, I κ B kinase; Tak1, TGF- β -activating kinase 1.

activity of Tak1. When expressed in 293 cells, both Tax and Tak1 were able to activate IKK, as shown by immunocomplex kinase assays (Fig 2A, panel 1). It is important to note that Tax significantly potentiated Tak1-mediated activation of IKK (Fig 2A, upper panel, lane 4). This effect was not due to an increase in Tak1 expression, as the level of Tak1 was even lower in the presence of Tax (Fig 2A, panel 3). Consistent with this biochemical result, Tax also synergized with Tak1 in the induction of a κ B-luciferase reporter (Fig 2B). Although it is likely that the Tax–Tak1 synergy might involve other factors, these data indicate the possibility that Tax might stimulate the catalytic activity of Tak1. This idea was confirmed by directly analysing the activity of Tak1 by kinase assays. Indeed, Tax potently stimulated the catalytic activity of Tak1 (Fig 2C, panel 1, lane 3) without increasing the level of Tak1 expression (Fig 2C, panels 2 and 3, lane 3).

A characteristic of HTLV-I-transformed T cells is the constitutive activation of IKK (Sun & Yamaoka, 2005). It was therefore important to determine whether Tak1 was also activated in these HTLV-I-infected T cells. Compared with the HTLV-negative T-cell line SupT1 (Fig 2D, lane 1), the HTLV-I-transformed T cells showed markedly higher levels of Tak1 kinase activity (Fig 2D, lanes 3,4). This deregulated activation of Tak1 seemed to be mediated by Tax, as it was also detected in a T-cell line

transformed by the Tax protein (Fig 2D, lane 5). To examine further this molecular connection, we expressed Tax or a control green fluorescent protein (GFP) in the HTLV-negative SupT1 cells by retroviral transduction. Indeed, Tax expression was sufficient for stimulating the catalytic activity of Tak1 in these T cells (Fig 2E). Thus, the retroviral oncoprotein Tax is an intracellular stimulator of Tak1.

Tax binds to Tak1 and induces Tak1–IKK association

Tax activation of NF- κ B does not require TRAFs or RIP1 (Geleziunas *et al*, 1998; Chu *et al*, 1999), which are immediate upstream regulators of Tak1 and IKK (Chen *et al*, 2006); therefore, it raises the intriguing question of whether Tax binds to Tak1 and mediates the Tak1–IKK association. This question was addressed by using both Tax-transfected and HTLV-I-transformed T cells. In transfected cells, Tax and Tak1 indeed formed a stable complex, which was precipitated by the Tak1 antibody (Fig 3A). It is important to note that this molecular interaction was also readily detected in HTLV-I-transformed T cells (Fig 3E; data not shown). As Tak1 exists in a complex, composed of the adaptor proteins TAB1 and TAB2 (Chen *et al*, 2006), it is unclear whether the Tax–Tak1 interaction is direct or through additional proteins, such as the Tak1-binding proteins (TABs). Our preliminary data showed

Fig 3 | Tax physically interacts with TGF- β -activating kinase 1 and induces the TGF- β -activating kinase 1-I κ B kinase association. (A) 293 cells were transfected with Tax either in the presence (+) or absence (–) of haemagglutinin (HA)-Tak1. The Tak1 protein complex was isolated by immunoprecipitation (IP) using Tak1 followed by immunoblotting (IB) to detect the Tak1-associated Tax (panel 1) and level of precipitated HA-Tak1 (panel 2). IB was also carried out to detect the expression of Tax and HA-Tak1 in cell lysates (panels 3,4). The small amount of Tax precipitated by anti-Tak1 from HA-Tak1-negative cells (panel 1, lane 1) might be attributable to the binding of Tax to endogenous Tak1. (B) 293 cells were transfected with either an empty vector (–) or expression vectors encoding wild-type (WT) Tax or a mutant form of Tax (M22) known to be defective in IKK activation. Endogenous Tak1 was isolated by IP followed by IB to detect the co-precipitated Tax and M22 (panel 1) and the amount of precipitated Tak1 (panel 2). The expression level of Tax in cell lysates was also monitored by IB (panel 3). (C) 293 cells were transfected as in (B). Endogenous Tak1 was isolated by IP and subjected to kinase assays (KA) using MKK6 as substrate (panel 1). IB was carried out to detect the indicated proteins (panels 2–4). (D) JNK1 was isolated by IP from the transfected cells used in (B) and subjected to kinase assays using glutathione S-transferase (GST)-cJun (1–79) as substrate (top panel). The expression levels of JNK1 isoforms and Tax were analysed by IB (lower two panels). (E) Tak1 complex was isolated from either SupT1 or the HTLV-I-transformed SLB-1 cells followed by IB to detect the Tak1-associated IKK γ (panel 1) and Tax (panel 3) and also the precipitated Tak1 (panel 2). IB was also carried out with cell lysates to monitor the indicated proteins (panels 2–4). (F) 293 cells were transfected with Tak1 (+) either in the absence (–) or presence (+) of Tax or Tax M22. IKK complex was isolated by IP using anti-IKK γ followed by IB to detect the co-precipitated Tak1 (panel 1) and IKK γ (panel 2). The expression of transfected proteins was monitored by IB using the cell lysates (panels 3,4). HTLV-I, human T-cell leukaemia virus type 1; IKK, I κ B kinase; JNK1, c-Jun amino-terminal kinase 1; Tak1, TGF- β -activating kinase 1.

that the glutathione S-transferase (GST)-Tax fusion protein was ineffective in pulling down *in vitro*-translated Tak1 from wheat germ extract (data not shown). Although this result indicates the requirement of accessory proteins in Tax-Tak1 binding, other possibilities should also be considered. For example, Tax undergoes phosphorylation in mammalian cells, which is required for its signalling function in NF- κ B activation (Bex *et al*, 1999). It is possible that post-translational modifications—such as phosphorylation—of Tax are required for its association with Tak1. Furthermore, it is also possible that the fusion of Tax to GST might interfere with its binding to Tak1. Nevertheless, our data clearly show that Tax and Tak1 are assembled into a stable complex *in vivo*.

To assess the functional significance of the Tak1-Tax interaction in Tax-mediated activation of Tak1 and IKK, we examined the Tak1-binding activity of a well-characterized Tax mutant, M22, known to be defective in the activation of IKK and NF- κ B (Smith & Greene, 1990; Sun & Ballard, 1999). Surprisingly, M22 was fully competent in binding to Tak1 (Fig 3B). Furthermore, like the wild-type Tax, M22 was able to stimulate the catalytic activity

of Tak1 (Fig 3C) and the downstream kinase JNK (Fig 3D). These results indicate that Tax activation of Tak1 is insufficient for triggering the activity of IKK.

The ability of Tax to bind to both Tak1 (Fig 3A) and IKK (Chu *et al*, 1999; Harhaj & Sun, 1999; Jin *et al*, 1999) suggested the possibility that Tax might promote the association of Tak1 with IKK. This possibility was first investigated by using control and HTLV-I-transformed T cells. Consistent with the requirement of cellular signals for triggering the Tak1-IKK association, Tak1 was not associated with IKK in the HTLV-negative SupT1 cells (Fig 3E, panel 1, lane 1). Remarkably, both IKK α (data not shown) and IKK β were co-precipitated with Tak1 in HTLV-I-transformed SLB-1 cells (Fig 3E, panel 1, lane 2), and also in HUT102 and Tax1 cells (data not shown); Tax was also assembled into this signalling complex (panel 3).

To confirm that Tax stimulates the Tak1-IKK association, we used a transfection model. Transfected Tak1 interacted weakly with endogenous IKK in the absence of Tax (Fig 3F, lane 2). It is important to note that this molecular interaction was strongly promoted by Tax (Fig 3F, lane 3). Conversely, the M22 mutant of

Fig 4 | Tax activation of nuclear factor- κ B does not require the ubiquitin-binding function of I κ B kinase. IKK γ -deficient Jurkat cells (JM4.5.2) were stably transfected with either wild-type (WT) IKK γ or the indicated IKK γ mutants defective in ubiquitin binding. These different cells were infected with retroviruses carrying either the pCLXSN(GFP) vector (–) or pCLXSN(GFP)-Tax. Approximately 30 h after infection, the cells were collected for preparation of nuclear and whole-cell extracts. Nuclear extracts were subjected to electrophoretic mobility shift assay using κ B or NF- γ oligonucleotide probes, and the whole-cell extracts were analysed by IB to detect the indicated proteins. EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; HA, haemagglutinin; IB, immunoblotting; IKK, I κ B kinase.

Tax failed to promote the binding of Tak1 to IKK (Fig 3F, lane 4). This finding explained why M22 was unable to activate IKK (Sun & Ballard, 1999) despite its ability to stimulate the catalytic activity of Tak1 (Fig 3C). As Tax M22 was known to be defective in IKK γ binding (Chu *et al*, 1999; Harhaj & Sun, 1999; Jin *et al*, 1999), it is logical to propose that Tax might function as an adaptor, mediating the Tak1–IKK association through binding to both of these cellular signalling components.

Mode of NF- κ B activation by Tax

Recent studies have shown a ubiquitin-dependent mechanism of IKK activation by immune stimuli (Chen *et al*, 2006). Specifically, the IKK regulatory subunit, IKK γ , has intrinsic ubiquitin-binding activity, which seems to be required for recruiting the IKK complex to upstream signalling molecules, such as RIP1 (Ea *et al*, 2006; Wu *et al*, 2006). Indeed, IKK γ mutants lacking the ubiquitin-binding function are unable to support the activation of NF- κ B when stably expressed in an IKK γ -deficient Jurkat T-cell line (Ea *et al*, 2006; Wu *et al*, 2006). Using the same approach, we examined whether the ubiquitin-binding function of IKK γ is required for Tax-mediated NF- κ B activation.

As we reported previously (Harhaj *et al*, 2000), Tax was unable to induce NF- κ B activation in the IKK γ -deficient Jurkat T cells (Fig 4, lane 2). However, this signalling defect could be rescued by stable expression of exogenous IKK γ (Fig 4, lane 4). Interestingly, despite their inability to mediate NF- κ B activation by TNF- α (Wu *et al*, 2006), the ubiquitin binding-defective IKK γ mutants, D311N and L329P, efficiently mediated Tax-stimulated NF- κ B activation (Fig 4, lanes 6,8). This result further suggests a unique mechanism that mediates the Tax-specific pathway of NF- κ B activation.

In conclusion, the data presented in this study identify a novel pathological mechanism of NF- κ B activation. The retroviral

oncoprotein Tax physically interacts with Tak1 and stimulates its catalytic activity. Consistently, Tak1 is essential for Tax-mediated activation of IKK and NF- κ B. However, it is clear that activation of Tak1 is only part of the mechanism by which Tax activates IKK. Another important part of the mechanism is Tax-mediated recruitment of IKK to Tak1. In keeping with these biochemical results, Tax activation of NF- κ B does not require the ubiquitin-binding function of IKK γ . This novel mechanism provides an explanation for how Tax persistently activates NF- κ B without the requirement of more upstream signalling factors.

METHODS

Cell lines. C8166, HUT102 and SLB-1 are interleukin-2-independent HTLV-I-transformed human T-cell lines, and Tax1 is an interleukin-2-dependent human T-cell clone immortalized by Tax in the context of a herpes saimiri vector. These cell lines have been described previously (Uhlir *et al*, 1998). Human embryonic kidney cell line 293 and an HTLV-I-negative leukaemia T-cell line SupT1 (Smith *et al*, 1984) were from ATCC. MEFs derived from Tak1-deficient (Tak1 $^{-/-}$) and wild-type control (Tak1 $^{+/+}$) mice were kindly provided by Dr Shizuo Akira (Sato *et al*, 2005). IKK γ -deficient Jurkat cells (JM4.5.2) were generated by somatic cell mutagenesis (Harhaj *et al*, 2000). JM4.5.2 cell clones stably transfected with wild-type IKK γ and IKK γ mutants, D311N and L329P, were provided by Dr Jonathan Ashwell (Wu *et al*, 2006).

Plasmid constructs, antibodies and other reagents. pCMV4-based expression vectors encoding Tax and Tax M22 were kindly provided by Dr Warner Greene (Smith & Greene, 1990). The GFP expression vector (pEGFP) was from CLONTECH (Mountain View, CA, USA). pCLXSN(GFP) retroviral vector was generated by replacing the neomycin-resistant gene of pCLXSN (Naviaux *et al*, 1996) with enhanced GFP complementary DNA. The pCLXSN(GFP)-Tax was cloned by inserting Tax cDNA into the *Bam*HI site of the pCLXSN(GFP) vector. The pCMV-HA-Tak1 vector and Tak1 antibody were kindly provided by Drs Kunihiro Matsumoto and Jun Ninomiya-Tsuji (Ninomiya-Tsuji *et al*, 1999). GST-I κ B α (1–54) and GST-c-Jun (1–79) have been described previously (Reiley *et al*, 2004). Recombinant MKK6 was from Upstate (Charlottesville, VA, USA). Horseradish peroxidase-conjugated haemagglutinin (HA) antibody (3F10) was from Roche Molecular Biochemicals (Indianapolis, IN, USA). Tax monoclonal antibody was prepared from a hybridoma (168B17-46-34) provided by the AIDS Research and Reference Program, NIAID, NIH. IKK α (H-744), IKK β (H470), IKK γ (FL-419), actin (C-2), JNK1 (C-17) and tubulin (TU-02) antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA).

Cell transfection and retroviral infection. MEFs and 293 cells were seeded in six-well plates and transfected using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA). Retroviral transduction was carried out using the pCLXSN system provided by Dr Inder Verma (Naviaux *et al*, 1996). The procedure for retrovirus production and infection was as described previously (Babu *et al*, 2006). The bulk of infected cells were used in experiments to prevent clonal variation.

Immunoblotting, immunoprecipitation and *in vitro* kinase assays. Cells were lysed in a buffer containing 20 mM HEPES (pH 7.6), 250 mM NaCl, 0.5% NP-40, 20 mM β -glycerophosphate, 1 mM EDTA, 20 mM *p*-nitrophenylphosphate, 0.1 mM Na₃VO₄, 1 mM dithiothreitol and 1 mM phenylmethylsulphonyl

fluoride. The protein complexes were isolated by immunoprecipitation and subjected to either immunoblotting or *in vitro* kinase assays essentially as described previously (Uhlík *et al*, 1998).

Electrophoresis mobility shift assay. Nuclear extracts were prepared and subjected to EMSA using a ³²P-radiolabelled κ B probe (5'-CAA CGG CAG GGG AAT TCC CCT CTC CTT-3') or a control probe containing the NF- κ B-binding site (5'-AAG AGA TTA ACC AAT CAC GTA CGG TCT-3') followed by resolving the DNA-protein complexes on native 5% polyacrylamide gels.

Reporter gene assays. 293 cells were seeded into 24-well plates and transfected using Lipofectamine-2000 (Invitrogen), with the indicated cDNA expression vectors together with 100 ng of κ B-luciferase reporter. For normalizing the transfection efficiency, the cells were also transfected with a control *Renilla* luciferase reporter driven by the constitutive thymidine kinase promoter (40 ng). At 36 h after transfection, the cells were collected for dual luciferase assays (Promega, Madison, WI, USA). The κ B-specific luciferase activity was normalized on the basis of the *Renilla* luciferase activity.

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