

# Tumour-necrosis-factor-receptor-associated factor 6, NF- $\kappa$ B-inducing kinase and I $\kappa$ B kinases mediate IgE isotype switching in response to CD40

Kevin BRADY<sup>1</sup>, Stephen FITZGERALD<sup>1</sup> and Paul N. MOYNAGH<sup>2</sup>

Department of Pharmacology, University College Dublin, Foster Avenue, Blackrock, Co. Dublin, Ireland

The process of IgE switching requires the prior transcription of the unrearranged C $\epsilon$  gene, which leads to its recombination with the VDJ region. The activation of NF- $\kappa$ B by CD40 is a key process in facilitating this transcription by promoting the activation of the C $\epsilon$  promoter. The present study explores the uncharacterized signalling pathways employed by CD40 in activating NF- $\kappa$ B by the overexpression of genes encoding wild-type and dominant-negative forms of the signalling components tumour-necrosis-factor-receptor-associated factor 6 (TRAF-6), NF- $\kappa$ B-inducing kinase (NIK), I $\kappa$ B kinase (IKK)-1 and IKK-2 in the BJAB B-cell line. The overexpression of TRAF-6 or NIK was sufficient to activate NF- $\kappa$ B and the C $\epsilon$  promoter, whereas their dominant-negative counterparts decreased the ability of

CD40 to activate NF- $\kappa$ B and the C $\epsilon$  promoter. The overexpression of wild-type IKK-1 or IKK-2 seemed to cause toxic effects on the cells, whereas the dominant-negative forms were selective in their blockade of NF- $\kappa$ B and the C $\epsilon$  promoter. These results suggest that CD40 employs TRAF-6, which presumably recruits NIK, which in turn employs IKK-1/IKK-2 to activate NF- $\kappa$ B and the C $\epsilon$  promoter, the prologue to IgE switching. Thus the findings define a crucially important pathway in the generation of allergic states.

**Key words:** allergy, B-cells, kinases, nuclear factor  $\kappa$ B, signal transduction.

## INTRODUCTION

Antibody isotype switching in B-cells is characterized by a deletional recombination event in which the VDJ gene segment is joined to a different heavy chain C region (C<sub>H</sub>) gene [1]. This switch recombination results in the production of an antibody with a different heavy-chain isotype but with the original antigen specificity. Because the C<sub>H</sub> region dictates the functional capabilities of an antibody, isotype switching is crucial in exploiting the various effector functions of the immune system. The process of switch recombination occurs between switch (S) regions located upstream of each C<sub>H</sub> gene, except C $\mu$ . However, the transcription of a particular germline (unrearranged) C<sub>H</sub> gene is a prerequisite for switch recombination to that specific C<sub>H</sub> gene [2–5]. Such transcription yields a sterile germline transcript starting at the I exon, upstream of the S region, and continuing through the S region and C<sub>H</sub> gene [6]. The S region is removed by splicing, resulting in the joining of the I exon and the C<sub>H</sub> region. Because the germline transcripts are key components in the targeting of switch recombination, much interest has focused on the mechanisms controlling their transcription. The latter is controlled primarily via the modulation of the activity of the proximal promoters in the C<sub>H</sub> genes by transcription factors. In isotype switching to IgE, which is central to the generation of allergic states, the induction of germline transcripts from the unrearranged C $\epsilon$  gene is facilitated primarily by the concerted binding of the transcription factors STAT-6 and NF- $\kappa$ B to their cognate binding sites in the C $\epsilon$  promoter [7–9]. The activities of these two transcription factors are regulated by two extracellular signals [8]. Interleukin 4 (IL-4), which is secreted by T lymphocytes, activates STAT-6 via a well-characterized signal transduction pathway [10–13]. The activation of NF- $\kappa$ B is achieved by

the cognate recognition of the B-cell membrane protein CD40 and the T-cell membrane CD40 ligand (CD40L) [8,14]. The central role of CD40L–CD40 interaction in B-cell isotype switching was initially demonstrated in hyper-IgM syndrome patients who have a mutated CD40L gene, and also in mice having a targeted disruption of the CD40L or CD40 gene. Hyper-IgM syndrome patients [15] and mice lacking CD40L [16] or CD40 [17] do not undergo isotype switching. CD40 thus emerges as a central component in regulating IgE production; the resolution of its signal transduction pathway promises to provide a valuable mode for controlling IgE synthesis.

The activation of NF- $\kappa$ B is a key step in mediating IgE switching in response to CD40 because it promotes the induction of germline transcripts from the unrearranged C $\epsilon$  gene, the prerequisite to IgE switching. The importance of NF- $\kappa$ B is emphasized by a study showing that NF- $\kappa$ B knock-out mice have defects in IgE class switching [18]. Latent NF- $\kappa$ B is the predominant form in most cell types and it is rendered inactive in the cytoplasm via its association with the I $\kappa$ B inhibitory proteins [19]. The activation of NF- $\kappa$ B necessitates the dissociation of I $\kappa$ B from NF- $\kappa$ B via serine phosphorylation [20] and subsequent proteasomal [21] proteolysis of the I $\kappa$ B inhibitory subunit, thus releasing an active NF- $\kappa$ B heterodimer that is translocated into the nucleus [22,23]. The signal transduction pathway used by CD40 in effecting the phosphorylation of the I $\kappa$ B forms in B-cells is currently unclear. However, valuable clues might be provided by the IL-1 and tumour necrosis factor (TNF) signal transduction pathways because both of these cytokines activate NF- $\kappa$ B; the signalling components used by both in activating NF- $\kappa$ B have recently been delineated. The pathways initially recruit specific members of the TNF-receptor-associated factor (TRAF) family. The TNF pathway employs TRAF-2 [24], whereas TRAF-6 is

Abbreviations used: CD40L, CD40 ligand; EGFP, enhanced green fluorescent protein; FCS, fetal calf serum; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IKK, I $\kappa$ B kinase; IL-4, interleukin 4; NIK, NF- $\kappa$ B-inducing kinase; RT-PCR, reverse-transcriptase-mediated PCR; S, switch; TNF, tumour necrosis factor; TRAF, TNF-receptor-associated factor.

<sup>1</sup> These authors contributed equally to the work.

<sup>2</sup> To whom correspondence should be addressed (e-mail pmoyagh@macollamh.ucd.ie).

recruited by the IL-1 receptor complex [25]. Both TRAF-2 and TRAF-6 subsequently interact with and activate the NF- $\kappa$ B-inducing kinase (NIK) [26,27]. The activated NIK in turn can activate the two forms of I $\kappa$ B kinase (IKK), IKK- $\alpha$ /IKK-1 and IKK- $\beta$ /IKK-2, which phosphorylate I $\kappa$ B, permitting the translocation of active NF- $\kappa$ B into the nucleus [27–32]. The role of these various signalling components in mediating the activation of NF- $\kappa$ B by CD40 is currently unknown. It is known that the cytoplasmic domain of CD40 can associate with various members of the TRAF family, including TRAF-2 and TRAF-6 [33–35]. In addition, reports have described a role for TRAF-2 in mediating the activation of NF- $\kappa$ B by CD40 [24,36,37], but recent findings have suggested that CD40 might activate NF- $\kappa$ B by a mechanism independent of TRAF-2 [38]. TRAF-6 has recently gained prominence as a crucial mediator of the activation of NF- $\kappa$ B by CD40 [39] in mice that were deficient in TRAF-6, being defective in CD40 signalling [40]. However, whereas TRAF-6 is a key early signalling component in CD40 signalling, the identities of the signalling components downstream of TRAF-6 remain to be defined. The present work was therefore aimed at mapping the CD40 signalling pathway from TRAF-6 to activation of NF- $\kappa$ B in B-cells, with a view to exploring the contribution of the components of this pathway to the induction of germline transcripts from the unrearranged *C $\epsilon$*  gene, the prologue to IgE switching. The findings confirm the crucial role of TRAF-6 in mediating the activation of NF- $\kappa$ B by CD40 in B-cells, but also define the involvement of the downstream signalling kinases, NIK, IKK-1 and IKK-2 in the activation process. In addition, the pathway formed from these components is also demonstrated to be crucial in mediating the activation of the *C $\epsilon$*  promoter by CD40, which leads to the induction of germline *C $\epsilon$*  transcripts and ultimately to IgE production. This study thus resolves a CD40 signal transduction pathway that is critical for IgE switching and might be of great value in identifying novel targets to exploit in our attempts to design innovative regimes to counter unwanted allergic responses.

## MATERIALS AND METHODS

### Materials

RPMI 1640 medium, fetal calf serum (FCS) and Superscript II reverse transcriptase were from Life Technologies (Paisley, Renfrewshire, U.K.). Glutamine, streptomycin and HAT medium (100  $\mu$ M hypoxanthine/0.4  $\mu$ M aminopterin/16  $\mu$ M thymidine) were from Sigma (Poole, Dorset, U.K.). *Taq* DNA polymerase, dNTPs, luciferase substrate and lysis buffer were from Promega Corp. (Madison, WI, U.S.A.). RNA Isolator was from Genosys Biotechnologies (Cambridge, U.K.). The FLAG (FLAG being Asp-Tyr-Lys-Asp-Asp-Asp-Lys) Western detection kit was from Stratagene (La Jolla, CA, U.S.A.). The anti-NIK antibody was from Calbiochem-Novabiochem Corp. (San Diego, CA, U.S.A.). The enhanced chemiluminescence development reagent was from New England Biolabs (Beverly, MA, U.S.A.). The Epstein-Barr-virus-negative human B-cell lymphoma cell line (BJAB) was a gift from Dr D. Walls (Dublin City University, Dublin, Ireland). CD32-transfected fibroblasts (A.T.C.C. no. CRL-10680) and the anti-CD40 were gifts from Professor C. Borrebaeck (Department of Immunotechnology, Lund University, Lund, Sweden). The  $\epsilon$ -luciferase reporter plasmid ( $\epsilon$ -luc) consisted of the germline *C $\epsilon$*  promoter (the region –162 to +53 relative to the first initiation site for germline  $\epsilon$ -transcripts) ligated upstream of the luciferase reporter gene and was a gift from Professor J. Stavnezer (University of Massachusetts Medical School, Worcester, MA, U.S.A.). The NF- $\kappa$ B-luciferase reporter plasmid ( $\kappa$ B-luc) consisted of five copies of the

NF- $\kappa$ B consensus site cloned into the luciferase reporter construct pGL3-Basic (Promega Corp.). pEGFP-N1 (in which EGFP stands for enhanced green fluorescent protein) was from Clontech Laboratories (Palo Alto, CA, U.S.A.). The expression vectors encoding TRAF6-Flag, dominant-negative mutant TRAF6 (289–522)-Flag, NIK, dominant-negative mutant ( $\Delta$ ) NIK(KK-AA), IKK1-Flag, dominant-negative mutant IKK1 (K44A)-Flag, IKK2-Flag and dominant-negative mutant IKK2 (K44A)-Flag were obtained from Tularik (San Francisco, CA, U.S.A.).

### Cell culture

Human BJAB cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin. CD32-transfected fibroblasts (A.T.C.C. no. CRL-10680) were cultured in RPMI 1640 supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and HAT medium supplement. Both cell types were grown at 37 °C in a humidified air/CO<sub>2</sub> (19:1) atmosphere.

### Transfection of BJAB cells and assay of expression of luciferase reporter genes

Aliquots (250  $\mu$ l) of BJAB cells ( $4 \times 10^7$  cells/ml) [in RPMI 1640 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin] were mixed on ice with 15  $\mu$ g of the designated expression vectors, 5  $\mu$ g of pEGFP-N1 and 5  $\mu$ g of  $\kappa$ B-luc or  $\epsilon$ -luc and electroporated (220 V, 960  $\mu$ F) by means of a Bio-Rad Gene Pulser equipped with a capacitance extender (Bio-Rad, Hercules, CA, U.S.A.). The electroporated cells were subsequently added to fresh medium (5 ml) and allowed to recover at 37 °C for 24 h in a humidified air/CO<sub>2</sub> (19:1) atmosphere. Aliquots (2.5 ml) of recovered cells were incubated in the absence or presence of anti-CD40 monoclonal antibody (500 ng/ml) and recombinant human IL-4 (100 i.u./ml) for 5 min at room temperature. Cells were subsequently mixed with aliquots (750  $\mu$ l;  $4 \times 10^5$  cells/ml) of CD32-transfected fibroblasts. Aliquots (250  $\mu$ l) of the mixed BJAB/fibroblast suspension were then seeded into 96-well plates and incubated at 37 °C for a further 24 h. Cell extracts were then generated and measured for luciferase activity by using a luciferase assay system with reporter lysis buffer from Promega Corporation.

### Quantification of transfection efficiency

BJAB cells were electroporated as above and allowed to recover at 37 °C for 48 h in a humidified air/CO<sub>2</sub> (19:1) atmosphere. The cells were then centrifuged at 1450 *g* for 5 min, washed once in PBS (5 ml) and finally resuspended in PBS (10<sup>6</sup> cells/ml). Transfection efficiency was measured by counting the number of cells expressing the EGFP by using a FACStar Plus Flow Cytometer (Becton Dickinson, Aalst, Belgium) with laser excitation at 488 nm by using a 530 nm bandpass filter to collect the green fluorescence.

### Western immunoblotting of transfected cells

BJAB cells were electroporated as above and allowed to recover at 37 °C for 24 h in a humidified air/CO<sub>2</sub> (19:1) atmosphere. Aliquots of cells (1 ml;  $2 \times 10^6$ ) were then centrifuged at 1450 *g* for 5 min, washed once in PBS (1 ml) and finally lysed in SDS/PAGE sample buffer (100  $\mu$ l) [50 mM Tris/HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v)

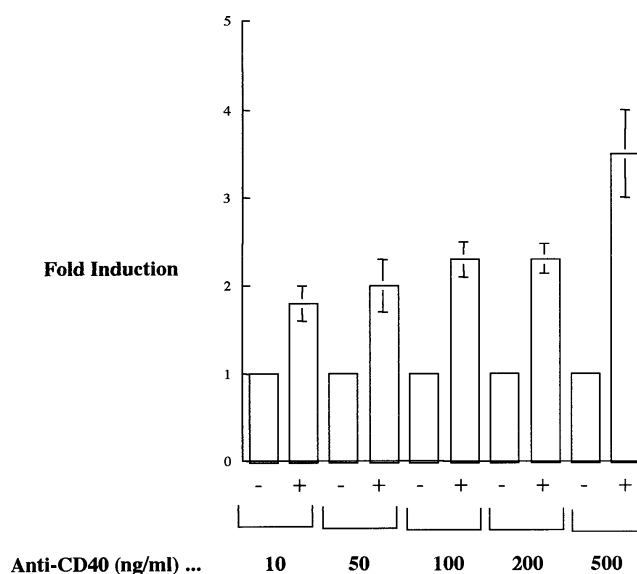
2-mercaptoethanol and 0.1% (w/v) Bromophenol Blue]. The lysates were sonicated for 10 s to decrease sample viscosity and then heated to 90 °C for 5 min. Lysates were cleared of particulate material by centrifugation at 20000 *g* for 5 min. Aliquots (25  $\mu$ l) of the clear supernatants were subjected to SDS/PAGE [6% (w/v) gel] and the separated proteins were transferred electrophoretically from the gels to nitrocellulose. The nitrocellulose was then probed for FLAG expression by using the alkaline phosphatase-based FLAG Western detection kit in accordance with the manufacturer's instructions. The expression of NIK was detected by probing the nitrocellulose with a rabbit polyclonal anti-NIK antibody (1:350 dilution); detection was with an enhanced chemiluminescence system, used in accordance with the manufacturer's instructions.

### RNA extraction and reverse transcriptase-mediated PCR (RT-PCR)

Aliquots (2.5 ml) of untransfected BJAB cells ( $4 \times 10^6$  cells/ml) were stimulated in the absence or presence of anti-CD40 monoclonal antibody and IL-4, as described above. Total cellular RNA was prepared by using the RNA Isolator in accordance with the manufacturer's instructions. First-strand cDNA synthesis was performed with Superscript II reverse transcriptase and random oligonucleotide primers; PCR amplification was performed with *Taq* DNA polymerase and primers to amplify selectively regions of the *C $\epsilon$*  and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA species. The sequences of the forward and reverse oligonucleotide primers and the sizes of the products were as follows: *C $\epsilon$* , 5'-CGTCTCGGGTGC-GTGGGC-3', 5'-CAGGTGATCGTGGGCGACT-3', 500 bp; GAPDH, 5'-ACCACAGTCCATGCCATC-3', 5'-TCCACCA-CCCTGTTGCTG-3', 452 bp. The forward and reverse *C $\epsilon$*  primers were designed to hybridize within the first and third exons of the *C $\epsilon$*  region [41] respectively. Amplification parameters were as follows: step 1, 30 cycles at 94 °C for 1 min, at 53 °C for 1 min and at 72 °C for 1 min; step 2, at 72 °C for 10 min. The PCR products were subjected to electrophoresis on a 1.5% (w/v) agarose gel containing ethidium bromide, then photographed.

### RESULTS AND DISCUSSION

To probe the signalling components that are employed by CD40 in its activation of NF- $\kappa$ B and induction of germline *C $\epsilon$*  transcripts in B-cells, the Epstein-Barr-virus-negative human B-cell lymphoma cell line BJAB was used as a model for naive B-cells. This cell line has been characterized as a valuable model system for exploring the regulation of expression of germline *C $\epsilon$*  transcripts [42]. The process of IgE switching requires the dual signals of IL-4 and activation of CD40; this was achieved by treating the BJAB cells with exogenous human recombinant IL-4 and an agonistic anti-CD40 antibody adsorbed on CD32-transfected fibroblasts. The latter has been shown to be an effective means of cross-linking and activating CD40 [43]. The efficacy of the anti-CD40 antibody in the present model system was initially examined by assessing its capacity, in conjunction with IL-4, to activate the promoter controlling the expression of the *C $\epsilon$*  germline transcripts. The activity of the *C $\epsilon$*  promoter was determined by measuring the expression of a transfected luciferase gene ligated downstream of the *C $\epsilon$*  promoter ( *$\epsilon$ -luc*) [8]. BJAB cells, in the absence of stimulation by IL-4 and CD40, but in the presence of various amounts of isotype antibody control for the anti-CD40 antibody, displayed basal expression of luciferase; any induction above this level is depicted as fold induction. The anti-CD40 antibody caused a dose-dependent activation of the *C $\epsilon$*  promoter as demonstrated by an induction

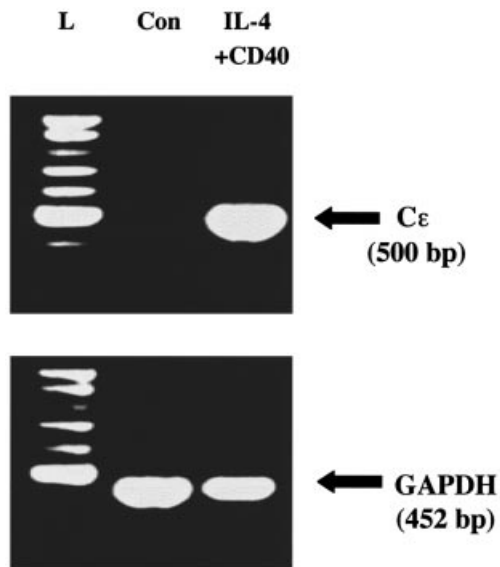


**Figure 1** Dose-dependent activation of the *C $\epsilon$*  promoter by agonistic anti-CD40 antibody in BJAB B-cells

BJAB cells ( $10^7$ ) were electroporated with  *$\epsilon$ -luc* (5  $\mu$ g), a plasmid encoding a luciferase reporter gene regulated by the *C $\epsilon$*  promoter. Electroporated cells were subsequently incubated for 24 h in the absence (–) and the presence (+) of IL-4 (100 units/ml) and various concentrations of anti-CD40 antibody (500 ng/ml) adsorbed on CD32-transfected fibroblasts. Unstimulated control cells (–) were treated with equivalent concentrations of isotype control antibody. Cell extracts were measured for luciferase activity and the results are expressed as fold induction of luciferase relative to cells that were incubated in the absence of IL-4 and anti-CD40 antibody. Results are means  $\pm$  S.E.M. for four independent experiments.

of  *$\epsilon$ -luc* (Figure 1). Maximal induction was apparent with the highest anti-CD40 dose (500 ng/ml). It was not practically feasible to assess the effect of higher concentrations of the antibody, so 500 ng/ml was employed for all further experiments. Attempts were then made to assess the ability of the combined treatment of IL-4 and anti-CD40 to induce IgE in BJAB cells. The two agents failed to induce detectable levels of IgE, as measured by a sandwich-based ELISA system for human IgE with a sensitivity limit in the pg/ml range (results not shown). However, the more sensitive approach of RT-PCR was used to show that the combined treatment of IL-4 and anti-CD40 induced the expression of germline *C $\epsilon$*  transcripts (Figure 2). The transcripts were assayed by RT-PCR by using primers to amplify the region within the first and third exons of the *C $\epsilon$*  gene [41]. The splicing of the intervening introns forms germline transcripts with a predicted size of 500 bp when amplified with the described RT-PCR protocol. The stimulation of BJAB cells with IL-4 and anti-CD40 generated increased levels of this product (Figure 2). As a control for the RT-PCR assay, primers were also used to amplify a 452 bp region of the housekeeping GAPDH gene. Both samples showed comparable levels of the resulting PCR product, thus confirming that the effects of IL-4 and anti-CD40 are selective for induction of the *C $\epsilon$*  transcripts. This confirms a previous finding [42] and importantly validates the BJAB cell line as a suitable paradigm for probing the early stages of IgE switching.

The identity of the signalling components that mediate the activation of NF- $\kappa$ B by CD40 in BJAB cells was initially explored. The activity of NF- $\kappa$ B was determined by measuring the induction of a transfected NF- $\kappa$ B-regulated luciferase gene ( *$\kappa$ B-luc*); the potential role of signalling molecules was assessed by

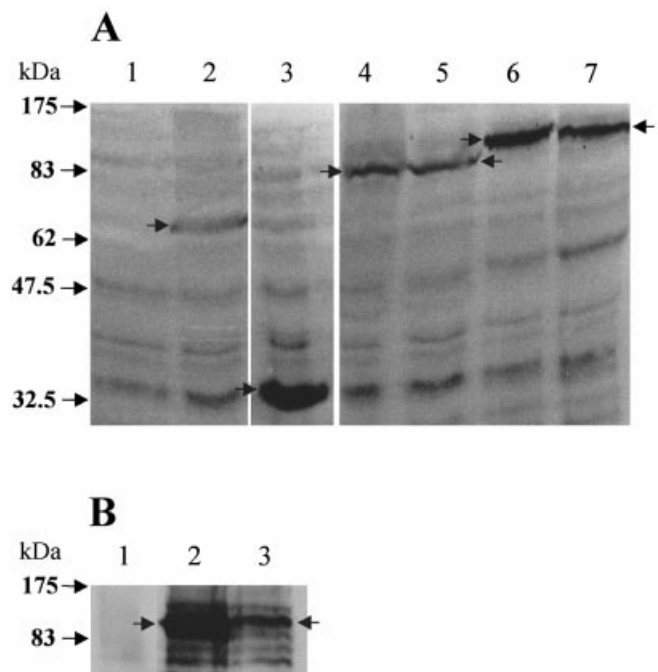


**Figure 2** Induction of  $C\epsilon$  germline transcripts by IL-4 and engagement of CD40 in BJAB B-cells

BJAB cells ( $4 \times 10^6$ ) were stimulated in the absence (Con) and presence of IL-4 (100 units/ml) and anti-CD40 antibody (500 ng/ml), after which total cellular RNA was prepared and subjected to first-strand cDNA synthesis with Superscript II reverse transcriptase and random oligonucleotide primers. PCR amplification was performed with *Taq* DNA polymerase and primers to selectively amplify regions of the  $C\epsilon$  and GAPDH cDNA species. This resulted in the generation of products with predicted sizes of 500 and 452 bp respectively. A base pair ladder (L) was used to calculate the sizes of the products.

determining the effects of co-transfected wild-type or dominant-negative forms of TRAF-6, NIK, IKK-1 and IKK-2 on the expression of  $\kappa$ B-luc. However, before exploring the functional consequences of expressing wild-type and dominant-negative forms of these components, it was first necessary to confirm the overexpression of the various signalling components. This was achieved by a Western immunoblotting analysis of extracts from transfected cell populations (Figure 3). The wild-type and dominant-negative forms of TRAF-6, IKK-1 and IKK-2 had been tagged with a FLAG epitope, permitting their detection with an anti-FLAG detection system, whereas the expression of the two forms of NIK, which lacked the FLAG epitope tag, was detected with an anti-NIK antibody. This analysis confirmed the overexpression of both forms of the four signalling components. Each component displayed an electrophoretic mobility consistent with its predicted molecular mass. The dominant-negative form of TRAF-6 is considerably smaller than its wild-type counterpart because the former was generated by the deletion of a region at the N-terminal domain of wild-type TRAF-6. The dominant-negative forms of NIK, IKK-1 and IKK-2 were derived by point mutations; their electrophoretic mobilities are therefore comparable with their wild-type equivalents. The demonstrable expression of the various signalling molecules facilitated an exploration of their potential roles in CD40 signalling in B-cells.

The role of TRAF-6 in mediating the activation of NF- $\kappa$ B by CD40 in B-cells was initially examined (Figure 4). The overexpression of wild-type TRAF-6 caused the induction of  $\kappa$ B-luc even in the absence of stimulation by CD40. This indicates that activation of TRAF-6, which is achieved by overexpression, is a sufficient stimulus to activate NF- $\kappa$ B in B-cells. This has previously been observed in other cell types [39]. The activation by TRAF-6 seems to represent a maximum level, because CD40 did

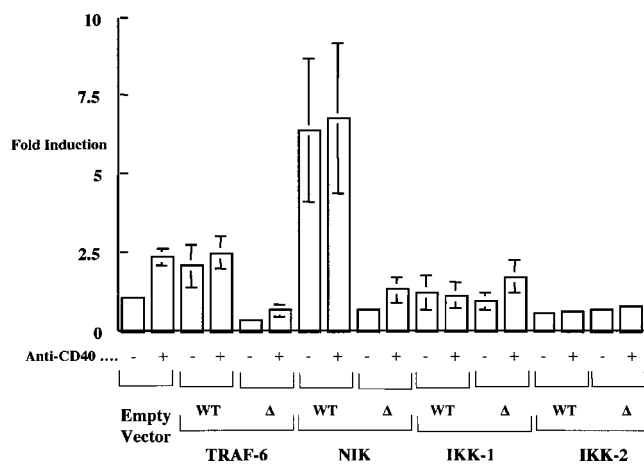


**Figure 3** Overexpression of wild-type and dominant-negative forms of NF- $\kappa$ B signalling components in BJAB B-cells

BJAB cells ( $10^7$ ) were electroporated with expression vectors (15  $\mu$ g of DNA) encoding wild-type and dominant-negative forms of TRAF-6, NIK, IKK-1 and IKK-2 and allowed to recover overnight at 37 °C. Whole cell lysates were subsequently prepared in SDS/PAGE sample buffer, subjected to SDS/PAGE [4% (w/v) gel] and electroblotted to nitrocellulose membrane. (A) The wild-type and dominant-negative forms of TRAF-6 (lanes 2 and 3 respectively), IKK-1 (lanes 4 and 5 respectively) and IKK-2 (lanes 6 and 7 respectively) were FLAG-tagged and were detected with an anti-FLAG antibody in conjunction with an alkaline-phosphatase-based detection system. Lane 1 contained an extract from cells that had been transfected with the empty expression vector. (B) Both wild-type and dominant-negative forms of NIK (lanes 2 and 3 respectively) were probed with a rabbit polyclonal anti-NIK antibody and were detected by enhanced chemiluminescence. Lane 1 contained an extract from cells that had been transfected with the empty expression vector. The arrows within the gels indicate the electrophoretic mobilities of the various proteins. The positions of molecular mass markers are indicated on the left.

not significantly enhance the expression of  $\kappa$ B-luc above this level. The overexpression of the dominant-negative form of TRAF-6 caused a marked inhibition of basal and CD40-induced expression of  $\kappa$ B-luc, confirming reports [39,40] of the crucial role of TRAF-6 in mediating the activation of NF- $\kappa$ B by CD40. This finding again justifies our experimental system as a suitable model for exploring CD40 signalling in B-cells.

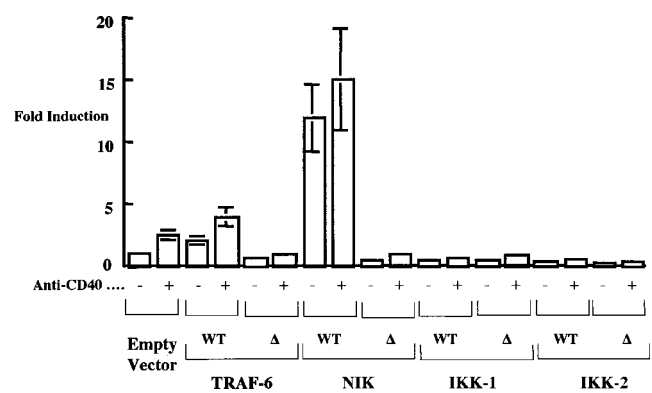
The role of lead candidate signalling molecules likely to act downstream of TRAF-6 was investigated next. The overexpression of wild-type NIK, like that of TRAF-6, was sufficient to activate NF- $\kappa$ B, even in the absence of CD40, as demonstrated by an increased expression of  $\kappa$ B-luc. Indeed, NIK is considerably more efficacious than TRAF-6 in its activation of NF- $\kappa$ B, promoting it as a key regulator of NF- $\kappa$ B in B-cells. The greater efficacy of NIK relative to TRAF-6 might suggest an additional TRAF-6-independent mechanism for activating NIK. However, the possibility cannot be excluded that the lower efficacy of TRAF-6 is due to limiting endogenous levels of NIK. The expression of the dominant-negative form of NIK blocked the ability of CD40 to induce  $\kappa$ B-luc. These findings thus indicate that NIK is a necessary and sufficient mediator in the activation of NF- $\kappa$ B by CD40. This suggests that, like the IL-1 receptor system [25], CD40 triggers the activation of NF- $\kappa$ B by recruiting



**Figure 4** Effect of wild-type and dominant-negative forms of NF- $\kappa$ B signalling components on the activation of NF- $\kappa$ B in BJAB cells

BJAB cells ( $10^7$ ) were co-transfected by electroporation with an expression vector ( $15 \mu\text{g}$  of DNA) encoding one of the wild-type (WT) or dominant-negative ( $\Delta$ ) forms of the various NF- $\kappa$ B signalling components, together with a plasmid encoding constitutively expressed EGFP ( $5 \mu\text{g}$  of DNA) and  $\kappa$ B-luc ( $5 \mu\text{g}$ ), a plasmid encoding a luciferase reporter gene regulated by a promoter containing five copies of the NF- $\kappa$ B consensus site. Electroporated cells were subsequently incubated for 24 h in the absence and the presence of IL-4 (100 units/ml) and anti-CD40 antibody (500 ng/ml) adsorbed on CD32-transfected fibroblasts. Unstimulated cells were treated with isotype control antibody (500 ng/ml). Cell extracts were measured for luciferase activity and the results are expressed as fold induction of luciferase relative to unstimulated cells that had been transfected, as above, with an empty expression vector. Results are means  $\pm$  S.E.M. for at least three independent experiments.

TRAF-6, which in turn activates NIK. Because NIK has previously been shown to activate IKK-1 and IKK-2, the role of the latter two kinases in CD40 signalling was examined. The dominant-negative forms of IKK-1 and IKK-2 caused a significant inhibition of basal expression and induction of  $\kappa$ B-luc by CD40. This demonstrates their key roles in the CD40 signalling pathway, which effects the activation of NF- $\kappa$ B in B-cells. Paradoxically, the wild-type forms of IKK-1 and IKK-2 also caused an apparent inhibition of basal and induced expression of  $\kappa$ B-luc. However, such inhibition might result from toxic effects of the overexpressed IKK-1 and IKK-2 on the BJAB cells. Clues from the latter emerge from an assessment of the effects of the various signalling components on the expression of a constitutively expressed reporter gene, namely EGFP. Such an experiment was originally designed to control for the effects of the different expression vectors on transfection efficiency. Cells transfected with an empty expression vector exhibited an average transfection efficiency of approx. 20%. This efficiency was comparable for all other transfections involving the various expression vectors, with the exception of those encoding the wild-type forms of IKK-1 and IKK-2. The transfection efficiency was decreased to 12% and 7% in the latter two cases respectively. The nature of such a decrease is not currently apparent but might be due to toxic effects on the BJAB cells. The amounts of wild-type IKK-1 and IKK-2 expression vectors in the transfection process were decreased to eliminate the effects on transfection efficiency. These lower amounts failed to affect the activity of NF- $\kappa$ B (results not shown), confirming that the inhibitory effects of wild-type IKK-1 and IKK-2 on NF- $\kappa$ B are related to their non-specific effects on transfection efficiency. Irrespective of the mechanism, the effects of the IKKs on transfection efficiency are dependent on their kinase activity because their catalytically inactive dominant-negative forms fail to affect the transfection



**Figure 5** Effect of wild-type and dominant-negative forms of NF- $\kappa$ B signalling components on the activation of C $\epsilon$  promoter in BJAB cells

BJAB cells ( $10^7$ ) were co-transfected by electroporation with an expression vector ( $15 \mu\text{g}$  of DNA) encoding one of the wild-type (WT) or dominant-negative ( $\Delta$ ) forms of the various NF- $\kappa$ B signalling components, together with a plasmid encoding constitutively expressed green fluorescent protein ( $5 \mu\text{g}$  of DNA) and with  $\epsilon$ -luc ( $5 \mu\text{g}$ ), a plasmid encoding a luciferase reporter gene regulated by the C $\epsilon$  promoter. Electroporated cells were subsequently incubated for 24 h in the absence or the presence of IL-4 (100 units/ml) and anti-CD40 antibody (500 ng/ml) adsorbed on CD32-transfected fibroblasts. Unstimulated cells were treated with isotype control antibody (500 ng/ml). Cell extracts were measured for luciferase activity and the results are expressed as fold induction of luciferase relative to unstimulated cells that had been transfected, as above, with an empty expression vector. Results are means  $\pm$  S.E.M. for at least three independent experiments.

efficiency. However, the role of NF- $\kappa$ B, which is presumably activated by IKK-1 and IKK-2, in mediating the inhibitory effects is questionable because the wild-type forms of TRAF-6 and NIK, which also activate NF- $\kappa$ B, fail to influence transfection efficiency. Thus the basis of the non-selective inhibitory effects of IKK-1 and IKK-2 on transfection efficiency awaits resolution. However, the lack of influence of the other overexpressed signalling components confirms their selective effects on the expression of  $\kappa$ B-luc. These studies indicate that CD40 activates NF- $\kappa$ B in B-cells via a TRAF-6–NIK–IKK-1/IKK-2 pathway in B-cells. The functional consequence of activating this pathway in B-cells was investigated next.

The proposed key role of NF- $\kappa$ B in promoting IgE switching by inducing C $\epsilon$  germline transcripts prompted an assessment of the involvement of the above signalling components in the induction of germline transcripts. This was performed with the wild-type and dominant-negative forms as above but by measuring their effects on the expression of  $\epsilon$ -luc (Figure 5). The results mirrored closely the findings from the equivalent studies with  $\kappa$ B-luc in that the dominant-negative forms of TRAF-6, NIK, IKK-1 and IKK-2 decreased the ability of CD40 to induce the expression of  $\epsilon$ -luc. This demonstrates the key role for the TRAF-6–NIK–IKK-1/IKK-2 pathway in mediating the induction of C $\epsilon$  germline transcripts. The wild-type forms of IKK-1 and IKK-2 again demonstrated unexpected inhibitory effects on the expression of  $\epsilon$ -luc but this was related to their inhibitory effects on transfection efficiency.

In summary, these findings identify a number of key components with crucial roles in the CD40 signalling pathway in B-cells. The stimulation of CD40 in B-cells leads to the engagement of the sequential TRAF-6–NIK–IKK-1/IKK-2 pathway, resulting in the activation of NF- $\kappa$ B. The latter, in conjunction with an IL-4 co-stimulus that activates STAT-6, induces the expression of C $\epsilon$  germline transcripts. Because this process is a crucial requirement for IgE switching, the present work identifies a

number of key targets that might be exploited in the development of novel anti-allergy therapies.

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