CD40 Employs p38 MAP Kinase in IgE Isotype Switching

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IgE switching requires the prior induction of Cε germline transcripts which is mediated by the concerted binding of STAT-6 and NFκB to the Cε promoter. These transcription factors are regulated by IL-4 and CD40, respectively. However the latter can effect other signaling pathways and the present study explores the role of p38 MAPK in induction of Cε germline transcripts. CD40 and IL-4, both alone and in synergy, were initially shown to activate the Cε promoter in a B cell lymphoma cell line. Under the same conditions CD40 caused activation of p38 MAPK, whereas IL-4 was ineffective. The p38 MAPK inhibitor, SB203580, and a dominant negative form of p38 MAPK decreased the CD40 activation of the Cε promoter by reducing the ability of CD40 to increase the transactivation potential of NFκB. This study suggests that p38 MAPK is crucially important in mediating CD40 activation of NFκB which acts to induce Cε germline transcripts, ultimately facilitating IgE switching. © 2001 Academic Press

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IgE is a central player in the generation of allergic diseases and thus the molecular mechanisms underlying IgE synthesis have become an area of intense interest (reviewed in 1). In order for a B lymphocyte to produce IgE, it needs to undergo a process known as isotype switching. This phenomenon involves the recombination of the VDJ gene region to a different heavy chain C (C_H) gene which will thus encode for another heavy chain isotype with different effector functions but with the same antigen-binding specificity (2). Such recombination takes place between the 5′ intronic segment, known as the switch (S) region, of the C_μ gene and the corresponding S region of the appropriate C_H gene (3). In the case of switching to IgE, recombination results in the formation of a Sμ/Sε junction, with deletion of the intervening DNA. However a necessary pre-requisite for this deletional recombination is the prior transcription of the Cε gene which starts at the Iε exon, upstream of the Sε region, and continues through the S region and Cε gene (4). The S region is removed by splicing, resulting in the joining of the Iε exon and Cε region which together form a germline sterile transcript (5). The induction of these Cε germline transcripts is a determining step in isotype switching to IgE since transcription through the Cε gene segment makes that region accessible to switch recombination (6). The induction of Cε germline transcripts is regulated by a promoter region, upstream of the Iε exon, and it is activated by the concerted binding of the transcription factors STAT-6 and NFκB (7–9). IL-4 regulates the activation of STAT-6 and the importance of this pathway in IgE synthesis is emphasised by the lack of IgE switching in mice deficient for STAT-6 (10). The engagement of the B cell transmembrane protein CD40 by its cognate ligand is sufficient to cause activation of NF-κB (8, 11) and this synergises with STAT-6 to activate the Cε promoter (8, 9). The crucial contribution of CD40 activation of NF-κB to IgE synthesis is reinforced by the observation that CD40 ligand-deficient patients (12), CD40 deficient mice (13) and NF-κB deficient mice (14) are all defective in IgE isotype switching and lack serum IgE. However the activation of NF-κB may not be the sole contributory pathway to mediating IgE switching in response to CD40. The engagement of the latter has also been shown to activate a plethora of intracellular signaling molecules including protein tyrosine kinases and PI-3 kinase (15). In addition much recent interest has focussed on the ability of CD40 to activate the mitogen-activated protein kinases (MAPKs), especially the c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (16, 17) and the p38 MAPK (18, 19). While the functional consequences of CD40 activation of these MAPKs remain to be fully resolved a recent report has demonstrated a role for p38 in mediating

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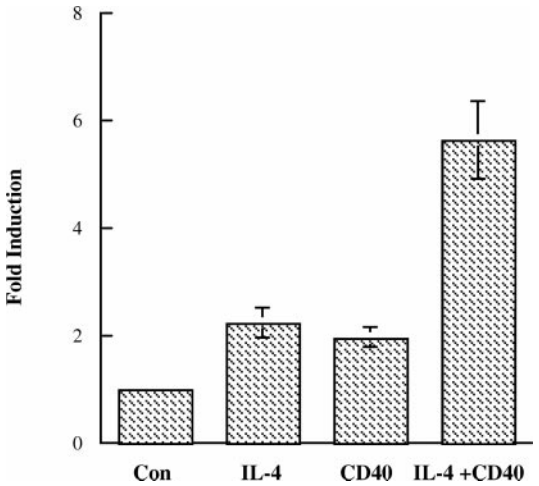

FIG. 1. Activation of Cϵ promoter by IL-4 and engagement of CD40 in BJAB B cells. BJAB cells (1×10^7 cells) were electroporated with ϵ-luc (10 μg), a plasmid encoding a luciferase reporter gene regulated by the Cϵ promoter. Electroporated cells were subsequently treated for 24 h in the absence (Con) and presence of IL-4 (100 U/ml) and/or an anti-CD40 antibody (500 ng/ml) adsorbed onto CD32-transfected fibroblasts. Cell extracts were measured for luciferase activity and the results are expressed as fold induction of luciferase relative to cells which were treated in the absence of IL-1 and anti-CD40 antibody. The data represent mean +/− SEM of four independent experiments.

the CD40-induced proliferation of B cells and its induction of genes such as ICAM-1 (20). Interestingly, the same report also described a lack of involvement of p38 in mediating the CD40 induction of a wide range of other proteins including CD40 itself, CD95/Fas, cIAP2, TRAF1, TRAF4, and DR3 and thus concluded that CD40 induces gene expression via both p38 MAPK-dependent and independent pathways. This prompted the present investigation into whether p38 is employed by CD40 in mediating one of its most important biological effects in B cells, namely its induction of Cϵ germline transcripts which acts as the key determining step in IgE isotype switching.

The study exploited the specific cell-permeable p38 MAPK inhibitor SB203580 (21) and a dominant negative form of p38 MAPK to show that p38 is crucially involved in mediating the CD40 activation of the Cϵ promoter which regulates induction of Cϵ germline transcripts. The results also suggest that p38 contributes to the activation of the promoter by increasing the transactivation potential of NF-κB. The study thus concludes that p38 MAPK is a crucial player in IgE isotype switching and may represent a valuable target in controlling unwanted IgE synthesis in allergic reactions.

MATERIALS AND METHODS

Materials. RPMI 1640 and FCS were from Life Technologies, Inc. (Paisley, UK). Glutamine, streptomycin, and HAT medium (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) were from Sigma (Poole, UK). IL-4 was from R & D (Oxon, UK). Luciferase substrate and lysis buffer were from Promega Corp. (Madison, WI). The PhosphoPlus p38 MAP Kinase antibody kit was from New England Biolabs (Beverly, MA). SB203580 was from Calbiochem-Novabiochem Ltd. (Nottingham, UK). The EBV negative human B cell lymphoma cell line was a gift from Dr. D. Walls, Dublin City University, Ireland. CD32-transfected fibroblasts (ATCC No. CRL-10680) and the anti-CD40 antibody are previously described (22). The ϵ-luciferase reporter plasmid (ϵ-luc) consists of the germline C_e promoter (the region −162/+53 relative to the first initiation site for germline ϵ-transcripts) ligated upstream of the luciferase reporter gene and was a gift from Professor J. Stavnezer (University of Massachusetts Medical School, MA). The NFκB-luciferase reporter plasmid (κB-luc) consists of five copies of the NFκB consensus site cloned into the luciferase reporter construct pGL3-Basic (Promega Corporation). The plasmid encoding dominant negative p38 MAPK was a gift from Professor R. J. Davis (University of Massachusetts Medical Center, MA) and has been described previously (23). PEGFP-N1 (in which EGFP stands for enhanced green fluorescent protein) was from Clontech Laboratories (Palo Alto, CA).

Cell culture. Human BJAB cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. CD32-transfected fibroblasts (ATCC No. CRL-10680) were cultured in RPMI 1640 supplemented

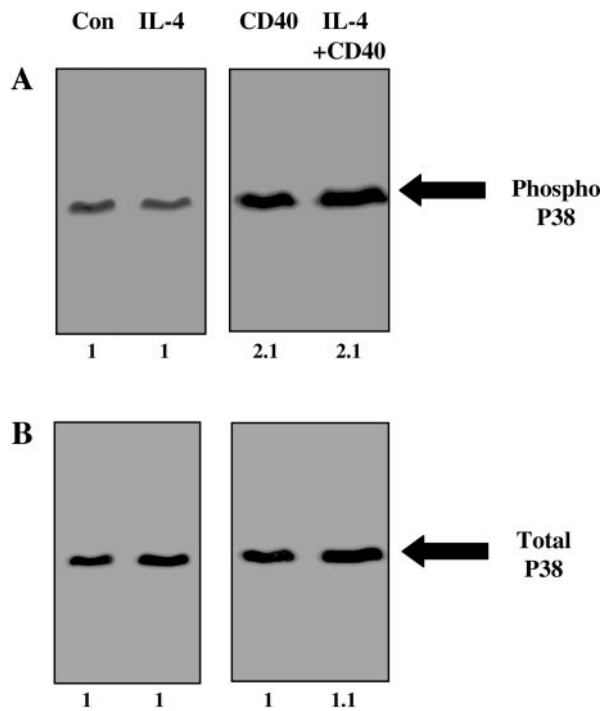

FIG. 2. Engagement of CD40 activates p38 MAPK in BJAB cells. BJAB cells (1×10^7 cells) were treated for 30 min in the absence and presence of IL-4 (100 U/ml) and/or an anti-CD40 antibody (500 ng/ml) adsorbed onto CD32-transfected fibroblasts. Cell lysates were prepared and analysed by Western immunoblotting for levels of (A) phosphorylated p38 MAPK and (B) total p38 MAPK using the PhosphoPlus p38 MAP kinase antibody kit (New England Biolabs). Immunoreactivity was visualised by enhanced chemiluminesence and band intensity quantitated by densitometric scanning on a Molecular Dynamics 300S computing densitometer (Sunnyvale, CA) using ImageQuant V3.0 software. The numbers under each lane represent the band intensity relative to the intensity of the band in cells which had been treated in the absence of IL-4 and anti-CD40.

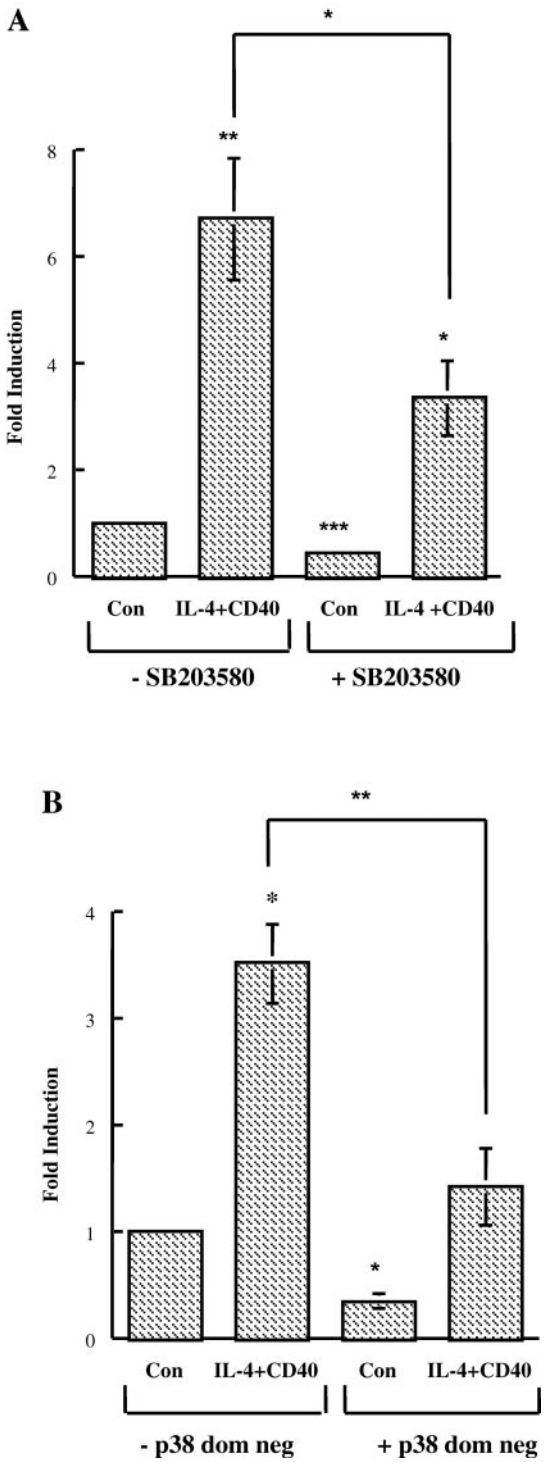

FIG. 3. SB203580 and dominant negative p38 MAPK inhibit CD40 activation of Cϵ promoter. (A) BJAB cells (1×10^7 cells) were electroporated with ϵ-luc (10 μg), a plasmid encoding a luciferase reporter gene regulated by the Cϵ promoter. Electroporated cells were treated with and without SB203580 (10 μM) for 1 h and subsequently stimulated for 24 h in the absence (Con) and presence (IL-4+CD40) of IL-4 (100 U/ml) and anti-CD40 antibody (500 ng/ml). (B) BJAB cells (1×10^7 cells) were cotransfected by electroporation with an empty expression vector (15 μg of DNA) or the same amount of expression vector encoding dominant negative p38 MAPK, together with a pEGFP-N1 plasmid encoding constitutively expressed EGFP (5 μg of DNA) and ϵ-luc (10 μg). Electroporated cells were subsequently incubated for 24 h as above in the absence and presence of IL-4 and anti-CD40. All cell extracts were measured for luciferase activity and the results are expressed as fold induction of luciferase relative to control cells which were treated in the absence of IL-4 and anti-CD40 antibody. The data represent mean +/− SEM of four independent experiments. Significantly different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) when compared to control values.

with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and HAT medium supplement. Both cell types were grown in a humidified atmosphere of 5% CO_2 at 37°C.

Transfection of BJAB cells and assay of expression of luciferase reporter genes. Aliquots (250 μl) of BJAB cells (4×10^7 cells/ml) were electroporated (220 V, 960 μF) with ϵ-luc (10 μg) or κB-luc (5 μg) in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin by means of a Bio-Rad Gene Pulser equipped with a capacitance extender (Bio-Rad, Hercules, CA). In some experiments BJAB cells were cotransfected with 15 μg of the expression vector encoding dominant negative p38 MAPK, 5 μg of pEGFP-N1 and ϵ-luc (10 μg) or κB-luc (5 μg). The electroporated cells were subsequently added to fresh medium (5 ml) and allowed to recover for 24 h in a humidified atmosphere of 5% CO_2 at 37°C. Aliquots (2.5 ml) of recovered cells were incubated in the absence or presence of SB203580 (10 μM) for 1 h prior to stimulation with or without anti-CD40 monoclonal antibody (500 ng/ml) and recombinant human IL-4 (100 U/ml) for 5 min at room temperature. Cells were subsequently mixed with aliquots (750 μl; 4×10^5 cells/ml) of CD32-transfected fibroblasts and aliquots (250 μl) of the mixed BJAB/fibroblast suspension then seeded into 96-well plates and incubated at 37°C for a further 24 h. Cells extracts were then generated and measured for luciferase activity using the Luciferase Assay System with Reporter Lysis buffer from Promega Corporation. Transfection efficiency was measured by counting the number of cells expressing the EGFP fluorescent protein using flow cytometry as described previously (24).

Western blot analysis of p38 phosphorylation. Aliquots (2.5 ml) of untransfected BJAB cells (4×10^6 cells/ml) were stimulated for 30 min in the absence or presence of anti-CD40 monoclonal antibody and IL-4, as described above. Stimulation was terminated by centrifuging the cells at 1450*g* for 5 min and washing the cell pellet once in PBS (1 ml). The cells were then lysed in SDS–PAGE sample buffer (100 μl) (50 mM Tris–HCl buffer, pH 6.8 containing 2% (*w/v*) SDS, 10% glycerol, 1% (*v/v*) 2-mercaptoethanol and 0.1% bromophenol blue). The lysates were sonicated for 10 s to reduce sample viscosity and then heated to 90°C for 5 min. Lysates were cleared of particulate material by centrifugation at 20,000*g* for 5 min. Aliqouts (25 μl) of the clear supernatants were subjected to SDS–PAGE on 12% polyacrylamide slab gels and the separated proteins electrophoretically transferred from the gels to nitrocellulose. The nitrocellulose was then probed for levels of phosphorylated and total p38 using the PhosphoPlus p38 MAP Kinase (Thr180/Tyr182) antibody kit as recommended by the manufacturers (New England Biolabs). Immunoreactivity was visualised by enhanced chemiluminesence. Densitometric scanning of enhanced chemiluminescence blots was performed on a Molecular Dynamics 300S computing densitometer (Sunnyvale, CA) using ImageQuant V3.0 software.

Statistical analysis. Significance was evaluated by Student's *t* test for unpaired data.

RESULTS AND DISCUSSION

The overall objective of this study was to explore the potential role of p38 in IgE isotype switching in B

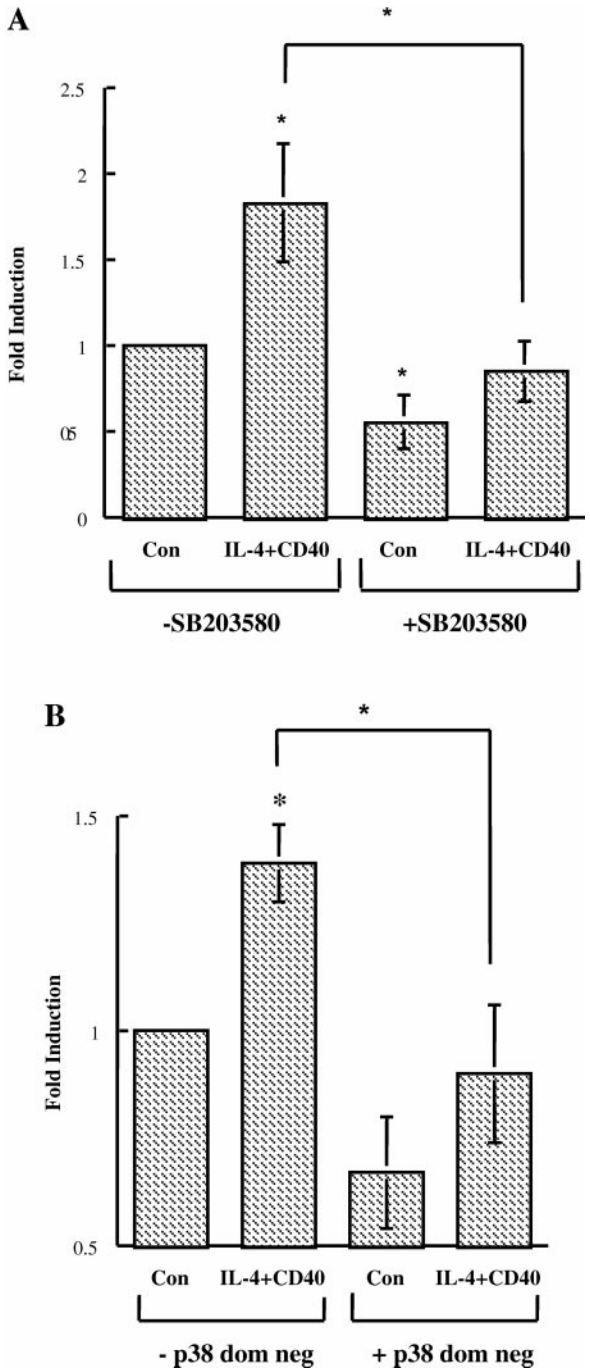

FIG. 4. SB203580 and dominant negative p38 MAPK inhibit CD40 induction of NFκB-regulated reporter gene. (A) BJAB cells (1×10^7 cells) were electroporated with κB-luc (5 μg), a plasmid encoding a luciferase reporter gene regulated by a promoter containing five copies of the NFκB consensus site. Electroporated cells were treated with and without SB203580 (10 μM) for 1 h and subsequently stimulated for 24 h in the absence (Con) and presence (IL-4+CD40) of IL-4 (100 U/ml) and anti-CD40 antibody (500 ng/ml). (B) BJAB cells (1×10^7 cells) were cotransfected by electroporation with an empty expression vector (15 μg of DNA) or the same amount of expression vector encoding dominant negative p38 MAPK, together with a pEGFP-N1 plasmid encoding constitutively expressed EGFP (5 μg of DNA) and κB-luc (5 μg). Electroporated cells were subsequently incubated for 24 h as above in the absence and presence of IL-4 and anti-CD40. All cell extracts were measured for luciferase activity and the results are expressed as fold induction of luciferase relative to control cells which were treated in the absence of IL-4 and anti-CD40 antibody. The data represent mean +/− SEM of four independent experiments. Significantly different (*$P < 0.05$) when compared to control values.

lymphocytes. Since the induction of Cϵ germline transcripts is the key step in initiating IgE switching this prompted an investigation into the importance of p38 in regulating this induction process. However, it was necessary to initially establish a suitable model system for investigation. The EBV negative B cell lymphoma BJAB cell line was used as the model human B cell system. The process of IgE switching requires the dual signals of IL-4 and activation of CD40 and this was achieved by treating the BJAB cells with exogenous human recombinant IL-4 and an agonistic anti-CD40 antibody adsorbed onto CD32-trasfected fibroblasts. The latter has been shown to be an effective means of cross-linking and activating CD40 (22). BJAB cells were initially examined for their responsiveness to stimulation by IL-4 and CD40 with respect to activation of the promoter controlling expression of the Cϵ germline transcripts. The activity of the Cϵ promoter was determined by measuring the expression of a transfected luciferase gene ligated downstream of the Cϵ promoter (ϵ-luc) (8). BJAB cells, in the absence of stimulation by IL-4 and CD40, displayed basal expression of luciferase and any induction above this level is displayed as fold induction. BJAB cells showed some basal activation of the Cϵ promoter but ϵ-luc expression was increased approximately twofold by stimulating the cells alone with either IL-4 or CD40 (Fig. 1). The combination of both stimuli caused a synergistic sixfold induction of luciferase. Such synergistic activation of the Cϵ promoter by IL-4 and CD40 is consistent with a previous report (8) and is explained by the concerted binding of STAT-6 and NF-κB to the Cϵ promoter (7–9). These findings further validate a previous report which promoted the BJAB cell line as a valuable model system for exploring the regulation of expression of germline Cϵ transcripts (25). Thus BJAB cells and the ϵ-luc reporter system were employed as a suitable paradigm to explore the role of p38 in mediating the induction of Cϵ germline transcripts in B cells. However prior to such an exploration it was necessary to show that CD40 could activate p38 in BJAB cells.

BJAB cells were stimulated in the presence and absence of IL-4 and agonistic anti-CD40 antibody and subsequently examined for activation of the p38 pathway. This was judged by probing the cells for immunoreactivity with an antibody which detects doubly phosphorylated threonine 180 and tyrosine 182 of p38 MAPK. Western immunoblotting demonstrated that cells in the absence of stimulation by IL-4 and CD40,

contained a band representing a protein of 38 kDa which reacted with the antibody indicative of some basal levels of phosphorylated p38 MAPK in BJAB cells (Fig. 2A). The engagement of CD40 alone was sufficient to at least double the intensity of the band thus demonstrating that CD40 can activate the p38 MAPK pathway in BJAB cells. The addition of IL-4 failed to affect the degree of basal or CD40 induced phosphorylation of p38 indicating that it does not contribute to the activation of the p38 MAPK pathway in B cells. As control for the assay, all samples showed equivalent levels of total p38 as judged by Western immunoblotting analysis using control antibodies which recognise p38 irrespective of its phosphorylation status (Fig. 2B). The ability of CD40 engagement to activate the p38 MAPK pathway in B cells confirms similar previous findings (18–20). The lack of effect of IL-4 on p38 is not surprising since its primary role is to activate the transcription factor STAT-6 via the unrelated JAK-3 kinase (26).

The demonstration that CD40 activates p38 in BJAB cells allowed for the exploration of the contribution of this pathway to mediating CD40 induction of Cϵ germline transcripts. This was achieved by initially evaluating the inhibitory effects of SB203580, the p38 MAPK inhibitor, on the activity of the Cϵ promoter, as judged by expression of the ϵ-luc reporter gene (Fig. 3A). SB203580 reduced the basal expression of ϵ-luc and inhibited by over 50% the ability of CD40 to induce ϵ-luc. This indicates a key role for p38 in mediating the CD40 induction of Cϵ germline transcripts, the prologue to IgE switching. This was further confirmed by probing the effect of cotransfected dominant negative p38 MAPK on the activity of the Cϵ promoter (Fig. 3B). Like SB203580, dominant negative p38 MAPK markedly reduced the basal and CD40-induced expression of ϵ-luc, confirming the crucial role of p38 MAPK in mediating the activation of the Cϵ promoter by CD40. Dominant negative p38 MAPK had no effect on transfection efficiency since cells transfected with dominant negative p38 and control cells transfected with an empty expression vector displayed comparable transfection efficiencies of 12%. The clear role of p38 in mediating the CD40 activation of the Cϵ promoter prompted an investigation into the underlying mechanism. The unequivocal importance of NFκB in the CD40 signaling pathway leading to IgE swithing (8, 9, 11, 14) coupled to recent reports describing a role for p38 in regulating the transactivation potential of NFκB (20, 27) warranted an analysis of the involvement of p38 in modulating the activity of NFκB in the present experimental system.

The activity of NFκB in the BJAB cells was determined by measuring the induction of a transfected NFκB-regulated luciferase gene (κB-luc) (Fig. 4). The stimulation of CD40 in BJAB cells was sufficient to induce an approximate twofold induction of luciferase. The pretreatment of cells with SB203580 dramatically reduced the ability of CD40 to induce κB-luc indicating an important role for p38 in mediating the CD40 activation of NFκB (Fig. 4A). This was further confirmed by the equally impressive inhibitory effects of cotransfected dominant negative p38 MAPK on the induction of κB-luc by CD40 (Fig. 4B). Since SB203580 fails to affect the ability of CD40 to effect nuclear translocation and DNA-binding of NFκB in BJAB cells (data not shown) these findings suggest that the CD40 activation of p38 facilitates increased NFκB activity by improving its transactivation potential. This confirms a recent report which proposes a similar mechanism by which CD40 activates NFκB and thus induces B cell proliferation (20).

In summary, the present study thus proposes that CD40 activates p38 MAPK in B cells which in turn increases the ability of nuclear translocated NFκB to activate the Cϵ promoter and induce Cϵ germline transcripts. This ultimately promotes IgE isotype switching and thus p38 is a major player in facilitating IgE synthesis. Such findings promote p38 as a novel target to exploit in the development of new therapeutic regimes to counter the production of IgE in allergy-based diseases.

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