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Cross-linking CD40 on B cells preferentially induces stress-activated protein kinases rather than mitogen-activated protein kinases

Ingolf Berberich[1,2,3], Geraldine Shu[1], Friederike Siebelt[4], James R.Woodgett[5], John M.Kyriakis[6] and Edward A.Clark[1,4]

[1]Department of Microbiology, SC-42 and [4]Regional Primate Research Center, University of Washington Medical Center, Seattle, WA 98195, USA, [5]The Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario M4X 1K9, Canada and [6]Diabetes Research Laboratory, Massachusetts General Hospital East, Charlestown, MA 02129, USA

[2]Present address: Institut für Virologie und Immunbiologie, Universität Würzburg, Versbacher Strasse 7, 97078 Würzburg, Germany

[3]Corresponding author

The B cell-associated surface molecule CD40 plays a key role in T cell-dependent B cell maturation, as individuals with defects in either CD40 or its ligand are impaired in immunoglobulin isotype class switching and germinal center formation. CD40 signaling activates downstream effectors, including the tyrosine protein kinase, Lyn, the phosphatidylinositol-3-kinase (PI-3 kinase), and the transcription factor, NF-κB. In this study, we demonstrate that stress-activated protein kinases (SAPK) are activated after CD40 cross-linking on various B cell lines or human tonsillar B cells. The activation is rapid and transient and is mediated through a cyclosporin A-insensitive pathway. Furthermore, this signaling pathway appears not to rely on protein kinase C. While CD40 ligation strongly activates the SAPKs (up to 25-fold), it does not affect members of the mitogen-activated protein kinase family (MAPK; ERK1 and ERK2). Consistent with these data, CD40 signals up-regulate c-*jun* but not c-*fos* mRNA and alter the transcription factor ATF2 but not the Raf-1 protein. In summary, CD40 signaling preferentially induces SAPK but not MAPK.

Keywords: B cells/CD40/JNK/MAPK/SAPK

Introduction

CD40 is a 45–50 kDa transmembrane glycoprotein originally identified in B cells, some B cell malignancies and carcinoma cell lines (Paulie *et al.*, 1985; Clark and Ledbetter, 1986). In addition to B cells, CD40 has also been detected in other cell types, including follicular dendritic cells (Schriever *et al.*, 1989), dendritic cells (Hart and McKenzie, 1988), activated macrophages (Alderson *et al.*, 1993) and thymic epithelial cells (Galy and Spits, 1992). It is a member of the tumor necrosis factor (TNF) receptor superfamily (Smith *et al.*, 1994). The ligand for CD40 (CD40L, also known as gp39, T-BAM or TRAP) is expressed on activated $CD4^+$ T cells (Armitage *et al.*, 1992). Contact-dependent signals mediated through CD40/CD40L stimulate B cell survival, growth and differentiation (Noelle *et al.*, 1992; Tsubata *et al.*, 1993; Klaus *et al.*, 1994b; Parry *et al.*, 1994). The key role of CD40 in the humoral immune response is evident from patients with X-linked hyper-IgM syndrome (HIM), where the cooperation between B and T cells has been disturbed due to a defective CD40L gene (Hill and Chapel, 1993). These patients do not form germinal centers and do not switch from producing IgM to other Ig classes. A similar phenotype has been found in mice with targeted disruption of either the CD40 (Kawabe *et al.*, 1994) or the CD40L (Xu *et al.*, 1994) gene. CD40 plays a critical role in activation, proliferation and differentiation of B cells, including immediate early gene expression, cell adhesion, interleukin (IL)-6 secretion, B cell proliferation and survival, and Ig isotype production. In addition, cross-linking CD40 promotes the survival of germinal center B cells and, in combination with IL-4, supports long-term B cell proliferation (see Banchereau *et al.*, 1994; Clark and Ledbetter, 1994; Klaus *et al.*, 1994a). It may also function to regulate dendritic cells and dendritic cell-dependent HIV-1 expression (Pinchuk *et al.*, 1994).

New protein tyrosine phosphorylation is induced very early after CD40 stimulation (Uckun *et al.*, 1991; Knox and Gordon, 1993; Faris *et al.*, 1994; Ren *et al.*, 1994) and is accompanied by an increase in Lyn protein tyrosine kinase activity and phosphatidylinositol-3-kinase (PI-3 kinase) activity (Ren *et al.*, 1994). We and others have found that CD40 ligation induces NF-κB (Berberich *et al.*, 1994; Francis *et al.*, 1995). Recently, protein(s) that interact directly with the CD40 cytoplasmic tail (called CD40bp, CRAF1 and LAP1) have been described by several investigators (Hu *et al.*, 1994; Cheng *et al.*, 1995; Mosialos *et al.*, 1995). Although the exact function of CD40 receptor-associated factor, CRAF1, is not known, an essential role for this protein in CD40 signaling has been demonstrated in a B cell line expressing a truncated version of CRAF1. These cells no longer up-regulate CD23, a low affinity Fc receptor for IgE, after signaling through CD40 (Cheng *et al.*, 1995). Whether this binding protein relays all signals to account for all phenotypic changes remains to be established.

Stress-activated protein kinases (SAPK; also known as JNK for c-Jun NH_2-terminal kinase), form a group of serine–threonine protein kinases (see Davis, 1994) related to the mitogen-activated protein kinases (MAPK; ERK1 and ERK2). Different isoforms of these kinases—with molecular weights of 46 (p46) or 54 kDa (p54)—have been cloned recently (Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994; Sluss *et al.*, 1994). In this study, we refer to the p46 SAPKs as SAPKs. Like ERK1 and ERK2, they are activated through phosphorylation of both a threonine and tyrosine residue in a highly conserved tripeptide motif (Dérijard *et al.*, 1994). Environmental stress, TNF-α, IL-1 and certain growth factors [i.e. epidermal growth factor

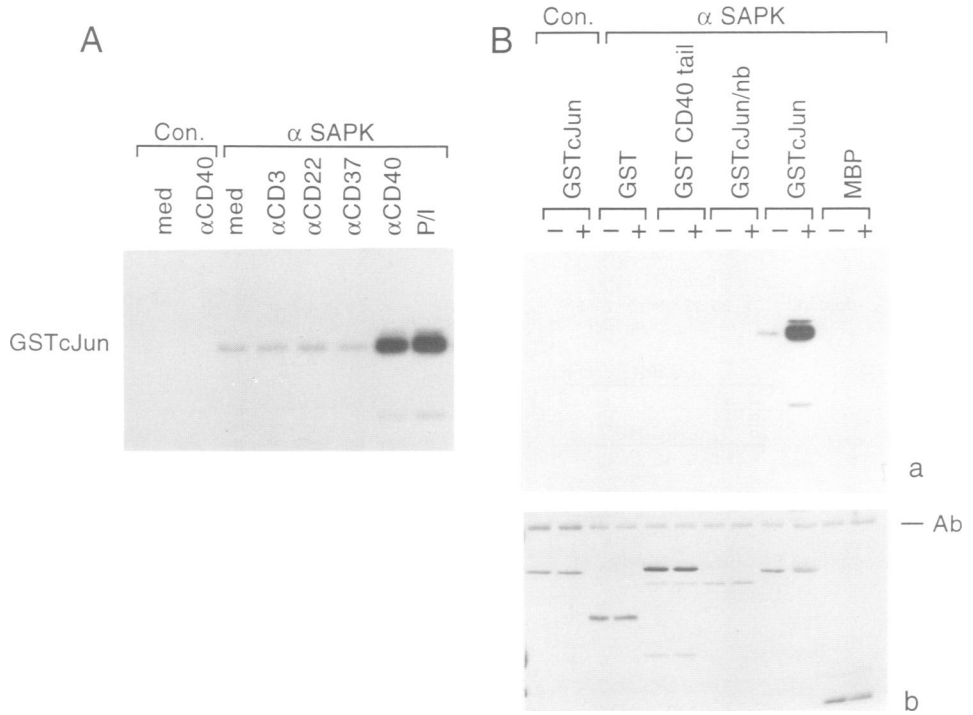

Fig. 1. Cross-linking CD40 activates SAPK. (**A**) Daudi cells were stimulated for 15 min as indicated with anti-CD40 mAb (0.5 μg/ml) or PMA (P, 50 ng/ml) and ionomycin (I, 0.25 μg/ml). SAPK were precipitated from the cell lysates and tested for activity in an immune complex *in vitro* kinase assay using GST–c-Jun (2 μg) as substrate. In control (con.) lanes, a control antiserum (anti-rac2) was used in the precipitation step. (**B**) Daudi cells were treated with either anti-CD3 (–) or anti-CD40 (+) mAb (0.5 μg/ml) for 15 min. (a) The substrate specificity of the precipitated SAPK was tested using GST fusion proteins or MBP as indicated in an immune complex kinase assay. (b) The Coomassie Blue staining of the gel. Ab, precipitating antibodies.

(EGF) and nerve growth factor (NGF)] are potent activators of SAPKs (Hibi *et al.*, 1993; Bird *et al.*, 1994; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994; Minden *et al.*, 1994b). Activation of SAPK probably involves Ras-dependent and Ras-independent pathways (Minden *et al.*, 1994a). MEKK (Yan *et al.*, 1994), a MAPK kinase kinase, seems to be a good candidate for transmitting the activation signal to SEK1, an immediate upstream regulator of SAPK (Sanchez *et al.*, 1994). The regulation of SAPK and MAPK can be both linked and executed separately. While EGF activates both the SAPKs and MAPKs (Minden *et al.*, 1994a), TNF-α and UV radiation target SAPKs much more efficiently (Kyriakis *et al.*, 1994; Minden *et al.*, 1994a). The transcription factors c-Jun and ATF2 are the best characterized substrates for SAPK to date. In both cases, two serines located proximal to the major transactivation domain become phosphorylated in response to SAPK agonists (Hibi *et al.*, 1993; Gupta *et al.*, 1995).

In the study reported here, we investigated whether CD40 signaling can modulate SAPK and/or MAPK activities. Our results indicate that cross-linking CD40 on B cells strongly activates SAPK but changes MAPK activity only marginally, if at all.

Results

Cross-linking CD40 on B cells activates SAPK

Initially, the effect of cross-linking CD40 on SAPK (p46, JNK1) activity was examined in the human B cell lymphoma line, Daudi. The activity of the SAPK after various stimuli was evaluated in immune complex kinase assays using a GST–c-Jun (amino acids 5–89) fusion protein as substrate. As shown in Figure 1A, cross-linking CD40 strongly activated SAPK, whereas treating the Daudi cells with several isotype-matched control antibodies—either binding (anti-CD22 and anti-CD37) or non-binding (anti-CD3)—did not influence the activity of SAPK compared with the medium control. Next, we tested the substrate specificity of the anti-SAPK precipitates. Whereas GST–c-Jun containing both the phosphorylation sites (Ser63 and Ser73) and the δ domain as binding domain for SAPK was phosphorylated, none of the other substrates tested was phosphorylated by the anti-SAPK precipitates (Figure 1B). Most importantly, a GST–c-Jun fusion protein containing both phosphorylation sites but lacking the δ domain (GST–c-Jun/nb), previously shown to be necessary for SAPK-dependent c-Jun phosphorylation (Adler *et al.*, 1992; Hibi *et al.*, 1993; Dérijard *et al.*, 1994), and myelin basic protein (MBP; a substrate for ERK1 and ERK2) could not serve as substrates for SAPK precipitates in immune complex kinase assays (Figure 1B).

Next, we determined the kinetics of SAPK activation after CD40 cross-linking on Daudi cells. SAPK activity was increased 10- to 20-fold compared with unstimulated cells within 5 min after the onset of signaling through CD40, reached its maximum (≥20 fold) after 10–15 min, and declined steadily thereafter (Figure 2A–C). Similar activation of SAPK was seen when soluble CD40L was used to ligate CD40 on Daudi cells (data not shown). The kinetics of activation were independent of the kinase assay used, i.e. *in vitro* immune complex kinase assays and *in vitro* in-gel kinase assays gave comparable results

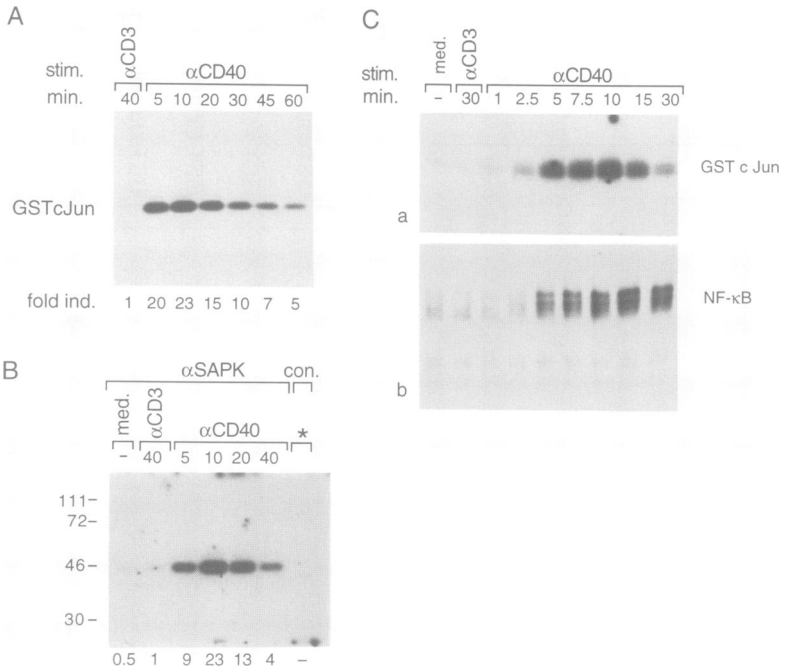

Fig. 2. Cross-linking CD40 transiently activates SAPK in Daudi cells. (**A**) Daudi cells were treated with medium or anti-CD3 or anti-CD40 mAb (0.5 μg/ml). The activities of the SAPKs were determined in an immune complex kinase assay using GST–c-Jun (2 μg) as substrate. Activation (fold ind.) of the SAPK in anti-CD40-stimulated versus control cells was evaluated by a PhosphorImager. (**B**) Daudi cells were stimulated as in (A). SAPKs were precipitated from the lysates and tested for their activity in an in-gel kinase assay using GST–c-Jun as substrate (40 μg/ml). In the control lane (con.), 1/7 of each of the other lysates was combined, immunoprecipitated with anti-rac2 antisera (*) as control antisera and tested in parallel with the other samples. Fold induction was assessed as in (A). Molecular weight standards are indicated in kDa. (**C**) Daudi cells were stimulated as in (A). SAPKs were precipitated from the cytoplasmic extracts and tested for their activity in immune complex kinase assays as in (A) (panel a). The nuclear extracts of the same cells were tested for induction of NF-κB binding activity in an electrophoretic mobility shift assay using the NF-κB binding site found in the promoter of invariant chain of MHC class II (Pessara *et al.*, 1990) (panel b).

(Figure 2A and B). The results of the in-gel kinase assays further support the specificity of the antiserum used because: (i) the molecular weight detected for SAPK correlates well with that published for SAPK (46 kDa) (Hibi *et al.*, 1993; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994); (ii) only one dominant band emerged in the autoradiograph (Figure 2B); and (iii) an anti-rac2 antiserum used as a control gave no signal (Figure 2B). While anti-CD40 strongly activated p46 SAPK (JNK1) in Daudi cells, it only weakly and transiently activated p54 SAPK (JNK2) (data not shown). Recently, we and others have shown that CD40 ligation activates the NF-κB system (Berberich *et al.*, 1994; Francis *et al.*, 1995). Since it is conceivable that SAPK is involved in the induction of these transcription factors, we also analyzed the onset of SAPK relative to the activation of NF-κB after CD40 ligation. SAPK slightly preceded the induction of NF-κB binding activity in Daudi cells (Figure 2C).

We next tested whether SAPK activation after CD40 cross-linking could be generalized to other B cell sources. Cross-linking CD40 was also sufficient to induce SAPK activity in the murine B cell lymphoma M12 (Figure 3A) and the murine immature B cell, WEHI 231 (Figure 3B). As in Daudi cells, the activation of the p54 SAPK (JNK2) isoform was weak and very transient (data not shown). In addition, in a variant of M12 stably transfected with human CD40 (M12/CD40), both human and murine CD40 molecules initiated SAPK activation, indicating that the CD40–SAPK signaling pathway is evolutionarily conserved (Figure 3A). The B cell lines tested varied in the strength of SAPK activity induced through CD40; for example, CD40 ligation produced only 2-fold induction of SAPK activity in DND39 and CESS cell lines (data not shown).

We also examined human tonsillar B cells for their responsiveness to CD40 signaling. Unlike the B cell lines tested, which gave highly reproducible results, the freshly isolated human tonsillar B cells yielded results that varied from donor to donor. In ~50% of all experiments, cross-linking CD40 alone was sufficient to activate SAPK. In most of these experiments, the activation was 2- to 4-fold but ranged as high as 12-fold over the medium controls (Figure 3C and data not shown). In other experiments, cross-linking CD40 was not sufficient to stimulate SAPK activity in untreated cells (Figure 3D). This suggested that the state of activation of the B cells might influence their response to CD40 ligation and the degree of SAPK activation. To test this possibility, we kept tonsillar cells in medium or pre-treated them with a low dose of phorbol myristate acetate (PMA) for 12 h before isolating the B cells. Pre-treatment with PMA consistently made tonsillar B cells more responsive to CD40-mediated SAPK activation (Figure 3D).

CD40 signaling preferentially targets SAPK compared with ERK1 and ERK2

Next, we asked whether CD40 ligation could also activate the related protein kinases ERK1 and ERK2, initially using the Daudi B cell line. Under conditions in which CD40 ligation clearly activated SAPK, the activity of ERK1 and ERK2 was not changed as measured by the

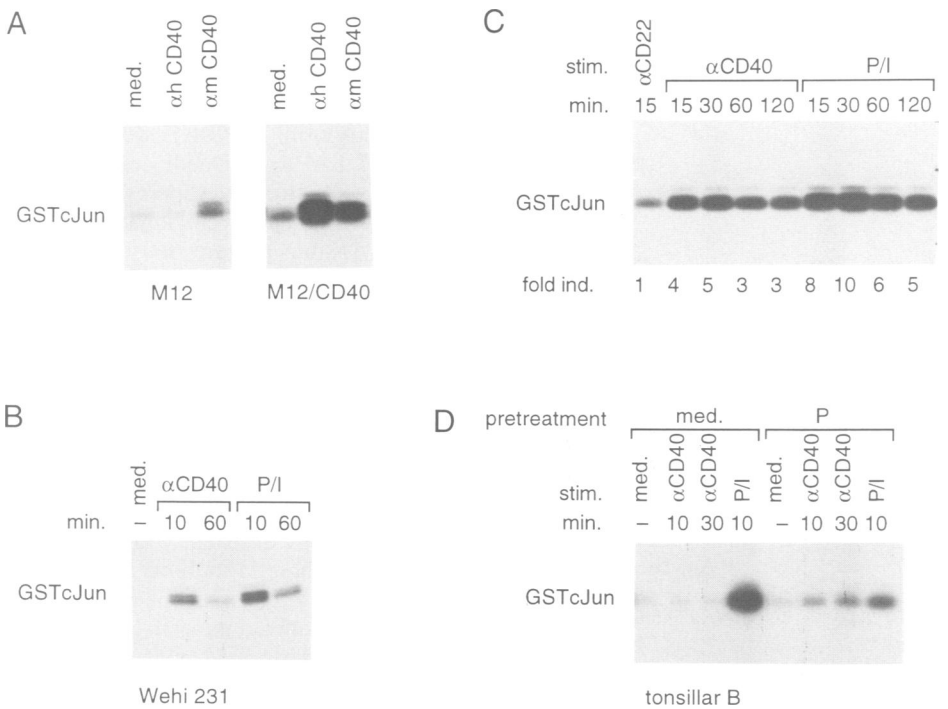

Fig. 3. Cross-linking CD40 activates SAPK in murine B cell lines and human tonsillar B cells. (**A–C**) The murine B cell lymphoma M12 (wild-type, M12) or a clone stably transfected with human CD40 (M12/CD40) (A), the murine immature B cell line WEHI 231 (B) and human tonsillar B cells (C) were stimulated for 15 min as indicated. SAPK activities were determined in immune complex assays with GST–c-Jun as substrate, as described in Materials and methods. A PhosphorImager was used to calculate fold induction. h CD40, human CD40; m CD40, mouse CD40. (**D**) Before tonsillar B cells were isolated, the whole tonsillar cell population was either pre-treated with PMA (P, 10 ng/ml) or kept in medium (med.) for 12 h at 37°C. Thereafter, the B cell population was enriched as described, stimulated as indicated and tested for SAPK activity as described in Figure 1A. The figure shows one of four representative experiments.

phosphorylation of MBP in an immune complex kinase assay (Figure 4A). The lack of CD40-dependent ERK1 and ERK2 activation was not due to the absence of these enzymes from Daudi cells, since activation of protein kinase C (PKC) by PMA and the calcium ionophore ionomycin did activate both ERK1 and ERK2. Kinetic analyses also revealed that ERK2 was not activated at earlier or later time points, up to 90 min after CD40 ligation (Figure 4B). ERK1 activity paralleled ERK2 activity in those cells but was not as strong (data not shown). Furthermore, no significant change in the activity of ERK1 and ERK2 was detected in the murine M12/CD40 and WEHI 231 cell lines (data not shown).

To test whether CD40 signaling might activate ERK1 and ERK2 at even later time points, we used a transient expression assay based on a reporter plasmid, 5× SRE–FOS–CAT/wt, which contains five copies of the serum response element (SRE) (Lee and Gilman, 1994) placed adjacent to a basic c-*fos* promoter and linked to CAT (chloramphenicol acetyltransferase). The SREs from the c-*fos* promoter bind the transcription factor $p62^{TCF}$ (in a ternary complex with SRE), which, on phosphorylation by ERK1 and ERK2, can initiate transcription of the reporter gene (Gille *et al.*, 1992; Marais *et al.*, 1993). Daudi cells were transfected with the relevant reporter constructs and stimulated with either anti-CD40 monoclonal antibody (mAb) or a combination of PMA and ionomycin during the last 16 h of the transfection assay. Owing to the high stability of the CAT enzyme, this assay should pick up and integrate any change in ERK1 and ERK2 activity during the whole period of stimulation. While PMA in combination with ionomycin strongly induced SRE-dependent gene expression, cross-linking CD40 induced little or no expression (Figure 5A and data not shown). The inability of the CD40 signal to induce TPA-response element (TRE)-dependent gene expression is most likely due to the lack of Fos and/or to the fact that phosphorylation of Jun by p46 SAPK alone is insufficient to activate the TRE. Again, transfection experiments with M12/CD40 cells yielded essentially the same results (data not shown).

Further support for the notion that CD40 signaling activates SAPK more efficiently than ERK1 and ERK2 came from the analyses of endogenous mRNA expression. While PMA and ionomycin increased both c-*jun* and c-*fos* mRNA levels in Daudi cells within 1 h after stimulation, CD40 stimulation augmented only c-*jun* mRNA expression (Figure 5B). The inability of CD40 ligation to activate increases in c-*fos* mRNA is consistent with the inability of CD40 ligation to activate MAPK since, if CD40 ligation could stimulate ERK1 and ERK2 activity, the SRE in the c-*fos* promoter should also be activated. Even in the presence of cycloheximide, which superinduces the expression of early response genes, no c-*fos* mRNA could be detected after signaling through CD40. Together, these data indicate that cross-linking CD40 activates SAPK but not MAPK in the B cell lines examined.

CD40-induced SAPK activation is not blocked by pre-treating B cells with high doses of phorbol ester or cyclosporin A

The data thus far suggested that PKC was probably not involved in the CD40-dependent SAPK activation, since

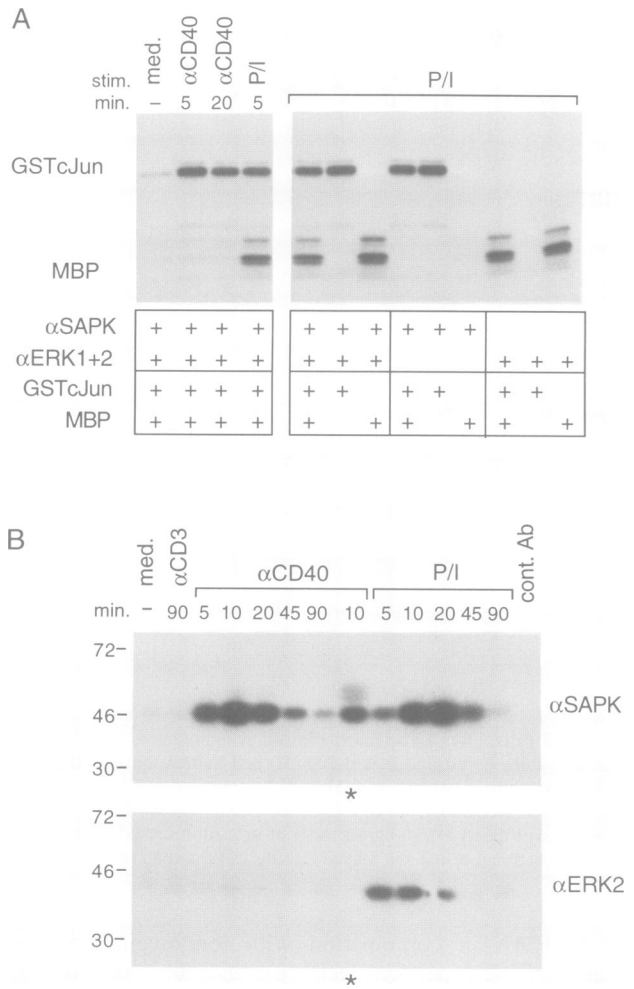

Fig. 4. Cross-linking CD40 preferentially activates SAPK compared with ERK1 and ERK2. (**A**) Daudi cells were stimulated with anti-CD40 mAb (0.5 μg/ml) or a combination of PMA (P, 50 ng/ml) and ionomycin (I, 0.25 μg/ml) for the times indicated. SAPK and/or ERK1 and ERK2 were precipitated and tested for their activities in immune complex kinase assays using GST–c-Jun and/or MBP as substrates in various combinations. In the immune complex kinase assays shown in the right panel, the various combinations of kinases and substrates were tested to ensure kinase/substrate specificity. (**B**) Daudi cells were treated as indicated. SAPK and ERK2 activities were determined in in-gel kinase assays with GST–c-Jun and MBP, respectively. In the upper panel, an asterisk marks the lane where a polyclonal antiserum directed against a GST–SAPK fusion protein (Kyriakis *et al.*, 1994) was used for comparison. In all the other lanes of this panel, the JNK1 (C-17) antiserum directed against a C-terminal peptide of SAPK was used. In the lower panel, an asterisk marks the lane where an antiserum against ERK1 was used for precipitation. Anti-rac2 antiserum was used to control for specificity (cont. Ab). Molecular weight standards are indicated in kDa.

induction of PKC normally activates ERK1 and ERK2 via Raf-1 (Kolch *et al.*, 1993). To test whether PKC is required for CD40 activation of SAPK, we pre-treated M12/CD40 cells with high doses of PMA (0.3 μg/ml) for 16 h to deplete PKC levels. PMA pre-treatment totally abrogated PKC-dependent PMA/ionomycin-induced SAPK and ERK2 activity at 10 and 60 min after stimulation but left CD40-stimulated SAPK activity untouched (Figure 6A). The same result was obtained when PKC-depleted Daudi cells were used (data not shown). Thus, PKC apparently is not required for the CD40 signaling pathway leading to SAPK activation.

Because SAPK activated by ligation of both CD3 and CD28 receptors on T cells has been shown to be sensitive to cyclosporin A (CsA), we examined whether CD40-induced SAPK in Daudi cells was influenced by CsA. While CsA strongly suppressed the induction of SAPK by PMA and ionomycin, it had no effect on CD40-induced SAPK 15 min (Figure 6B) or 30 min (data not shown) post-stimulation. Furthermore, the CD40–SAPK pathway was also insensitive to CsA in murine M12/CD40 cells (data not shown).

Cross-linking CD40 changes the electrophoretic mobility of ATF2 but not of Raf-1

It has been reported that ATF2 is phosphorylated by activated SAPK and that phosphorylation of ATF2 correlates with a shift in ATF2 mobility in SDS–PAGE (Gupta *et al.*, 1995). Consistent with those results, we found that the electrophoretic mobility of ATF2 in Daudi cells was also altered after cross-linking CD40, and this change in mobility paralleled the activation of SAPK (Figure 7a and b). We also performed an analogous experiment for Raf-1. In contrast to ATF2, Raf-1 mobility was influenced not by CD40 signaling but by PMA and ionomycin (Figure 7c). These results suggest that CD40-induced SAPK may phosphorylate and thereby activate ATF2, implying that this transcription factor may function as a downstream mediator of CD40 signaling. In addition, these data indicate that the CD40 pathway does not involve Raf-1 activation.

Discussion

In this study, we evaluated whether cross-linking CD40 on B cell lines and human tonsillar B cells activates SAPKs and MAPKs (ERK1 and ERK2). Our results clearly demonstrate that signaling through CD40 can up-regulate the activity of SAPK while affecting ERK1 and ERK2 activity only marginally, if at all. Several lines of evidence indicate that ERK1 and ERK2 are not significantly activated by CD40: (i) no significant phosphorylation of MBP was detected with anti-ERK1 and ERK2 precipitates in *in vitro* kinase assays after CD40 cross-linking for various lengths of time; (ii) no significant SRE-dependent reporter gene expression was detected after CD40 ligation, which should pick up any ERK1 and ERK2 activity via an activated $p62^{TCF}$ (Gille *et al.*, 1992; Marais *et al.*, 1993); and (iii) no endogenous c-*fos* mRNA was expressed after CD40 signaling. However, the CD40-induced SAPK response could be quite strong, with 15- to 25-fold increases in activity in Daudi cells. The activity peaked at ~10–15 min after the engagement of CD40 and declined thereafter, but persisted for several hours. Although p46 SAPK is preferentially activated compared with p54 SAPK and the ERK1/2 kinases in the B cells we have studied, it is possible that in other cell types or stages ligating CD40 may produce a different pattern of p46/p54 SAPK and/or ERK1/2 activation. For example, Rafiee *et al.* (1995) recently found that TNF-α, which preferentially induces SAPK activation in a number of cell types (Kyriakis *et al.*, 1994), did activate ERK in human neutrophils.

SAPK apparently may be activated via either Ras-

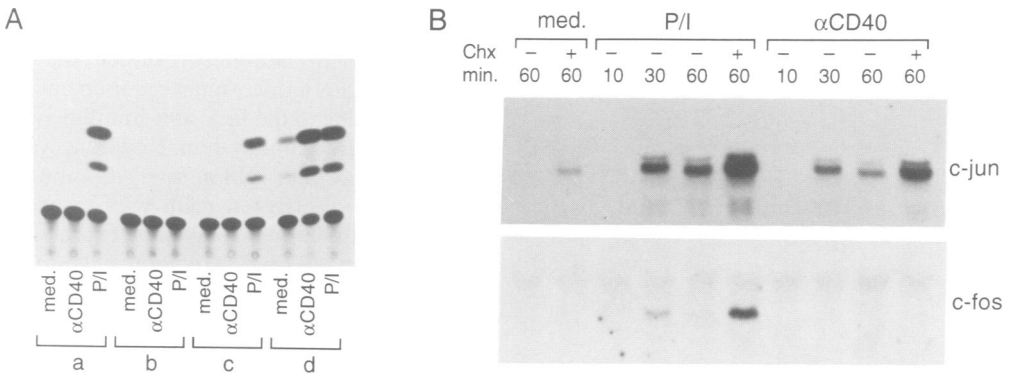

Fig. 5. Gene expression after cross-linking CD40. (**A**) Daudi cells were transiently transfected with CAT reporter constructs [5× SRE–FOS–CAT/wt (a), 5× SRE–FOS–CAT/mp12 (b), 5× TREcol (c) or pBL2 7+ (contains NF-κB binding sites; d)]. One day after transfection, cells were split and stimulated for 16 h with the stimulus indicated [anti-CD40 mAb, 0.5 μg/ml; PMA (P), 50 ng/ml; and ionomycin (I) 0.25 μg/ml]. CAT assays were performed as described in Materials and methods. (**B**) Daudi cells were treated as indicated [mAb, 0.5 μg/ml; PMA (P), 50 ng/ml; ionomycin (I), 0.25 μg/ml; and cycloheximide (Chx), 20 μg/ml]. Total RNA was isolated and tested for c-*jun* and c-*fos* mRNA levels. Equal loading was confirmed by rehybridizing the membranes with a β-actin probe.

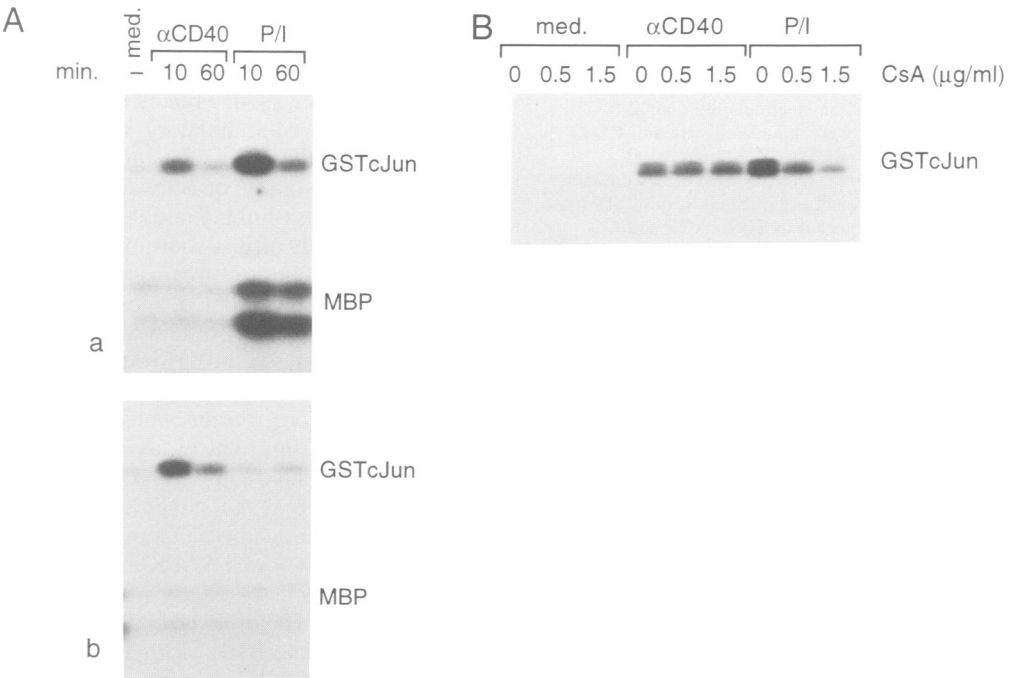

Fig. 6. CD40-dependent activation of SAPK is not blocked by pre-treating M12/CD40 cells with high doses of PMA or by the inhibitor cyclosporin A (CsA). (**A**) M12/CD40 cells were cultured in medium alone (a) or with PMA (300 ng/ml) (b) for 16 h. Cells were then stimulated as indicated [anti-CD40, 0.5 μg/ml; PMA (P), 50 ng/ml; and ionomycin (I) 0.25 μg/ml]. SAPK and ERK2 were precipitated and tested for their activity in immune complex kinase assays including GST–c-Jun and MBP as substrates. (**B**) Daudi cells were either pre-treated with CsA for 1 h as indicated or left untreated prior to stimulation [concentrations as in (A)]. Lysis and testing for SAPK activity were performed as described in Materials and methods.

dependent or Ras-independent pathways (Bird *et al.*, 1994; Minden *et al.*, 1994a). We have no direct data supporting one pathway or the other, but two findings argue in favor of the Ras-independent pathway. First, cross-linking CD40 does not lead to the changes in Raf-1 mobility (Figure 7c) that one might expect after activation. Second, the related TNF-α receptors (TNF-αRs) and CD40 share a number of highly conserved features: all three surface molecules belong to the same protein family (Smith *et al.*, 1994); CD40 and TNF-αR p75 bind highly homologous proteins with their cytoplasmic tail—TRAF2 for the TNF-αR p75 (Rothe *et al.*, 1994) and CRAF1 for CD40 (Hu *et al.*, 1994; Cheng *et al.*, 1995; Mosialos *et al.*, 1995); both CD40 and TNF-αR p55 activate NF-κB (Kruppa *et al.*, 1992; Berberich *et al.*, 1994); and CD40 and the TNF-αR(s), p55 and/or p75, both target SAPK (Figures 1–4; see also Kyriakis *et al.*, 1994; Minden *et al.*, 1994a), with the TNF-αR(s) acting in a Ras-independent way. Thus, it is likely that CD40 may signal in a similar manner.

Cross-linking CD40 leads to c-*jun* mRNA expression in Daudi cells (Figure 5B). There was a good correlation between the strength of SAPK activity and the amount of c-*jun* mRNA expressed. Nevertheless, it is not yet clear whether the transcription factors known to be effectors of SAPK, i.e. c-Jun (Hibi *et al.*, 1993) and ATF2 (Gupta *et al.*, 1995), are in fact responsible and sufficient to

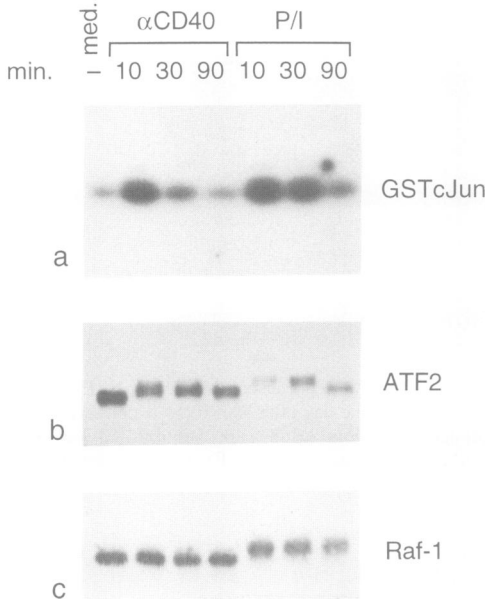

Fig. 7. Cross-linking CD40 changes the electrophoretic mobility of ATF2 but not of Raf-1. Daudi cells were stimulated with anti-CD40 mAb (0.5 μg/ml) or a combination of PMA (P, 50 ng/ml) and ionomycin (I, 0.25 μg/ml) for the times indicated. Extracts were then immunoprecipitated with polyclonal antisera directed against ATF2 or Raf-1. Immunoprecipitates were subjected to SDS–PAGE and visualized by Western blotting (**b** and **c**). SAPK activity of the cells was monitored by means of an immune complex kinase assay (**a**).

account for the up-regulation of the c-*jun* promoter. After CD40 cross-linking, c-*fos* mRNA was not detectable (Figure 5B) and a reporter gene dependent on the classical AP-1 (Jun/Fos) heterodimer was not expressed significantly (Figure 5A; see also Berberich *et al.*, 1994). Thus, it seems unlikely that AP-1, a potent regulator of the c-*jun* promoter (Angel *et al.*, 1988), contributes to the increase in c-*jun* mRNA found after CD40 cross-linking. Jun2/TRE is another regulatory site in the 5′ region of the c-*jun* gene. It has been shown to bind ATF2/c-Jun heterodimers (van Dam *et al.*, 1993) and to exert a regulatory function in ATF2-dependent c-*jun* promoter activity (Livingstone *et al.* 1995; van Dam *et al.*, 1995). Since CD40 ligation induces a migrating shift in ATF2 (Figure 7b), and ATF2 contains a phosphorylation-dependent transcription activation domain (Livingstone *et al.*, 1995; van Dam *et al.*, 1995), Jun2/TRE seems a likely target site for SAPK-dependent c-*jun* up-regulation. Recently, Francis *et al.* (1995) found some increase in the TRE (AP-1 site) binding activity after cross-linking CD40 on primary murine B cells. Thus, as yet uncharacterized TRE site binding activity may influence transcripitional activity of the c-*jun* gene. Further transcripton factors could also be involved in CD40-dependent c-*jun* expression. NF-κB could be a potential candidate because: (i) it is strongly induced by CD40 signals (Berberich *et al.*, 1994); (ii) NF-κB and ATF2 have been shown to interact *in vitro* (Du *et al.*, 1993); and (iii) DNA sequences differing in only one residue from an NF-κB consensus site are located in the c-*jun* promoter region close to the ATF2 binding site (D.Morris, personal communication).

Consistent with data published by Gille *et al.* (1995), c-*fos* mRNA expression in B cells correlates with ERK activity (Figure 5B and data not shown). So far, we have been unable to definitely exclude the involvement of SAPK and thus a dual signal requirement for c-*fos* mRNA expression due to the lack of a stimulus which exclusively activates ERK in our system. However, we have compared anti-IgM with anti-IgM/anti-CD40 stimulation in WEHI 231 cells and analyzed them with respect to SAPK and ERK activation and c-*fos* mRNA expression. Both stimuli induced identical ERK activation and c-*fos* mRNA expression, even though there was a dramatically higher SAPK activation in doubly stimulated cells (Siebelt *et al.*, 1995). Thus, at least the level of SAPK activity did not influence ERK-dependent c-*fos* mRNA expression. The ERK dependency of c-*fos* expression was demonstrated by stimulating the cells with anti-CD40 alone, which again led to strong SAPK induction—comparable with doubly stimulated cells—but neither ERK activation nor c-*fos* mRNA expression (Siebelt *et al.*, 1995).

CD40 signals lead to changes in a variety of downstream signal transducers including protein tyrosine kinases (Faris *et al.*, 1994), the PI-3 kinase (Ren *et al.*, 1994), the SAPK (Figures 1–4) and the transcription factors NF-κB (Berberich *et al.*, 1994; Francis *et al.*, 1995) and NF-AT; for the latter a co-stimulatory signal may be necessary (Choi *et al.*, 1994; Francis *et al.*, 1995). Intriguingly, in Daudi cells, the onsets of activation of NF-κB and SAPK after CD40 cross-linking are basically parallel (Figure 2C). On the basis of this coordinated action, it is possible that the SAPK lies upstream on a pathway leading to phosphorylation of IκB (-α and/or -β) and subsequently to activation of NF-κB, or that SAPK *per se* is the kinase that phosphorylates IκB(s). However, we have not detected phosphorylation of IκB-α with activated SAPK (unpublished observation). Furthermore, pre-treatment of Daudi cells with the anti-oxidant pyrrolidine dithiocarbamate totally inhibits CD40-induced NF-κB activation (Berberich *et al.*, 1994) but, at the same doses, activates the SAPK by itself (data not shown). Thus, our data at this juncture favor a model in which SAPK and NF-κB are located on parallel pathways.

In contrast to Daudi, M12 and WEHI 231 cells, another human B cell line, B104 (Ishigami *et al.*, 1992), showed no increase in SAPK activity after CD40 cross-linking, even though SAPK was present and activatable by phorbol ester and ionomycin in these cells (data not shown). Furthermore, freshly isolated human tonsillar B cell preparations varied in their response to CD40; responsiveness to CD40 ligation may depend on the maturation or activation stage of B cells, i.e. the CD40–SAPK linkage may not exist (physically or functionally) at every B cell stage. Pre-activation of tonsillar B cells with PMA induced otherwise unresponsive B cells to be sensitive to CD40-dependent SAPK activation. Pre-treating tonsillar B cells with either heterologous $F(ab')_2$ anti-IgM antisera or IL-4 had the same effect (data not shown). This model is supported further by our preliminary data that buoyant human tonsillar B cells are more responsive to CD40-dependent SAPK activation than dense cells. To define how activation signals make normal B cells more responsive to CD40-induced SAPK activation will require further study. For now we can speculate that a component of the CD40–SAPK pathway may be missing at certain activation stages of B cell maturation, or that the pathway is inhibited.

We proposed previously that CD40 on antigen-presenting cells and CD28 on T cells have similar signaling properties and can activate each other's pathways in a reciprocal dialog (Clark and Ledbetter, 1994). Here we found additional similarities in these pathways: CD40 (Figure 5B) and CD28 ligation (Chatta *et al.*, 1994) both activate increases in c-*jun* and not c-*fos* mRNA, and both CD40 (Figures 1–4) and CD28 (Su *et al.*, 1994) are involved in SAPK activation. In addition, similar to CD40 on certain human tonsillar B cells, ligation of CD28 by itself is not sufficient to activate SAPK in the T cells analyzed so far but depends on simultaneous T cell receptor (TCR) signals (Su *et al.*, 1994). However, in contrast to B cells, no situation has been described for T cells in which CD28 ligaton by itself could stimulate SAPK. Also, unlike in T cells, in WEHI 231 cells the engagement of the antigen receptor is sufficient for SAPK activation (Siebelt *et al.*, 1995). In addition, CD40-dependent activation of SAPK seems to be insensitive to CsA (Figure 6B), whereas the combination of TCR/CD28 ligation leading to SAPK activation is sensitive to this drug (Su *et al.*, 1994). The data, however, are not yet sufficient to determine whether the differences we observe for SAPK recruitment in B and T cells reflect differences in T versus B cells or are due to distinct requirements for induction of SAPK at specific activation/maturation stages. The finding that CD40 and the BCR are not always strong inducers of SAPK by themselves and can cooperate in certain B cells supports the latter notion.

Materials and methods

Cells

Cell culture conditions were described previously (Berberich *et al.*, 1994). Daudi, CESS and WEHI 231 were obtained from American Type Culture Collection. DND39 (Ichiki *et al.*, 1992) and B104 (Ishigami *et al.*, 1992) cells were kindly provided by Dr T.Watanabe and Dr M.Mayumi, respectively. The M12 and M12/CD40 B cell lines are described elsewhere (Inui *et al.*, 1990). Tonsils from children were minced, then live mononuclear cells were collected on a Ficoll gradient and, after two washes, plated at $2.5–5\times10^7$ cells/ml in RPMI 1640 supplemented as described (Clark *et al.*, 1989; Berberich *et al.*, 1994). Cells were kept at 25 or 37°C with or without PMA stimulus for 12–16 h. Then, T cells were rosetted with 2-aminoethylisothiouranium bromide (AET)-treated sheep red blood cells as described (Clark *et al.*, 1989) and removed by Ficoll gradient. The remaining cells were size fractionated on a discontinuous Percoll gradient. Cells ≥55% were used in the experiments. B cells were kept at 37°C in medium for 2–3 h before stimulation.

Reagents

The following mAbs were used in this study: G28-5 mAb (IgG1) to CD40 (Clark and Ledbetter, 1986), G19-4 (IgG1) to CD3 (Ledbetter *et al.*, 1985), G28-7 (IgG1) to anti-CD22 (Leprince *et al.*, 1993) and G28-1 (IgG1) to CD37 (Ledbetter *et al.*, 1987). 1C10 to anti-mouse CD40 is described by Heath *et al.* (1993). The polyclonal antisera [JNK1 (C-17), JNK2 (C-18), ERK1 (C-16), ERK2 (C-14), ATF2 (C-19) and rac2 (C-11)] used for protein kinase assays and Western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), except for the anti-p54 SAPK polyclonal antisera (Kyriakis *et al.*, 1994). Soluble mouse CD40L was prepared using a vector kindly provided by Dr Peter Lane as described (Lane *et al.*, 1993). MBP was obtained from UBI. CsA was a gift from Sandoz.

Transfection and CAT assay

Daudi and M12/CD40 cells were transiently transfected by means of the DEAE–dextran method (Berberich *et al.*, 1994). Routinely, cells were stimulated 20–24 h after transfection and harvested 16–24 h later. For the CAT assay, transfected cells were lysed in reporter lysis buffer (100 µl; Promega) for 15 min at room temperature. Lysates were cleared by centrifugation and incubated for up to 8 h in a reaction mixture (125 µl) containing 200 µM acetyl coenzyme A and 25 nCi [^{14}C]chloramphenicol (25 µCi/ml). [^{14}C]Chloramphenicol was extracted from the aqueous solution with ethylacetate (400 µl), vacuum dried and dissolved in 20 µl of ethylacetate. The diversely acetylated forms of [^{14}C]chloramphenicol were separated by thin layer chromotography. The following plasmids were used: 5× SRE–FOS–CAT/wt (five copies of the SRE of the c-*fos* promoter linked to a minimal c-*fos* promoter), 5× SRE–FOS–CAT/pm12 (as wt except that the serum response factor binding site of the SRE was mutated) (Lee and Gilman, 1994), pBL2 7+ [contains seven copies of the NF-κB binding site from the promoter of the invariant chain of MHC class II in front of the thymidine kinase (tk) minimal promoter (Pessara and Koch, 1990)], and 5× TREcol (five copies of the TRE site found in the human collagenase promoter linked to tk and CAT).

Immunoprecipitations, protein kinase assays and Western blotting

Immune complex kinase assay. Following stimulation, cells ($3–10\times10^6$) were lysed in 300–500 µl of kinase lysis buffer [20 mM HEPES, pH 7.4; 2 mM EGTA; 50 mM β-glycerophosphate; 1% Triton X-100; 10% glycerol; 1 mM dithiothreitol (DTT); 2 mM phenylmethylsulfonyl fluoride (PMSF); 1 µg/ml leupeptin; 1 µg/ml aprotinin; 2 mM Na_3VO_4; and 10 mM NaF] for 15 min on ice. [To detect SAPK activity in the cytoplasm, cells were lysed in 50 µl lysis buffer (Dignam *et al.*, 1983). Nuclei were removed by centrifugation and the cytoplasmic extract was combined with 450 µl of kinase lysis buffer.] Cell debris was removed by centrifugation at 10 000 *g* for 10 min at 4°C. Kinases (SAPK and/or ERK1 and ERK2) were immunoprecipitated with 0.5 µg of polyclonal antisera (in the case of SAPK, always JNK1 C-17, except for part of the experiment shown in Figure 4B) and 20 µl of protein A–Sepharose beads for 3–16 h. Beads were washed twice each with kinase lysis buffer, high salt buffer (100 mM Tris pH 7.6, 500 mM LiCl, 0.1% Triton X-100 and 1 mM DTT), and assay buffer (20 mM MOPS pH 7.2; 2 mM EGTA; 10 mM $MgCl_2$; 0.1% Triton X-100 and 1 mM DTT). After the last wash, beads were left as a 1+1 suspension in assay buffer. Kinase reactions were carried out at 30°C for 20 min after addition of 20 µl of substrate (1–3 µg of fusion protein or MBP in assay buffer) and 15 µl of ATP mix (50 mM $MgCl_2$, 200 µM ATP and 5–10 µCi [γ-^{32}P]ATP). Reactions were stopped by adding 25 µl of 6× Laemmli buffer and boiling for 5 min. Samples were fractionated on an SDS–polyacrylamide gel. Autoradiography was performed after the gels were dry.

In-gel kinase assay. Lysis and immunoprecipitation were done as described above. Kinase precipitates were fractionated on an SDS–polyacrylamide gel casted with the respective substrate(s) (GST–c-Jun, GST–c-Jun/nb and/or MBP: 40 µg/ml). The remaining steps (washes, denaturing and renaturing steps and protein kinase assay) were done as described by Hibi *et al.* (1993).

Western blotting. Cells were lysed in RIPA buffer (10 mM Tris pH 8.0, 50 mM β-glycerophosphate, 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) supplemented with PMSF (2 mM), pepstatin (1 µg/ml), leupeptin (1 µg/ml), aprotinin (1 µg/ml), soybean trypsin inhibitor (100 µg/ml), Na_3VO_4 (2 mM), NaF (10 mM) and DTT (1 mM). Immunoprecipitation and SDS–PAGE were performed as outlined above. Proteins were transferred to nitrocellulose membranes using standard techniques, and specific proteins were detected using the ECL kit (Amersham) according to the manufacturer's manual.

Northern blots

RNA extractions were performed as described by Chomczynski and Sacchi (1987). Up to 5×10^6 cells were lysed in 0.5 ml of 4 M guanidinium thiocyanate. Isolated RNA was denatured in formaldehyde/formamide buffer, fractionated on a conventional formaldehyde–agarose gel, and transferred to a membrane. Hybridization was performed in 10% dextran sulfate at 65°C with ^{32}P-labeled cDNA probes (random labeling kit; Boehringer Mannheim) specific for c-*jun*, c-*fos* and β-actin.

Production and purification of GST fusion proteins

GST–c-Jun (5–89) and GST–c-Jun/nb [as GST–c-Jun (5–89) but with the δ domain deleted] are described elsewhere (Kyriakis *et al.*, 1994). The cytoplasmic tail of mouse CD40 (amino acids 216–289) was amplified from the mCD40 cDNA-N clone (Torres and Clark, 1992) by PCR and was cloned in-frame into the pGEX-2T expression vector (Pharmacia) using the *Eco*RI and *Bam*HI restriction sites. Fusion proteins were expressed in *Escherichia coli* BL21(DE3). Expression of the fusion

proteins was induced in exponentially growing bacteria (OD_{590} ~0.5) with 0.1 mM IPTG for 2–4 h. After centrifugation, bacteria were resuspended in lysis buffer [PBS, 0.1% Triton X-100 with PMSF (2 mM), pepstatin (1 μg/ml), leupeptin (1 μg/ml), aprotinin (1 μg/ml), soybean trypsin inhibitor (100 μg/ml) and DTT (1 mM)] before sonication. Fusion proteins were collected from the cleared lysate by glutathione–agarose chromatography and released from the agarose with 5 mM glutathione. Proteins were dialyzed against 50 mM Tris pH 7.9 and stored as 50% glycerol solution at –20°C.

Nuclear extracts and electrophoretic mobility shift sssay

Nuclear extracts were prepared as described by Dignam *et al.* (1983). Electrophoretic mobility shift assays were performed as previously described (Berberich *et al.*, 1994).

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