Opposing regulation of B cell receptor-induced activation of mitogen-activated protein kinases by CD45

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Received 7 November 2000; revised 15 December 2000; accepted 15 December 2000

First published online 28 December 2000

Edited by Giulio Superti-Furga

Abstract In this study, we examined the contribution made by CD45 to B cell antigen receptor (BCR)-induced activation of mitogen-activated protein kinase (MAPK) family members. We found that CD45 negatively regulated BCR-induced c-Jun NH_2-terminal kinase (JNK) and p38 activation in immature WEHI-231 cells, whereas in mature BAL-17 cells, CD45 positively regulated JNK and p38 activation and negatively regulated extracellular signal-regulated kinase activity. Furthermore, cooperative action of JNK and p38 dictated BCR-induced inhibition of growth. Thus, CD45 appears to differentially regulate BCR-induced activation of MAPK members, and can exert opposing effects on JNK and p38 in different cellular milieu, controlling the B cell fate.

Key words: B lymphocyte; CD45; Mitogen-activated protein kinase; Protein tyrosine phosphatase; Signal transduction

1. Introduction

Engagement of B cell antigen receptor (BCR) by anti-IgM antibody (Ab) or multivalent antigens rapidly induces tyrosine phosphorylation of a number of cellular proteins [1]. Signals are then propagated downstream, ultimately leading to control gene expression and to B cell activation, death or anergy, depending upon the cell's developmental stage, the antigen structure, and the presence or absence of co-stimulatory signals [2,3]. Regardless of the context within which stimulation occurs, BCR signaling is dependent upon the proximal activation of protein tyrosine kinases (PTKs), in particular Src-family PTKs (Lyn, Blk, Lck, Fyn and Fgr), Syk and Btk. Accumulating evidence suggests that one of the crucial regulators of BCR signaling is the receptor-type protein tyrosine phosphatase (PTP), CD45 [4–6]. CD45 has been implicated in both T and B cell activation based on experiments using CD45-deficient cells [7–10] and cells from CD45 gene-targeted mice [11,12]. Although it is now clear that Src-family PTKs serve as targets for CD45, the precise mode of CD45 action remains controversial [13–15]. Furthermore, it is still unclear as to how CD45-mediated activation or inactivation of Src-family PTKs is transduced into downstream signaling pathways.

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine-specific protein kinases activated by a wide variety of extracellular signals [16,17]. In mammalian cells, three related but distinct members of the MAPK family have been identified: extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPK (p38). Integration of signals from any single member of this family may dictate cellular proliferation, differentiation or apoptosis among other cellular processes [16,17].

Our previous studies demonstrated that CD45 exerts opposing effects on BCR-induced signaling depending on the state of B cell maturation [9,10]. In CD45-negative clones from immature WEHI-231 cells, Ca^{2+} mobilization was delayed but enhanced, and growth inhibition and apoptosis were more striking [9]. In mature BAL-17 cells, by contrast, BCR-induced Ca^{2+} mobilization and growth inhibition are positively regulated by CD45 [10]. To elucidate the molecular basis of the differential regulation of growth by CD45, in this study, we focussed on the MAPK pathways. We found that the effects of CD45 on BCR-induced activation of JNK and p38 were opposing in WEHI-231 and BAL-17 and correlated with growth regulation in respective cells. We further observed that the cooperative action of JNK and p38 dictates the fate of B cells triggered by BCR ligation via a process that is dependent upon CD45.

2. Materials and methods

2.1. Cells

The murine, immature B cell line WEHI-231, its CD45-deficient clone, 10-5, the mature B cell line BAL-17, and its CD45-deficient clone, 44, were all described in previous reports [9,10]. These cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 50 mM 2-ME, 100 mg/ml streptomycin and 100 U/ml penicillin (complete medium).

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Abbreviations: BCR, B cell antigen receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase

0014-5793/01/$20.00
PII: S0014-5793(00)02416-9

2.2. Antibodies and reagents

Goat F(ab′)$_2$ fragments of anti-mouse IgM Ab were purchased from ICN Pharmaceuticals, Inc. (Aurora, OH, USA). PD98059 and SB203580 were obtained from New England BioLabs, Inc. (Beverly, MA, USA) and Calbiochem-Novabiochem Corp. (La Jolla, CA, USA), respectively. Rabbit anti-mouse phospho-specific JNK Ab was purchased from New England BioLabs. Rabbit anti-mouse ERK-2 Ab, anti-JNK Ab, anti-p38 Ab, and purified ATF-2 protein were all from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.3. Cell stimulation and Western blot analysis

Cells were initially incubated for 3 h at 37°C and then stimulated with 20 mg/ml F(ab′)$_2$ fragments of anti-IgM Ab for 5–30 min, after which the reactions were terminated with ice-cold PBS containing 2 mM Na_3VO_4 and 2 mM EDTA (PBS-VE). The cells were solubilized in TNE lysis buffer (1% Nonidet P-40, 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM Na_3VO_4, 2 mM EDTA, 1 mM NaF, 10 mM sodium pyrophosphate) supplemented with protease inhibitors. The lysates were centrifuged at 10 000×*g* at 4°C for 30 min, and the supernatants were separated on 10% SDS–PAGE gels and transferred to nitrocellulose membrane. Western blots were incubated with Abs against ERK, JNK and p38 and then with mouse anti-rabbit IgG-alkaline phosphatase (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) or horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology). The blots were visualized using an Alkaline Phosphatase Conjugate Substrate kit (Bio-Rad Laboratories, Hercules, CA, USA), or ECL Western blotting detection reagent (Amersham Pharmacia Biotech, Ltd., Buckinghamshire, UK).

2.4. In vitro kinase assays for MAPKs and MAPKAPK-2

Cell lysates were immunoprecipitated with protein G-Sepharose beads bound with Abs against ERK2, JNK, p38 and MAPKAPK-2. After washing with TNE lysis buffer and kinase buffer for MAPKs (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 100 mM Na_3VO_4, 100 mM ATP) or kinase buffer for MAPKAPK-2 (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 75 mM $MgCl_2$, 1 mM DTT, and 1 mM Na_3VO_4), the immunoprecipitates were incubated for 20–30 min at 30°C in kinase buffer containing 5–10 mCi of [γ-^{32}P]ATP, 500 mM ATP and the appropriate substrates: myelin basic protein (MBP) for ERK, glutathione *S*-transferase (GST)-c-Jun1-79 fusion protein (a gift from Dr. G. Koretzky) for JNK, purified ATF-2 protein for p38, and murine heat shock protein (hsp) 25 (Sigma Chemical Co., St. Louis, MO, USA). The reactions were terminated by adding Laemmli sample buffer, after which the samples were subjected to SDS–PAGE. The phosphorylated substrates were visualized by autoradiography. The intensities of bands were measured with a Bio-Rad imaging densitometer, and the results were expressed as percent control activity.

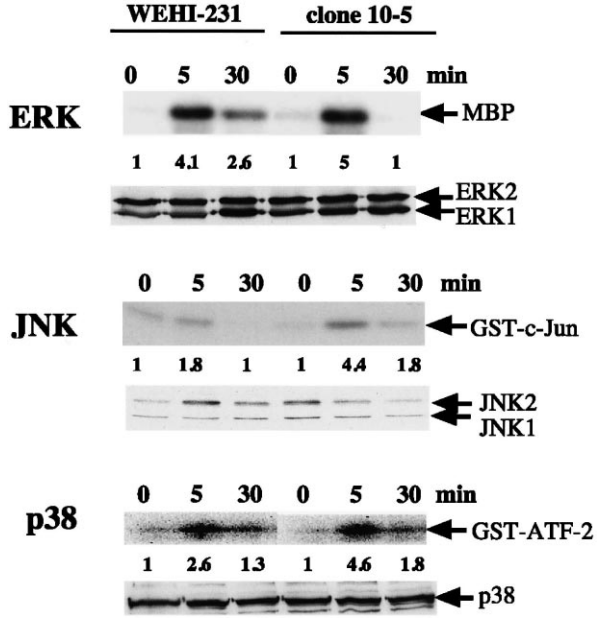


Fig. 1. BCR-induced activation of MAPKs in WEHI-231 cells and its CD45-deficient clone. WEHI-231 and clone 10-5 cells were cultured with 20 μg/ml F(ab′)$_2$ fragments of anti-IgM Ab for 5–30 min, and in vitro immune complex kinase assays were performed for ERK, JNK and p38 using MBP, GST-c-Jun and GST-ATF-2, respectively, as substrates. Total cell lysates were immunoblotted with Abs against ERK, JNK and p38. The results are representative of four independent experiments.

2.5. Transfection

Cells were suspended in 1 ml of complete cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, pH 7.6, 25 mM HEPES, pH 7.6, 2 mM EGTA, pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, and 5 mM glutathione). Aliquots of cell suspension (400 μl; 2×10^7 cells) were added to a cuvette together with 30 μg of either pEFII expression vector containing a gene encoding a HA-tagged dominant-negative SEK1 mutant (SEK-AL) [18], which had been excised from pcDNA3 Zeo, or the empty vector (a gift from Dr. G. Koretzky). The cells were transfected by electroporation at 270 V, 950 μF for 20 ms, after which they were gently mixed, cultured in complete culture medium for 24 h, and subjected to functional analysis.

2.6. Assay for DNA synthesis

Cells (5×10^3) were incubated in triplicate for 24 h in 0.2 ml of complete medium with or without F(ab′)$_2$ fragments of goat anti-mouse IgM Ab. To assess DNA synthesis, 0.5 μCi of [^{3}H]thymidine was added to each well for the final 4 h. The cells were then harvested on glassfiber filters using a semi-automatic Skatron harvester, and thymidine incorporation was measured in a Beckman liquid scintillation counter. The results were expressed as percent control DNA synthesis.

3. Results

3.1. Regulation of BCR-induced MAPK activation by CD45 in WEHI-231 cells

To define the molecular basis of the differential regulation by CD45, we first examined the effect of CD45 deficiency on the activation of ERK, JNK and p38 in WEHI-231 cells. WEHI-231 cells and its CD45-deficient, clone 10-5 were stimulated with 20 μg/ml F(ab′)$_2$ fragments of anti-IgM Ab for 5–30 min, and then in vitro kinase assays were performed with appropriate substrates. Results of a representative experiment are shown in Fig. 1. BCR-induced ERK activation in the parent peaked within 5 min and was still detected after 30 min, whereas ERK activity returned to baseline by 30 min in clone 10-5 cells. However, this difference in kinetics was not statistically significant in four independent experiments (Fig. 3). By contrast, BCR-induced activation of JNK and p38 was significantly more pronounced in clone 10-5 than in the parent (Figs. 1 and 3). These results suggest that in WEHI-231 cells, CD45 may negatively affect activation of both JNK and p38.

3.2. Regulation of BCR-induced MAPK activation by CD45 in BAL-17 cells

BCR-induced MAPK activation was then examined in BAL-17 and the CD45-negative, clone 44. As shown in Fig. 2, a representative experiment demonstrates that BCR stimulation elicited larger increases in ERK activity in clone 44 cells than in the parent. Conversely, although JNK activity was strongly induced by BCR ligation in the parent, it was completely ablated in clone 44. Similarly, activation of p38 was significantly reduced in clone 44 as compared with the parent. As shown in Fig. 3, all the differences were statistically significant. Thus, in BAL-17 cells, CD45 seems to negatively

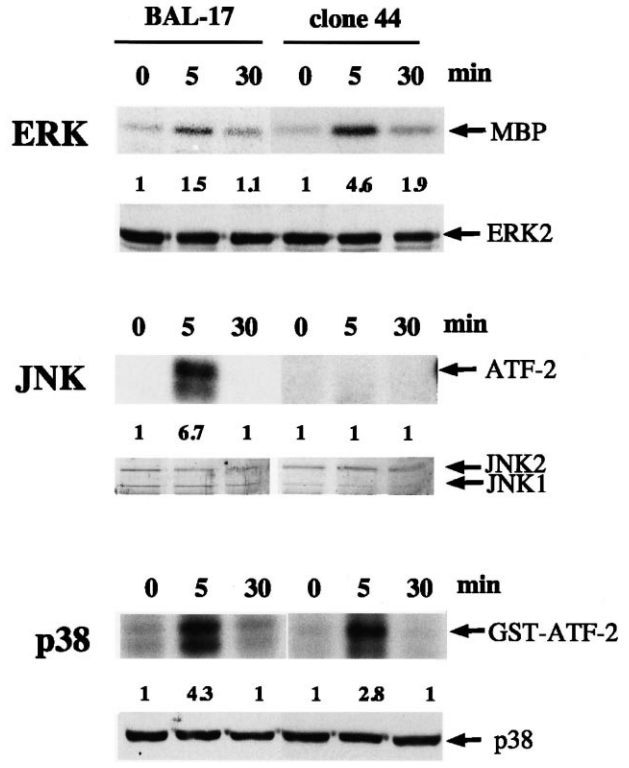


Fig. 2. BCR-induced activation of MAPKs in BAL-17 cells and its CD45-deficient clone. BAL-17 and clone 44 cells were cultured with 20 μg/ml F(ab′)$_2$ fragments of anti-IgM Ab for 5–30 min, and in vitro immune complex kinase assays were performed for ERK, JNK and p38 using MBP, ATF-2 and GST-ATF-2, respectively, as substrates. The total cell lysates were immunoblotted with Abs against ERK, JNK and p38. The results are representative of four independent experiments.

regulate BCR-induced activation of ERK but positively regulate activation of JNK and p38.

Taken two sets of experiments together, the results suggest that the regulatory effect of CD45 on BCR-induced activation of ERK differ from its effects on activation of JNK and p38, and more importantly, the regulatory effects of CD45 seem to be opposing in WEHI-231 and BAL-17 cells (Fig. 3). Given that growth inhibition is negatively regulated by CD45 in WEHI-231 cells [9] and positively regulated in BAL-17 cells [10], it is of particular note that the regulatory effect of CD45 on the final outcome for the cells correlates well with its effect on activation of JNK and p38.

3.3. ERK or p38 alone does not contribute to BCR-induced growth inhibition

To more directly explore the respective contributions of each MAPK family member to BCR-induced growth inhibition, we first examined the effect of PD98059, a specific inhibitor of MEK. BAL-17 cells were preincubated for 1 h with graded concentrations of PD98059, after which they were cultured with anti-IgM Ab in the presence of PD98059 for 24 h. As shown in Fig. 4A, in vitro kinase assays revealed that PD98059 inhibited BCR-induced activation of ERK by 74% at 100 μM, the highest dose that could be tested. However, PD98059 at 100 μM had no effect on growth inhibition (Fig. 4A), indicating that ERK activation contributed little if at all to BCR-induced inhibition of BAL-17 cell growth.

We then examined the role of p38 in growth inhibition using SB203580, a specific inhibitor for p38. BAL-17 cells were cultured with SB203580 as above. Preincubation for 1 h with 40 mM SB203580, the highest non-toxic dose, reduced subsequent BCR-induced p38 activation by 71% as measured by the activity of MAPKAP kinase-2 (MAPKAPK-2) and had a minimal inhibitory effect on growth inhibition. Thus, p38 activation alone may not contribute significantly to growth inhibition in BAL-17.

3.4. JNK acts cooperatively with p38 to dictate BCR-induced growth inhibition

To evaluate the importance of JNK, BAL-17 cells were transfected with a plasmid containing a dominant-negative form (DN) of SEK1 (DN-SEK1) [18], an upstream activator of JNK (Fig. 5A). 24 h later, the transfected cells were cultured with or without SB203580 or PD98059 and then stimulated with anti-IgM Ab for 24 h, after which DNA synthesis and the activation of ERK, JNK and p38 was assayed. Timing of the treatment with SB203580 and PD98059 was the same as described above. The transfection of DN-SEK1 reduced JNK activity by 54% with little or no effect on ERK and p38 activities. Under this condition, growth inhibition was reduced by about 20% (Fig. 5B). Strikingly, when the DN-SEK1 transfectants were treated with 10 μM SB203580,

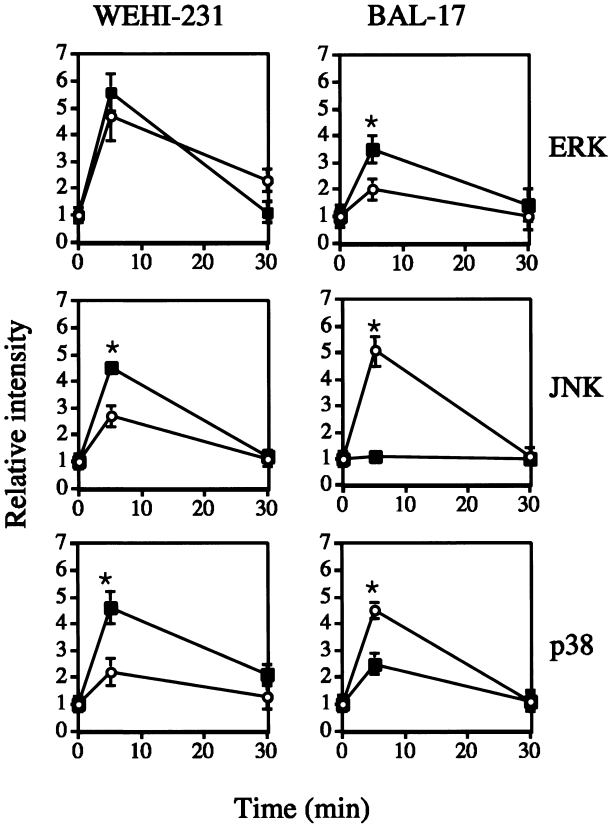


Fig. 3. CD45 exerts opposing effects on MAPK family members depending on the maturational stage of B cells. Data obtained from four independent in vitro kinase assays were subjected to densitometric analysis, and the intensity of each band was normalized to the amount of kinase assayed. The mean relative intensities ± S.E.M. of the parental cells (○) and CD45-deficient clones (■) were plotted as a function of time; the intensity of an unstimulated group arbitrarily set to 1. *Represents $P < 0.05$ by Student's t-test.

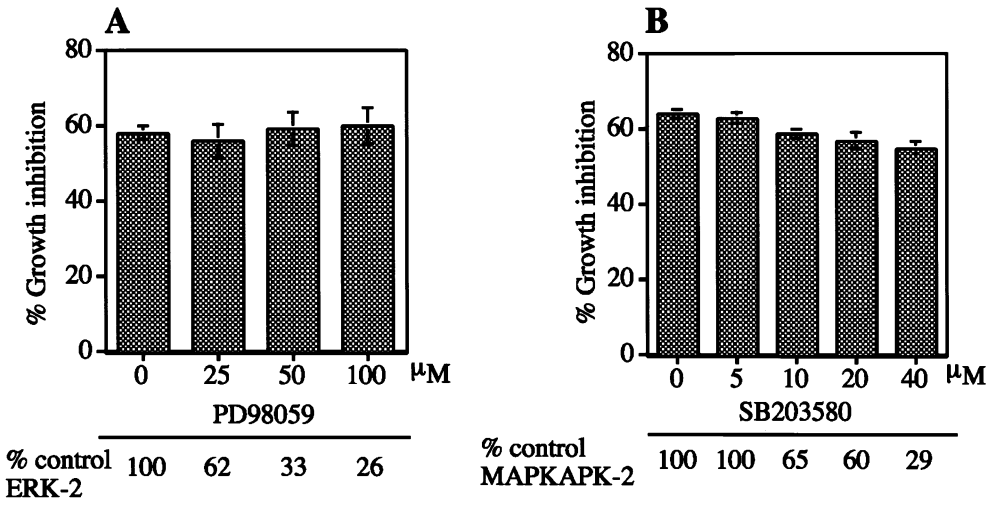


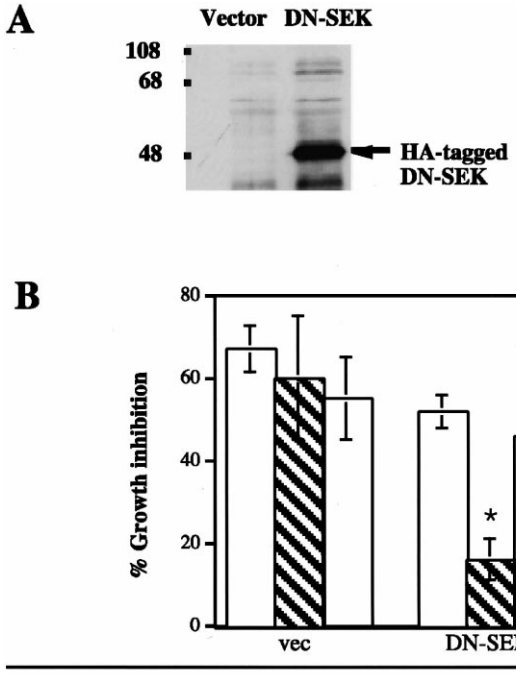
Fig. 4. Reduction in ERK and p38 activities does not affect BCR-induced growth inhibition. BAL-17 cells were pretreated for 1 h with graded concentrations of the MEK1-specific inhibitor, PD98059 (A) and the p38-specific inhibitor, SB203580 (B), after which they were cultured with 20 μg/ml $F(ab')_2$ fragments of anti-IgM Ab for 24 h. DNA synthesis was measured as described in Section 2. The results were expressed as mean percent growth inhibition $\{=100\ [1-(\text{anti-IgM Ab added})/(\text{no Ab added})]\} \pm$ S.E.M. of three independent experiments. Control cpms (without anti-IgM Ab) were not significantly different among groups. The effects of PD98059 and SB203580 on the activation of ERK-2 and p38 were determined by in vitro kinase assays of ERK and MAPKAPK-2 using MBP and hsp 25, respectively, as substrates. The results were expressed as mean control kinase activity of three experiments with the activity of untreated cells being 100%.

but not 50 μM PD98059, the BCR-induced growth inhibition was significantly rescued (Fig. 5B). Given that either DN-SEK1 transfection or SB203580 alone did not have significant effects on this process, the results may mean that BCR-induced growth inhibition is dictated by actions of JNK and p38.

4. Discussion

Three major findings are presented herein: first, the regulatory effects of CD45 on BCR-induced activation of ERK may differ from the effects on JNK and p38 in both WEHI-231 and BAL-17 cells; second, the effects of CD45 on JNK and p38 seem to be opposing in WEHI-231 and BAL-17 cells; and third, activation of both JNK and p38 is required for BCR-initiated inhibition of growth in BAL-17 cells.

We have previously reported that in WEHI-231 cells, CD45 negatively regulates Lyn kinase activity [19,20], and BCR-induced growth inhibition and apoptosis [9]. The present study further demonstrates that negative regulation of Lyn by CD45 correlates with the regulatory mode of downstream events such as activation of JNK and p38 and induction of growth



anti-IgM	+	+	+	+	+	+
SB203580	-	+	-	-	+	-
PD98059	-	-	+	-	-	+
% control activity						
ERK	100	100	30	90	106	23
JNK	100	100	100	46	45	70
MAPKAPK-2	100	60	90	100	57	100

→

Fig. 5. JNK and p38 synergistically rescue BCR-induced inhibition of BAL-17 cell growth. A: Expression of HA-tagged DN-SEK1. BAL-17 cells were transfected with HA-tagged DN-SEK1 or an empty vector, and the expression of DN-SEK1 was assessed by immunoblotting with anti-HA mAb. B: Transfection of DN-SEK1 and exposure to SB203580, but not to PD98059, significantly reduced BCR-induced inhibition of BAL-17 cell growth. Cells transfected with DN-SEK1 or empty vector were cultured with 20 μg/ml $F(ab')_2$ fragments of anti-IgM Ab in the presence or absence of 50 mM PD98059 or 10 μM SB203580 for 24 h, and DNA synthesis was assayed as described as above. The results were expressed as mean percent growth inhibition ± S.E.M. of four independent experiments. *Represents a statistically significant difference ($P < 0.05$ by Student's *t*-test) between vector and DN-SEK transfectants. Activation of ERK and p38 was measured by kinase assays using MBP and hsp 25, respectively, as substrates. Activation of JNK was examined by immunoblotting with anti-pJNK Ab. Mean percent control values of four experiments were calculated by densitometric analysis. S.E.M. of mean percent control kinase activities were all < 2.

inhibition. Consistent with these findings, one recent study conducted in the chicken DT40 B cell line suggested that BCR-evoked MAPK activation is regulated at the level of PTKs [21]. What is more, CD45 regulation of JNK and p38 activation parallels the induction of growth inhibition in BAL-17 cells [10]. Our preliminary results demonstrated that Fyn, but not Lyn, is a substrate for CD45 in BAL-17 cells. Thus, one possibility is that opposing effects of CD45 in WEHI-231 and BAL-17 cells may be dependent on which Src-family kinase is initially regulated by CD45. Alternatively, there may be molecules among substrates for Lyn and Fyn or other CD45 substrates that act as switches for positive or negative signaling.

Data from transgenic mouse models indicate that one of the mechanisms regulating positive and negative immune responses involves differences in the nuclear signals activated [22]. In naive B cells, a foreign Ag induces activation of ERK, JNK, NF-κB and nuclear factor of activated T cells (NFAT), whereas self Ag activates NFAT and ERK but not NF-κB or JNK. Additionally, CD45-deficient, tolerant B cells do not induce any of these signals [22]. Several studies have addressed the question of which members of MAPK family are critical to BCR-induced apoptosis or activation [21,23–28]. For example, it has been shown that activation of ERK appears to correlate with BCR-induced apoptosis in WEHI-231 cells [23,24], while activation of all three members, ERK, JNK and p38, correlates with cell survival [23]. In contrast, other studies demonstrated that activation of JNK and p38 correlates with anti-IgM-induced apoptosis in human B104 B lymphoma cells, which express both IgM and IgD on their cell surfaces [25,26]. In chicken DT40 B cells, BCR-induced apoptosis was blocked in Syk- and Btk-deficient cells, where JNK was not activated [21,27,28], but not in wild-type or Lyn-deficient cells, where JNK was activated [21,27]. However, all these data only suggest correlation between MAPK activation and the final outcomes.

To examine the contribution of JNK to BCR-induced signaling, we used DN-SEK1. One of the reasons is that kinase-dead, DN-JNK is not as potent an inhibitor as DN-SEK1. Moreover, DN-SEK1 is just as selective as DN-JNK since it does not interfere with p38 MAPKs nor does it tie up regulation of nuclear targets. Use of dominant negatives does have limitations but at present there are no good alternatives for the JNK pathway. Our result demonstrated that exposure of DN-SEK1 transfectants of BAL-17 to a specific p38, but not MEK1, inhibitor synergistically rescued BCR-induced growth inhibition, indicating for the first time a causal relationship between activation of both JNK and p38 and induction of growth inhibition. Because CD45 crucially regulates BCR-induced growth inhibition in BAL-17 cells [10], the effect of CD45 on the final outcome of BCR signaling is likely mediated in part through JNK and p38 pathways. What molecules are then linking CD45 to the activation of MAPKs? The fact that two small G proteins, Ras and Rac/Cdc42, respectively regulate ERK and JNK/p38 pathways makes it reasonable to hypothesize that CD45 may exert its differential effects via Ras and Rac/Cdc42. It is also possible that CD45 affects targets situated downstream of the pathways, for example, MAPKKKs or MAPKKs. How substrate PTKs regulated by CD45 in turn contribute to MAPK activation in a member-specific manner is a question for future study.

In conclusion, our results suggest that signals generated through CD45 may differentially regulate BCR-induced activation of MAPK family members, eliciting opposing effects in immature and mature B cell lines. Furthermore, our data indicate that JNK and p38 cooperatively dictate the BCR-induced fate of B cells, a process to which CD45 contributes positively.

Acknowledgements: We thank Dr. Gary Koretzky for valuable reagents and Dr. Gema Alonso for critical technical advice. This work was supported in part by Grants-in-Aid for Scientific Research and for International Scientific Research from the Ministry of Education, Science, Sports and Culture.

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