CD45 Is Required for CD40-induced Inhibition of DNA Synthesis and Regulation of c-Jun NH₂-terminal Kinase and p38 in BAL-17 B Cells*

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Stimulation of B cell antigen receptor (BCR) may induce proliferation, differentiation, or apoptosis, depending upon the maturational stage of the cell and the presence or absence of signals transmitted via coreceptors. One such signal is delivered via CD40; for instance, ligation of CD40 rescues B cells from BCR-induced apoptosis. Here we show that, in contrast to WEHI-231 cells, CD40 ligation did not reverse BCR-induced growth inhibition in the BAL-17 mature B cell line and CD40 ligation itself inhibited proliferation. This inhibitory signaling was not observed in CD45-deficient cells. Further analyses demonstrate that transfection of dominant-negative form of SEK1 or treatment with SB203580 strongly reduced CD40-induced inhibition of BAL-17 proliferation, suggesting a requirement for c-Jun NH₂-terminal kinase and p38 in CD40-induced inhibition of proliferation. Interestingly, CD40-initiated activation of c-Jun NH₂-terminal kinase and p38 was enhanced and sustained in CD45-deficient cells, and these phenotypes were reversed by transfecting CD45 gene. However, CD40-mediated induction of cell surface molecules was not affected in CD45-deficient cells. Taken collectively, these results suggest that CD45 exerts a decisive effect on selective sets of CD40-mediated signaling pathways, dictating B cell fate.

The fate of B cells is determined by the signals from antigen receptor (BCR). These signals may be influenced by a variety of factors including antigen binding strength and the maturational stage of the cell. Accumulating evidence indicates that signals transmitted via coreceptors are also critical to the final outcome of B cells; one such coreceptor that appears to be functionally important is CD40 (1–3). CD40, a member of tumor necrosis factor receptor/nerv growth factor receptor family, has a cysteine-rich extracellular domain and a short cytoplasmic tail without enzymatic activity and is expressed on a variety of cells including B cells, dendritic cells, and epithelial cells. In B cells, CD40 is known to mediate proliferation, maturation, memory cell induction, germinal center formation, and class switching of immunoglobulin (Ig) gene (1–5).

Signaling via CD40 not only protects germinal center B cells and several B lymphoma cells from spontaneous and BCR-induced apoptosis, respectively (6–9), but also suppresses the growth of B lymphoma cells (10–12) as well as mesenchymal-epithelial cells (13–15). Thus, CD40 may transmit both positive and negative signals depending upon the cell type, implying a complex regulation of CD40 signal transduction.

Numerous studies have been performed in determining specific pathways of CD40-initiated positive signaling. CD40 ligation has been demonstrated to activate several different signaling pathways, for example, activation of tyrosine kinases (Lyn, Fyn, and Syk), phosphatidylinositol 3-kinase, phospholipase C-γ2, Jak3-signal transducer and activators of transcription 3, and nuclear factor κB (NF-κB) (16–21). CD40 signaling also enhances the expression of P-ac, Bcl-xL, Cdk4, and Cdk6 (22, 23), as well as various membrane molecules including CD23 (FceRII) (24), CD45 (ICAM-1) (25), CD80 (B7–1) (26), CD86 (B7–2) (27), CD95 (Fas) (28), and MHC class II (29).

Furthermore, members of mitogen-activated protein kinase (MAPK) family are differentially activated by CD40 ligation. Three MAPK members have been identified: extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK), or stress-activated protein kinase), and the p38 MAPK. Although CD40-mediated activation of ERK is dependent on the cell types, JNK and p38 are activated in B cells and B cell lines by ligation via CD40 (30–33), and p38 is reported to be required for CD40-induced B cell proliferation and NF-κB activation in B cell lines and tonsillar B cells (34). However, the molecular basis for the negative effects of CD40 remains to be elucidated.

In the present study, we show that, in contrast to WEHI-231 cells, BCR-induced growth inhibition was not rescued by CD40 ligation in BAL-17 cells; indeed, CD40 ligation itself inhibited proliferation of BAL-17 cells in a dose-dependent manner. Significantly, in CD45-deficient cells, the CD40-induced inhibition of proliferation was not observed and activation of JNK and p38 was enhanced and sustained. The phenotype of CD45-deficient cells was reversed by transfecting CD45 cDNA, suggesting a decisive role for CD45 in these processes. Further analyses revealed that transfection of dominant-negative form of SEK1 into BAL-17 or treatment with a p38-specific inhibitor
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reduced CD40-induced inhibition of proliferation, suggesting that activation of JNK or p38 is crucial to CD40-initiated proliferative regulation. Additionally, induction of cell surface molecules upon CD40 ligation was not affected in CD45-deficient cells. Thus, these results suggest that CD45 critically regulates selective sets of signaling events induced by CD40 ligation, determining the fate of BAL-17 cells.

EXPERIMENTAL PROCEDURES

Cells—The WEHI-231 and BAL-17 murine B cell lines and the BAL-17-derived, CD45-deficient clone 44 were all described previously (35, 36). All cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 50 μg 2-mercaptoethanol, 100 μg/ml streptomycin, and 100 units/ml penicillin (complete medium). To assess DNA synthesis, 0.5 × 10⁶ triplicates in 0.2 ml of complete medium with or without anti-CD40 mAb or anti-IgM Ab for 24–48 h. To assess DNA synthesis, 0.5 × 10⁶ triplicates in 0.2 ml of complete medium with or without anti-CD40 mAb or anti-IgM Ab for 24–48 h. To assess DNA synthesis, 0.5 × 10⁶ triplicates in 0.2 ml of complete medium with or without anti-CD40 mAb or anti-IgM Ab were incubated with specific Abs for overnight at 4 °C in 0.5% gelatin (Crozatier, University of Pennsylvania, Philadelphia, PA). The cells were then resuspended in complete medium and incubated for 24–36 h. After incubation in complete medium for 1 h

Immunoblot Analysis—After incubation in complete medium for 1 h at 37 °C, 5–10 × 10⁶ cells were stimulated with 0.5 μg/ml of P(αb)γ fragments of goat anti-IgM antibody (Ab) and fluorescent isothiocyanate-conjugated (FL) goat anti-IgM Ab were purchased from mature B cell line BAL-17, as compared with immature B cell line WEHI-231. WEHI-231 and BAL-17 cells were cultured with 1–20 μg/ml anti-CD40 mAb in the presence or absence of P(αb)γ fragments of anti-IgM Ab for 48 h, and DNA synthesis was assayed. As shown in Fig. 1, stimulation with 1–20 μg/ml anti-CD40 mAb did not significantly affect the proliferative capacity of WEHI-231 cells and completely reversed anti-IgM-induced growth arrest, as reported previously (9). In BAL-17 cells, by contrast, anti-CD40 mAb stimulation did not rescue cells in anti-IgM-induced growth inhibition, but actually enhanced it. Moreover, CD40 ligation itself significantly and dose-dependently inhibited DNA synthesis of BAL-17 cells (Fig. 1).

CD40-induced Inhibition of BAL-17 Proliferation Is Strictly Dependent on CD45—CD40 ligation is known to inhibit proliferation not only in B lineage cells (10–12) but also in nonlymphoid cells (13–15). However, mechanisms underlying these phenomena are still not completely understood. We attempted to examine the role of CD45 in CD40-mediated signaling in BAL-17 cells. For this purpose, we utilized a CD45-deficient clone previously generated from BAL-17 cells (36). Flow cytometric analysis showed that, in clone 44 cells, expression of CD45 was completely absent, but that surface IgM levels and CD40 expression were comparable to those of the parent cells (Fig. 2). Expression of other molecules including sIgD, MHC class I and class II, Src family protein-tyrosine kinases (Lyn, Fyn, Blk, Lck), and Syk was also comparable in clone 44 to that in parental cells (36). No significant differences in protein tyrosine phosphorylation induced by CD40 ligation were observed between BAL-17 and clone 44 cells (data not shown).

We first asked whether CD40-evoked inhibition of DNA synthesis was observed in CD45-deficient, clone 44 cells, and as shown in Fig. 1, anti-CD40 mAb had no effect on the proliferative response of WEHI-231 cells, however, CD40-mediated effects on BAL-17 cells were not altered in CD45-deficient cells (data not shown). To confirm that the effect of anti-CD40 mAb in BAL-17 cells was mediated by CD45, we transiently transected Sce-CD45 cDNA or an empty vector back into clone 44 cells. We used Sac-CD45 cDNA, because it has been demonstrated that Src-CD45 is sufficient for reconstituting the function of CD45-
deficient T cell clones (38) and is more efficiently expressed than the full length. The transfectants efficiently expressed the CD45, as assessed by immunoblotting with anti-c-Src Ab (Fig. 3B). Control transfection with a vector containing enhanced green fluorescence protein revealed that transfection efficiency was 25–30%. Once CD45 gene was transfected, clone 44 cells were as susceptible to CD40-induced inhibition of proliferation as were BAL-17 cells (Fig. 3A).

Thus, in contrast to WEHI-231 cells, where CD40 transmits positive signals thereby reversing BCR-induced growth inhibition, in BAL-17 cells, CD40 exerts a negative effect on proliferation and on BCR-induced growth inhibition. More significantly, the data show that CD40-induced inhibition of BAL-17 proliferation was mediated through CD45.

CD40-induced Up-regulation of Cell Surface Molecules Is Not Controlled by CD45—CD40 ligation by CD40 mAb has been shown to up-regulate a variety of cell surface molecules. Therefore, to investigate signaling pathways governed by CD45, we first examined the capacity of anti-CD40 mAb to induce cell surface molecules on BAL-17 and clone 44 cells. When the cells were cultured with 10 μg/ml anti-CD40 mAb for 2 days and changes in the expression of CD54, CD80, CD86, CD95, and I-A molecules were assessed by flow cytometry, no difference in the degree to which these molecules were up-regulated by CD40 ligation in CD45-positive and CD45-deficient cells were found (Table I). In addition, reverse transcriptase-polymerase chain reaction analysis revealed that induction of the dual-specificity phosphatase, Pac-1, was also not significantly different in BAL-17 and clone 44 cells (data not shown).

CD40-induced Activation of JNK and p38 Is under the Control of CD45—To examine the extent to which CD45 regulates activation of MAPK family members, BAL-17 and clone 44 cells were cultured with 10 μg/ml anti-CD40 mAb for 5–60 min, and the lysates were subjected to immunoblotting with Abs against phosphorylated (activated) forms of ERK, JNK, and p38. CD40 ligation only marginally elevated ERK activation in BAL-17
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Introduction of CD45 gene into clone 44 cells reverses CD40-induced proliferation pattern. A, a vector containing Src-CD45 cDNA and an empty vector were transiently transfected into clone 44. Proliferation inhibition induced by 10 μg/ml anti-CD40 mAb was then assessed in BAL-17 (BAL), clone 44 (44), CD45-transfected clone 44 (S45), and vector-transfected clone 44 (Vec). The results are representative of three separate experiments and expressed as percentage of inhibition of DNA synthesis ± S.E. B, expression of Src-CD45 was examined in untreated clone 44 (−), CD45-transfected clone 44 (S45), and vector-transfected clone 44 (Vec) by immunoblotting with anti-c-Src Ab. Exogenous CD45 was detected only in S45 cells, as indicated by an arrow.

Table I

Anti-CD40-mediated induction of cell surface molecules in BAL-17 and its CD45-deficient clone

BAL-17 cells and its CD45-deficient clone 44 were cultured for 2 days in the presence (+) or absence (−) of 10 μg/ml anti-CD40 mAb. After culture, cells were labeled with mAbs against CD54, CD80, CD86, CD95, and I-A<sup>A</sup>, and then subjected to flow cytometric analysis. The results are expressed as mean fluorescence intensities and are representative of three separate experiments.

| CD40 stimulation | BAL-17  | Clone 44  |
|------------------|--------|-----------|
|                  | (−)    | (+)       |
| CD54 (ICAM-1)    | 12.4   | 10.3      |
| CD80 (B7–1)      | 7.3    | 9.0       |
| CD86 (B7–2)      | 3.6    | 1.5       |
| CD95 (Fas)       | 8.5    | 14.2      |
| I-A<sup>A</sup>  | 7.8    | 9.1       |

DISCUSSION

There have been several reports that negative signaling evoked by CD40 ligation inhibits proliferation of B lymphoma cells (10–12). However, compared with the role played by CD40 in B cell survival and rescue from apoptosis, the molecular mechanisms by which CD40 inhibits proliferation have not been extensively studied. In the present study, we demonstrated that, in contrast to WEHI-231 cells, CD40 ligation in the mature BAL-17 cells does not reverse BCR-initiated inhibition of proliferation, but instead inhibits proliferation further (Fig. 1). Interestingly, CD40 ligation itself induced inhibition of BAL-17 cell proliferation and such inhibition was not observed in CD45-deficient clone 44 cells (Fig. 1). Transfection of the CD45 gene into the clone reversed this phenotype (Fig. 3), indicating a decisive role for CD45 in CD40-induced inhibition of BAL-17 cell proliferation.

To elucidate the mechanisms by which CD45 exerts its regulatory effects on CD40-induced inhibition of proliferation, we examined several signaling events mediated by CD40 ligation in BAL-17 and its CD45-deficient clone. As one of the activation events, CD40 ligation by anti-CD40 mAb was shown to up-regulate a number of cell surface molecules (24–29), and our findings suggest that CD45 is not involved in the CD40-induced up-regulation of CD54, CD80, CD86, CD95, and MHC class II (Table I). On the other hand, CD40-mediated activation of JNK and p38 was augmented and sustained in the CD45-deficient clone (Fig. 4), and introduction of the CD45 gene resulted in recovery of the parental phenotype (Fig. 5). Thus, CD45 negatively regulates CD40-mediated activation of JNK and p38 in BAL-17 cells.

The question now arises as to whether activation of JNK and p38 directly contributes to CD40-induced inhibition of proliferation in BAL-17 cells. Our results showed that inhibition of JNK and p38, respectively, by transfecting DN-SEK1 into...
BAL-17 cells and treatment with a p38-specific inhibitor, SB203580, blocks proliferation inhibition triggered by anti-CD40 mAb (Fig. 6), suggesting that activation of JNK or p38 is required for CD40-initiated inhibition of proliferation in BAL-17 cells. Given that CD40 ligation did not induce inhibition of proliferation in clone 44 cells, where JNK and p38 activities were enhanced and sustained, it is possible that the level and duration of JNK and p38 activation may need to fall within a narrow window for optimal CD40 signaling, meaning that CD40-induced inhibition of proliferation would be blocked when JNK and p38 are activated either above or below a certain threshold at a certain time. It has been shown that activation of JNK by CD40 ligation requires TRAF2 (40, 41). One signaling cascade leading to JNK activation is believed to proceed from membrane proximal molecule, Rac → MEKK1 → SEK1/MKK4 → JNK; where and how the signal from TRAF2 converges into this pathway is still unknown. The signal from CD40 is also capable of activating NF-κB through TRAF2 and NF-κB inducing kinase to the IκB kinase/IκB complex (20, 42–44). In addition, MEKK1 has been reported to be a common upstream activator of both JNK and NF-κB (45). It is thus also possible that CD45-generated signals leading to proliferation inhibition may not be mediated solely by JNK or p38 but by a concerted action of multiple signaling molecules including MAPKs and NF-κB, for example.

Role of CD45 in CD40 signaling has been investigated previously using CD45 mAb. One such study demonstrated that cross-linking CD45 inhibits CD40-induced proliferation of human peripheral blood B cells and small tonsillar B cells, but has no effect on large tonsillar B cells (46), suggesting that CD45 exerts its inhibitory effects on CD40-induced growth regulation in a cell type-dependent manner. Another study showed that CD45 mAb blocked tyrosine phosphorylation evoked by CD40 ligation in human Raji cells (16). However, these experiments do not enable one to draw any conclusion as to how CD45 may affect CD40 signaling. It was reported recently that CD40-induced proliferation was partially impaired in splenic B cells isolated from CD45 knockout mice (47). Given that CD45 is not involved in the regulation of CD40 signaling in WEHI-231 cells, the effect of CD45 on CD40 signaling may be dependent on the cell type or the maturational stage of B cells. One possible factor for differential effects is NF-κB. The NF-κB complexes in WEHI-231 and BAL-17 cells consist predominantly of c-Rel/p50 (48)

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mediated through activation of JNK and p38 MAPK, and CD40-induced activation of JNK and p38, but not induction of cell surface molecules, is under strict control of CD45. Thus, CD45 is involved in the regulation of selective sets of CD40-induced signaling pathways, determining the final outcome of BAL-17 B cells.

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Fig. 6. Activation of JNK and p38 is required for CD40-induced inhibition of proliferation. A, BAL-17 cells were transfected with a plasmid containing DN-SEK1 or an empty vector, and 24 h later the transfected cells were stimulated with 10 μg/ml anti-CD40 mAb for 10 min. Activation of JNK was then assessed by immunoblotting with anti-phospho-JNK and anti-JNK Abs. Numbers shown below indicate the fold increase of phospho-JNK upon CD40 ligation, with the intensity of the specific band normalized to that of the nonstimulated group set to 1. B, BAL-17 cells, untreated and transfected with DN-SEK1 or with an empty vector, were stimulated with 10 -fold increases of phospho-JNK upon CD40 ligation, with the intensity of the specific band normalized to that of the nonstimulated group set to 1. C, BAL-17 B cells were pretreated with 5–20 μg/ml anti-CD40 mAb for 24 h, after which DNA synthesis was measured as described in Fig. 1. The results are shown as percentage of inhibition of DNA synthesis ± S.E. and are representative of three experiments. C, BAL-17 cells were pretreated with 5–20 μg/ml anti-CD40 mAb for 24 h, after which DNA synthesis was measured as described in Fig. 1. The results are shown as percentage of inhibition of DNA synthesis ± S.E. and are representative of three experiments. C, BAL-17 cells were pretreated with 5–20 μg/ml anti-CD40 mAb for 24 h, after which DNA synthesis was measured as described in Fig. 1. The results are shown as percentage of inhibition of DNA synthesis ± S.E. and are representative of three experiments. C, BAL-17 cells were pretreated with 5–20 μg/ml anti-CD40 mAb for 24 h, after which DNA synthesis was measured as described in Fig. 1. The results are shown as percentage of inhibition of DNA synthesis ± S.E. and are representative of three experiments.

and c-Rel/p65, respectively, and the specific combination of NF-κB family members may strongly affect total transcriptional activity. Indeed, transcriptional activity was different between WEHI-231 and BAL-17 cells. Additionally, our preliminary studies indicate that CD40 ligation-mediated recruitment of TRAFs differs in WEHI-231 and BAL-17 cells. These differences may contribute to the final outcome of CD40 signaling in the two cell lines.

In summary, our results demonstrate that, in mature BAL-17 B cells, CD40-mediated signaling is unable to reverse BCR-induced inhibition of proliferation, and that CD40 ligation itself inhibits proliferation. Such inhibition is strictly regulated by CD45. Furthermore, CD40-initiated growth inhibition is

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