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Involvement of a Common 10-Amino-Acid Segment in the Cytoplasmic Region of CD40 but Different MAP Kinases in Different CD40-Mediated Responses

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CD40-mediated signals can induce cell aggregation, proliferation and rescue from apoptosis in WEHI231. To define which segment of cytoplasmic domain of CD40 and how signals are involved in those events, we generated mutant CD40 transfectants. We demonstrated the same 10 amino acid segment that could bind to tumor necrosis factor receptor associated factor-2 and -3 mediated all those responses. However, activation pattern of mitogen activated protein kinases was different. Immunoglobulin M-mediated apoptosis was inhibited by CD40-mediated signal that activated c-Jun aminoterminal kinase synergistically. While, CD40 stimulus through the 10 amino acid segment alone that induced cell aggregation and proliferation resulted in activation of extracellular signal-regulated protein kinase 2.

The function of CD40 has been extensively studied in B cells, where it is believed to play an important role in regulation of proliferation (1), apoptosis (2,3), Ig class switching (4), generation of memory B cells (5), activation of NFkB (6-9), modification of gene expression (6-9), upregulation of surface adhesion molecules (10), induction of homotypic cell aggregation (10,11), and germinal center formation (4,5). It was reported that amino acid segments around Thr^{234} in the cytoplasmic portion of CD40 was important for several CD40-mediated signals (3,12-18). Novel ring finger proteins, TRAF-2, -3, and -5 is associated with CD40 around Thr^{234} and important for CD40-mediated NFkB activation or up regulation of CD23 molecules (8,13,15,17,19). Recently, it was reported that different cytoplasmic segments were involved in different CD40-mediated responses (16,20). The purposes of this report was to investigate (i) which segment in the cytoplasmic domain of CD40 is necessary for CD40 mediated-cellular responses, such as inhibition of IgM-mediated apoptosis, cell aggregation, or proliferation and (ii) whether different MAP kinases are used in those CD40-mediated cellular responses. We demonstrated that (i) the 10 amino acid segment having Thr^{234} in the cytoplasmic region of CD40 was associated with both TRAF-2 and -3, (ii) this 10 amino acid segment was important for all CD40-mediated biological responses examined here and (iii) JNK was activated synergistically by CD40 stimulation through the 10 amino acid segment in the presence of IgM signaling. But ERK2 was activated by CD40 signal through the 10 amino acid segment in CD40-mediated cell aggregation and proliferation.

MATERIALS AND METHODS

Antibodies. Anti-human CD40 mAb, 5C3 was provided from Dr. Kikutani (Osaka University, Osaka, Japan). Anti-mouse IgM was purchased from Cappel (Durmam, NC). FITC-anti-mouse kappa chain Ab and FITC-anti-goat Ig Ab were purchased from IBL (Gunma, Japan), and Jackson Immuno-Research Lab (West Grove, PA), respectively. Anti-HA (Babco, Richmond, CA), anti-TRAF2, anti-TRAF3 (Santa Cruz, CA), HRP-anti-rabbit Ig (Jackson Immuno Research Laboratories, Inc.), anti-FLAG (Eastman Kodak Co., New Haven, CT), and avidin-HRP (Bio-Rad, Tokyo, Japan) were purchased.

Construction of mutant human CD40 cDNA and transfection. Human CD40 cDNA was amplified using forward primer CD40-5′: 5′ GCGAATTCCTAGACA 3′, and 4 different reverse primers, CD40-850-3′: 5′AAGCGGCCGCTCACTCTTTGCCATCCTC 3′, CD40-820-

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Abbreviations used: Ig, immunoglobulin; NFκB, nuclear factor kappa B; Thr, threonine; TRAF, tumor necrosis factor receptor-associated factor; MAP, mitogen activated protein kinase; JNK, c-Jun aminoterminal kinase; ERK, extracellular signal-regulated protein kinase; mAb, monoclonal antibody; Ab, antibody; FITC, fluorescein isothiocyanate conjugated; HA, hemaggulutinin; HRP, horseradish peroxidase; FcγR, Fcγ receptor; FCS, fetal calf serum; 2ME, 2-mercaptoethanol; Wt, wild type; PI, propidum iodide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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3′: 5′AAGCGGCCGCTCAGCATCCATGTAAAGT 3′, CD40-790-3′: 5′AAGCGGCCGCTCAAGCAGTGTTGGAGCC 3′, and CD40-760-3′: 5′AAGCGGCCGCTCAAAAATTGATCTCCTG3′. The resulted cDNA fragments were sequenced, reconstructed into pME18S and transfected to WEHI231 cells with pSV2neo. WEHI231 transfectants were selected as described previously (2).

Flow cytometric analysis. Transfectants was pretreated with anti-FcγR mAb, incubated with anti-human CD40 mAb followed by FITC-anti-mouse Ig Ab and analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

In vitro growth assay. In vitro growth assays were achieved as described (2). For CD40-mediated proliferation assay, cells were starved in RPMI1640 for 5 hours and then cultured in RPMI plus 10% of FCS without 2ME for 96 hours as described (2).

Surface labeling and immunoprecipitation. Surface iodination and immunoprecipitation were achieved as described (21).

In vitro kinase assay. Cells were starved in RPMI1640 for 5 hours and stimulated with anti-mouse IgM Ab or/and anti-human CD40 mAb for 8 minutes, and cellular extracts were prepared as described (22). The solid-state JNK assay was performed as described (22). ERK2 activity was assayed as described (23).

Western blot analysis. HA-tagged TRAF3-pCDNA3 plasmid or FLAG-tagged TRAF2-pRK5 were transfected with/without hCD40 mutants-pME18S into COS7 cells. Immunoprecipitation and western blotting analysis were performed as described (15).

RESULTS

Construction and expression of mutant human CD40 molecules. Four mutant human CD40 cDNA, CD40-850, -820, -790, and -760 which encoded truncated CD40 that had 248, 238, 228, and 218 amino acids (wild type hCD40 had 257 amino acids) were constructed and transfected to WEHI231 cells (Figure 1A). WEHI231 transfectants expressed human CD40 and mouse IgM molecules. Additionally, human CD40 molecules were immunoprecipitated on the expected positions (Figure 1B, C and data not shown).

The role of 10 amino acid segment in the cytoplasmic region of CD40 for abrogation of IgM-mediated apoptosis, induction of cell aggregation, and proliferation. Anti-mouse IgM Ab treatment inhibited cell growth in all WEHI231 transfectants and these growth inhibition was completely abrogated in the presence of anti-human CD40 mAb in WEHI-wt, -850, and -820 but not in WEHI231, WEHI-790, and -760 cells (Figure 2A and data not shown) indicating that IgM-induced cell death is inhibited by signaling through the 10 amino acid segment between CD40-790 and -820 molecules. This conclusion was confirmed by a quantitative assay for

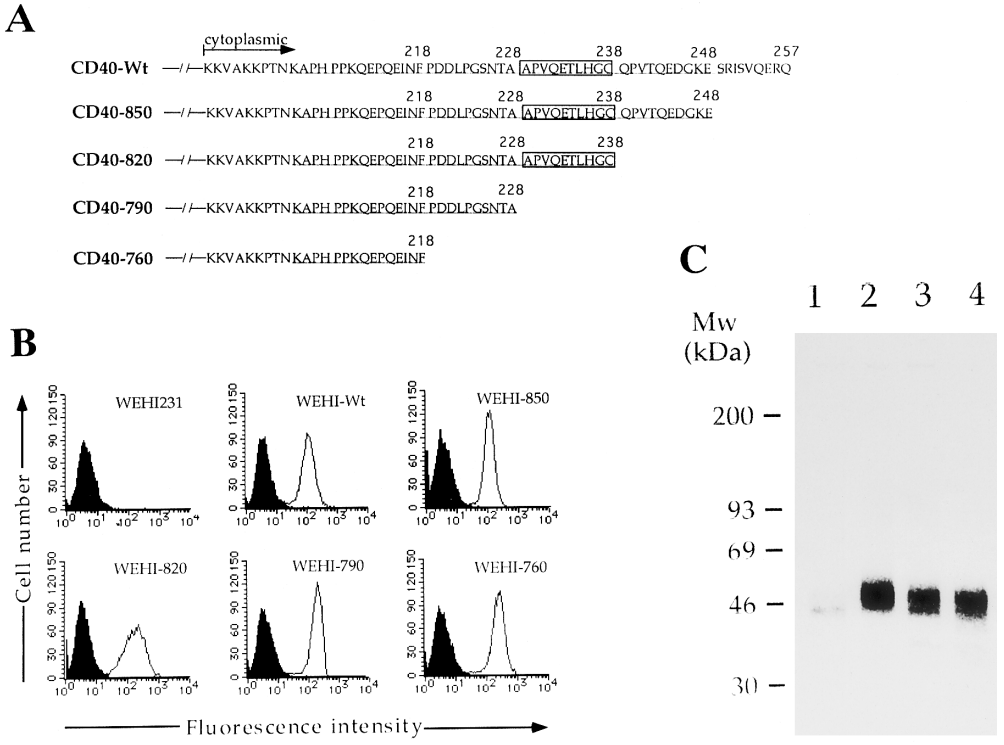

FIG. 1. (A) Schematic structure of mutant human CD40 molecules. Wild type and four mutant human CD40 molecules, CD40-850, -820, -790, and -760 having translational termination codons introduced at the position of the 258, 249, 239, 229, and 219th amino acid were constructed. (B) FACS analysis of human CD40 molecules. Cells were pretreated with anti-mouse FcγR mAb, 2.4G2 incubated with or without anti-human CD40 mAb followed by FITC-anti-mouse Ab and then analyzed by FACScan. (C) Immunoprecipitation of mutant CD40 molecules. WEHI231 (lane 1), WEHI-wt (lane 2), -820 (lane 3), and -790 (lane 4) cells were surface-iodinated, lysed in NP40 buffer and immunoprecipitated with anti-human CD40 mAb. Samples were analyzed under reducing condition on SDS-PAGE.

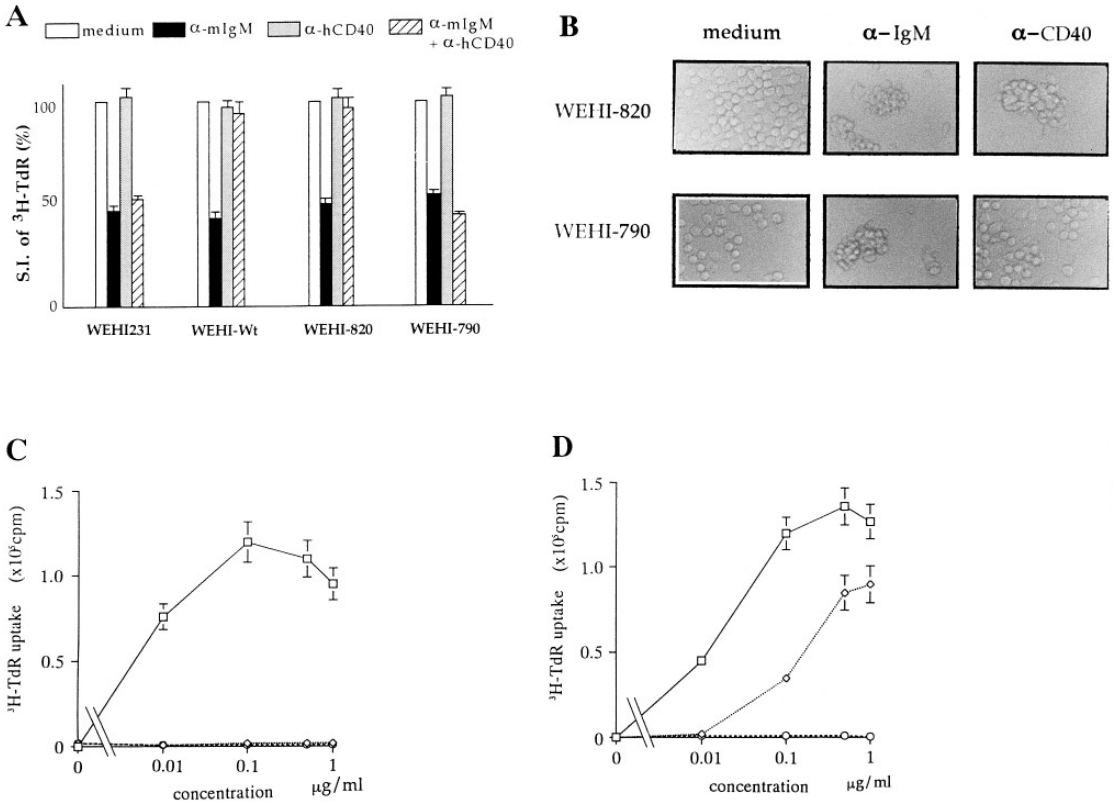

FIG. 2. The 10 amino acid segment was important for CD40-mediated responses. (A) CD40-mediated abrogation from IgM-dependent growth inhibition. WEHI231 transfectants were cultured with medium only (open column), anti-mouse IgM Ab (closed column), anti-human CD40 mAb (dotted column), or both anti-mouse IgM and anti-human CD40 antibodies (hatched column). Incorporated [^{3}H]-thymidine was measured. Data represent the stimulation index (S.I.), calculated from the incorporated radioactivity without antibodies. (B) CD40-mediated cell aggregation. WEHI-820 and -790 were cultured with anti-human CD40 mAb or anti-mouse IgM Ab for 24 hours. Then cell aggregation was analyzed by microscopy. (C) and (D) CD40-mediated cell proliferation. WEHI-790 (C) and -820 (D) cells were cultured with anti-mouse (square) or human (diamond) CD40 mAb or control mIgG (circle) for 96 hours. [^{3}H]thymidine uptake was measured.

apoptosis using propidium iodide (PI) (data not shown). Next, we investigated whether the 10 amino acid segment was important for CD40-mediated cell aggregation. WEHI-790 and -820 cells were incubated with anti-human CD40 mAb or anti-IgM Ab and cell aggregation was analyzed. As shown in Figure 2B, cell aggregation was observed in WEHI-820 but not in -790 cells upon treatment with anti-human CD40 mAb. On the other hand, using anti-mouse IgM Ab, both transfectants were aggregated (Figure 2B). To examine whether the same 10 amino acid segment was important for CD40-mediated cell proliferation, WEHI231 transfectants were cultured with anti-human or -mouse CD40 mAb. As shown in Figure 2C and D, although both transfectants proliferated in the presence of anti-mouse CD40 mAb, treatment with anti-human CD40 mAb induced dose-dependent proliferation in WEHI-820 cells but not in -790 cells.

Activation of MAP kinases by the same 10 amino acid segment. We investigated how CD40 signals through the 10 amino acid segment affect MAP kinases. JNK assay demonstrated that while incubation of WEHI-820 cells with either anti-mouse IgM Ab or anti-human CD40 mAb alone had weak or no effect on JNK activity (Figure 3), incubation with both antibodies resulted in synergistic activation of JNK. On the other hand, in WEHI-790 cells this synergistic activation could not be detected. ERK2 activity was enhanced by anti-mouse IgM Ab treatment in both WEHI-820 and -790 cells however anti-human CD40 mAb treatment induced significant activation of ERK2 only in WEHI-820 but not in -790 cells (Figure 3).

TRAF-2 and -3 were associated with the same 10 amino acid segment. We observed that WEHI231 cells expressed both TRAF-2 and -3 molecules (Figure 4A). Next, we examined whether TRAF-2 and -3 molecules bound to the 10 amino acid segment between CD40-790 and -820 molecules. As shown in Figure 4B, TRAF-2 and -3 molecules associated with wild type, CD40-850, and -820 but not with -790, and -760 molecules.

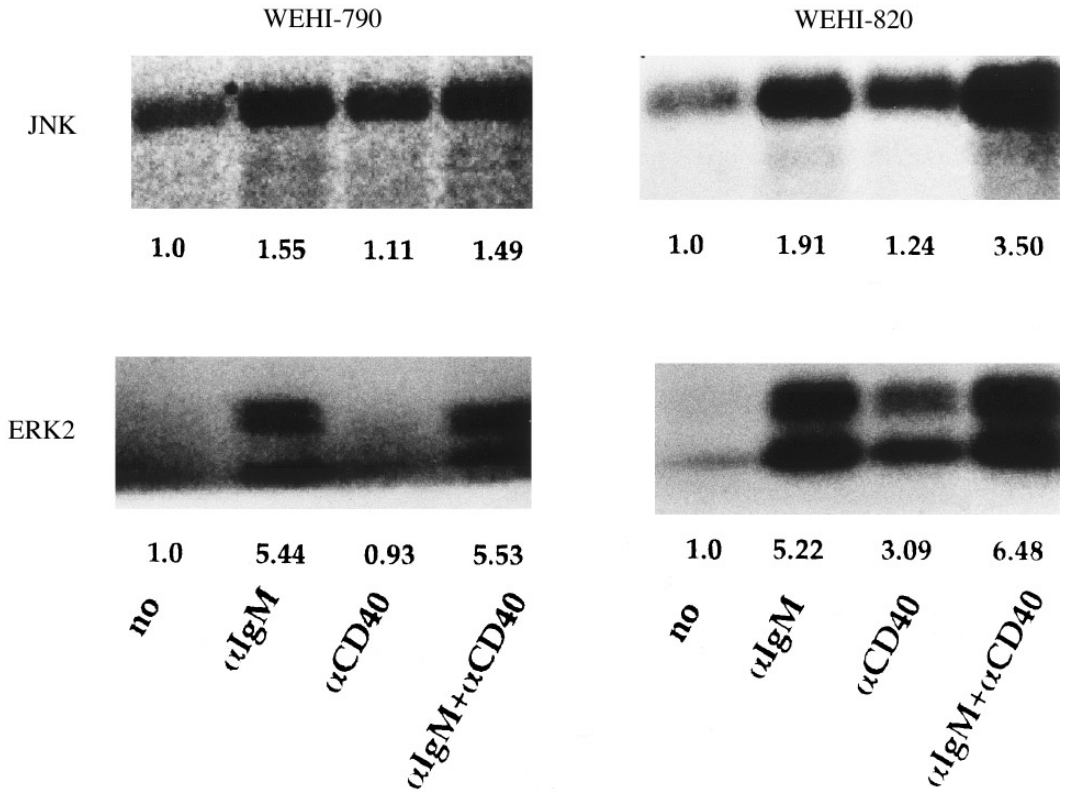

FIG. 3. In vitro kinase assay of JNK and ERK2. WEH-790 and -820 cells were cultured with anti-mouse IgM Ab or/and anti-human CD40 mAb. Kinase activities of JNK and ERK2 were analyzed as described in materials and methods. Relative densitometric indexes were indicated below.

DISCUSSION

The first issue to be discussed in this paper is the critical region of CD40 for its signal transduction. Mutagenesis studies suggested that amino acid segments having several phosphorylatable residues including Thr^{234} in the cytoplasmic region of CD40 molecules was essential for CD40-mediated (i) growth inhibition (12), (ii) expression of adhesion molecules (16), (iii) rescue from apoptosis (3), (iv) TRAF-2, -3, and -5 association (13,15,17). Additionally, it was speculated that phosphorylatable residues in the cytoplasmic tail of CD40 molecule was important for association of TRAF-2 and -3 (16). However, more recently several investigators pointed out that different cytoplasmic segments, one does not include Thr^{234} and the other includes Thr^{234} mediated different CD40-dependent responses (16) and CD40-mediated NFkB activation was induced by the association of TRAF-6 in the proximal cytoplasmic region to transmembrane portion of CD40 molecules (not including the 10 amino acid segment described in this paper) (20). We demonstrated that the 10 amino acid segment in the cytoplasmic region of CD40 that was associated with TRAF-2 and -3 is critical for at least 4 different CD40-mediated events, abrogation of IgM-mediated apoptosis, cell aggregation, proliferation, and ERK2 and JNK activation.

The next important issue to be discussed here is the involvement of kinases in different CD40-mediated events through the 10 amino acid segment. It was reported that MAP kinases, ERK, JNK, and p38 were activated by CD40-mediated signal (24-27), but there was no report describing which segment of the cytoplasmic domain of CD40 was important for activation of the MAP kinases. Here we clearly demonstrated (i) CD40 stimulation alone activated ERK2 and (ii) CD40 signal in the presence of IgM stimulation activated JNK synergistically through the 10 amino acid segment. Because IgM-mediated apoptosis was prevented by additional CD40 stimulation through the 10 amino acid segment, activation of JNK may be associated with suppression of apoptosis. Additionally, because not only CD40 mediated-ERK2 activation but also aggregation and proliferation were observed by CD40 signaling alone in WEHI-820 but not in -790 cells, it was reasonable to speculate that activation of ERK2 may be associated with CD40-mediated aggregation and proliferation.

It was reported that CD40-mediated activation of NFkB requires association of TRAF-2 or -5 molecules

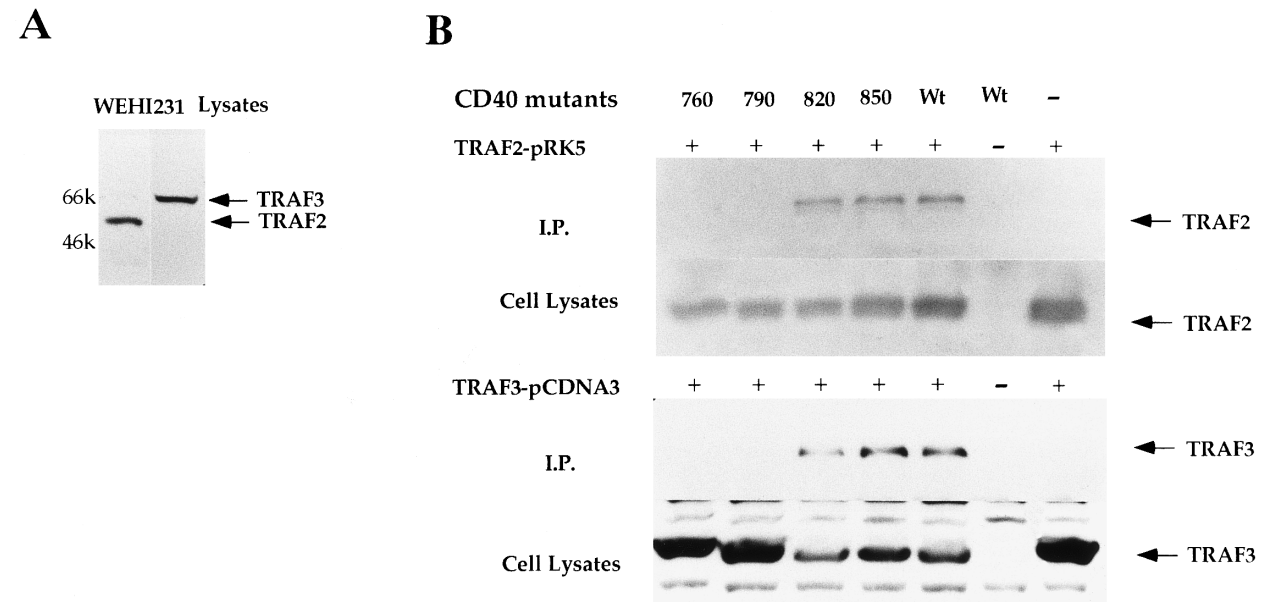

FIG. 4. TRAF-2 and -3 were associated with the 10 amino acid segment. (A) Expression of TRAF-2 and -3 in WEHI231 cells. Lysate of WEHI231 cells was immunoblotted using anti-TRAF-2 or -3 Ab as indicated. (B) Interaction of TRAF-2 or -3 with the 10 amino acid segment. COS7 cells were co-transfected with mutant CD40 and FLAG-tagged TRAF2 or HA-tagged TRAF3 as indicated. Cell lysates were immunoprecipitated with anti-CD40 mAb. Co-immunoprecipitated TRAF2 or TRAF3 were detected by western blot analysis using anti-FLAG or anti-HA Ab.

around Thr^{234} (8,17), and NFkB activity regulates many B cellular responses (9,28). Taken together, it is possible to speculate that in WEHI231 cells three different CD40-dependent responses, (i) aggregation, (ii) proliferation and (iii) rescue from apoptosis induced by IgM signaling are mediated by TRAF-2 and/or -3 and/or may be -5 through different MAP kinases, JNK (29) and ERK2 and then these cascades up-regulated transcription of anti-apoptotic or cell cycle related genes such as bcl-xL, cdk4, cdk6, c-myc, A20 (3,9,14) through activation of NFkB (6-9).

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REFERENCES

1. Clark, E. A., and Ledbetter, J. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4494–4498.
2. Tsubata, T., Wu, J., and Honjo, T. (1993) *Nature* **364,** 645–648.
3. Ishida, T., Kobayashi, N., Tojo, T., Ishida, S., Yamamoto, T., and Inoue, J. (1995) *J. Immunol.* **155,** 5527–5535.
4. Kawabe, T., Naka, T., Yoshida, K., Tanaka, T., Fujiwara, H., Suematsu, S., Yoshida, N., Kishimoto, T., and Kikutani, H. (1994) *Immunity* **1,** 167–178.
5. Foy, T. M., Laman, J. D., Ledbetter, J. A., Aruffo, A., Claassen, E., and Noelle, R. J. (1994) *J. Exp. Med.* **180,** 157–163.
6. Berberich, I., Shu, G. L., and Clark, E. A. (1994) *J. Immunol.* **153,** 4357–4366.
7. Francis, D. A., Karras, J. G., Ke, X., Sen, R., and Rothstein, T. L. (1995) *Int. Immunol.* **7,** 151–161.
8. Rothe, M., Sarma, V., Dixit, V. M., and Goeddel, D. V. (1995) *Science* **269,** 1424–1427.
9. Shauer, S. L., Wang, Z., Sonenshein, G. E., and Rothstein, T. L. (1996) *J. Immunol.* **157,** 81–86.
10. Barrett, T. B., Shu, G., and Clark, E. A. (1991) *J. Immunol.* **146,** 1722–1729.
11. Kansas, G. S., and Tedder, T. F. (1991) *J. Immunol.* **147,** 4094–4102.
12. Inui, S., Kaisho, T., Kikutani, H., Stamenkovic, I., Seed, B., Clark, E. A., and Kishimoto, T. (1990) *Eur. J. Immunol.* **20,** 1747–1753.
13. Hu, H. M., O'Rourke, K., Boguski, M. S., and Dixit, V. M. (1994) *J. Biol. Chem.* **269,** 30069–30072.
14. Sarma, V., Lin, Z., Clark, L., Rust, B. M., Tewari, M., Noelle, R. J., and Dixit, V. M. (1995) *J. Biol. Chem.* **270,** 12343–12346.
15. Cheng, G., and Baltimore, D. (1996) *Genes. Dev.* **10,** 963–973.
16. Hostanger, B. S., Hsing, Y., Harms, D. E., and Bishop, G. A. (1996) *J. Immunol.* **157,** 1047–1053.
17. Ishida, T., Tojo, T., Aoki, T., Kobayashi, N., Ohishi, T., Watanabe, T., Yamamoto, T., and Inoue, J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9437–9442.
18. Goldstein, M. D., and Watts, T. H. (1996) *J. Immunol.* **157,** 2837–2843.
19. Cheng, G., Ceary, A. M., Ye, Z., Hong, D. I., Lederman, S., and Baltimore, D. (1995) *Science* **267,** 1494–1498.

20. Ishida, T., Mizushima, S., Azuma, S., Kobayashi, N., Tojo, T., Suzuki, K., Aizawa, S., Watanabe, T., Mosialos, G., Kieff, E., Yamamoto, T., and Inoue, J. (1996) *J. Biol. Chem.* **271,** 28745–28748.

21. Murakami, M., Takahashi, Y., Isashi, Y., Kon, S., Inobe, M., Abe, R., and Uede, T. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7838–7842.

22. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes. Dev.* **7,** 2135–2148.

23. Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S., and Brugge, J. S. (1992) *Cell* **68,** 1031–1040.

24. Berberich, I., Shu, G., Siebelt, F., Woodgett, J. R., Kyriakis, J. M., and Clark, E. A. (1996) *EMBO J.* **15,** 92–101.

25. Kashiwada, M., Kaneko, Y., Yagita, H., Okumura, K., and Takemori, T. (1996) *Eur. J. Immunol.* **26,** 1451–1458.

26. Li, Y. Y., Baccam, M., Waters, S. B., Pessin, J. E., Bishop, G. A., and Koretzky, G. A. (1996) *J. Immunol.* **157,** 1440–1447.

27. Sutherland, C. L., Heath, A. W., Pelech, S. L., Young, P. R., and Gold, M. R. (1996) *J. Immunol.* **157,** 3381–3390.

28. Arsura, M., Wu, M., and Sonenshein, G. E. (1996) *Immunity* **5,** 31–40.

29. Liu, Z., Hsu, H., Goeddel, D. V., and Karin, M. (1996) *Cell* **87,** 565–576.