

B-Raf Contributes to Sustained Extracellular Signal-regulated Kinase Activation Associated with Interleukin-2 Production Stimulated through the T Cell Receptor*

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A T cell receptor (TCR) recognizes and responds to an antigenic peptide in the context of major histocompatibility complex-encoded molecules. This provokes T cells to produce interleukin-2 (IL-2) through extracellular signal-regulated kinase (ERK) activation. We investigated the roles of B-Raf in TCR-mediated IL-2 production coupled with ERK activation in the Jurkat human T cell line. We found that TCR cross-linking could induce up-regulation of both B-Raf and Raf-1 activities, but Raf-1 activity was decreased rapidly. On the other hand, TCR-stimulated kinase activity of B-Raf was sustained. Expression of a dominant-negative mutant of B-Raf abrogated sustained but not transient TCR-mediated MEK/ERK activation. The inhibition of sustained ERK activation by either expression of a dominant-negative B-Raf or treatment with a MEK inhibitor resulted in a decrease of the TCR-stimulated nuclear factor of activated T cells (NFAT) activity and IL-2 production. Collectively, our data provide the first direct evidence that B-Raf is a positive regulator of TCR-mediated sustained ERK activation, which is required for NFAT activation and the full production of IL-2.

T cells recognize self or non-self peptides in the context of major histocompatibility complex (MHC)¹-encoded molecules via T cell receptors (TCRs), and the signals are then transduced into the nucleus. These signals determine the fate of T cells and induce cytokine production, cytolytic activity, survival, apoptosis, and proliferation (1). Within seconds of MHC-peptide engagement, TCR components initiate phosphorylation cascades that trigger multiple branching signaling pathways. One well studied key switch is the activation signal of extracellular signal-regulated kinase 1/2 (ERK1/2), which is mediated by the small GTP-binding proteins, Ras (2, 3) and Rap1 (4, 5). Current models suggest that TCR stimulation with the agonistic peptide-MHC complex activates the conversion of Ras from the

GDP- to GTP-bound form (2, 6). Activated Ras subsequently recruits the serine/threonine kinase Raf-1 to the plasma membrane, resulting in its activation. Activated Raf-1 then activates ERK kinase (MEK), which directly phosphorylates tyrosine and threonine residues (TEY motif) on ERK1/2 to activate them (6). These signals combine to activate multiple transcription factors, including nuclear factor of activated T cells (NFAT), NF-κB, and activating protein-1 (AP-1), all of which contribute toward the production of IL-2 (7–9).

ERK1/2 are involved in a diverse array of cellular functions including cell growth and apoptosis of T cells (10–12). In ERK1-deficient mice, the thymocyte differentiation from CD4⁺CD8⁺ double positive to the CD4⁺/CD8⁺ single positive stage is impaired; thus, ERK activation by TCR ligation plays important roles in T cell development (13). Experiments using pharmacological inhibitors of MEK and dominant negative MEK also provided evidence that ERK1/2 are critical for thymocyte differentiation (11, 14) and for induction of TCR-mediated mitogenic signals and IL-2 production in mature T cells (7, 15). Hence, it is important to understand how the strength and duration of ERK activity is regulated in TCR-mediated activation and fate decisions of T cells.

The functions of ERK signaling are regulated by its upstream elements, in particular by members of the Raf family, in various cell types, and three Raf isoforms, Raf-1, A-Raf, and B-Raf, are expressed in mammalian cells (16, 17). Whereas Raf-1 is ubiquitously expressed, B-Raf shows a more restricted expression pattern (18, 19). Mice deficient in the different Raf isoforms exhibit different developmental defects, suggesting the nonredundant function(s) of each Raf isoform (20). A different phenotype of each Raf-deficient mouse is expected to be due, at least in part, to their distinct expression pattern. It was reported that B-Raf exhibits a much more basal kinase activity and a higher affinity toward MEK than does Raf-1 *in vitro* (21). Despite these differences, the specific function(s) *in vivo*, if any, of each Raf isoform is poorly understood. B-Raf was reported to be one component of the receptor-mediated MEK/ERK activation pathway in fibroblasts, B cell lines, and PC12 cells (21–26). Moreover, B-Raf expression in T cells is controversial; in this study, we detected B-Raf protein in Jurkat cells and primary human T cells, whereas others did not (4).

Although Raf-1 is a well characterized effector molecule for ERK activation in the TCR-mediated signaling cascade and IL-2 production in T cells (27), much less attention has been directed to the roles of B-Raf in T cells. We now report that interaction of B-Raf with MEK and B-Raf activity are induced in a TCR stimulation-dependent manner in Jurkat cells. Our data suggest that MEK/ERK activity are selectively regulated through the Ras/B-Raf signaling pathway and that the sustained B-Raf/MEK/ERK activation is indispensable for the

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¹ The abbreviations used are: MHC, major histocompatibility complex; AP-1, activating protein-1; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HA, hemagglutinin; IL-2, interleukin-2; MEK, mitogen-activated protein kinase/ERK kinase; NFAT, nuclear factor of activated T cells; TCR, T cell receptor; GFP, green fluorescent protein.

translocation of NFAT into the nucleus and for the production of IL-2.

EXPERIMENTAL PROCEDURES

Cell Preparations and Reagents—Jurkat cell clone, E6–1 from the American Type Culture Collection, and Jurkat cells expressing simian virus 40 large T antigen (TAg-Jurkat) (28) were maintained in RPMI 1640 medium (RPMI) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin (100 units/ml and 100 µg/ml, respectively). Jurkat cells stably expressing a wild-type or a dominant negative form of B-Raf were established and maintained in RPMI plus 10% fetal calf serum with 2 mg/ml G418. The human CD4⁺ T cell clone, YN 5–32 and peripheral blood mononuclear cells were prepared as described (29, 30). For transient and stable transfection, 2 × 10⁷ Jurkat cells were resuspended in 500 µl of cytomix (31) with the appropriate cDNAs. The amount of plasmid DNA was held at 40 µg constant by the addition of the pcDNA3 vector control. Cells were electroporated in 310 V at a capacitance of 960 microfarads. Transfectants were analyzed for CD3 and CD28 expression using flow cytometry (BD Biosciences). Anti-CD3 (clone UCHT-1) antibody, anti-CD28 (clone L923) antibody, and rabbit polyclonal anti-GFP antibody were purchased from Pharmingen. Anti-mouse IgG (Fab-specific) antibody was from Sigma. Mouse monoclonal anti-NFAT1 and anti-NFAT2 antibodies and rabbit polyclonal antibodies specific to Raf-1, B-Raf, MEK-1, c-Fos, and Lamin B were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies specific to MEK, phospho-ERK, phospho-p38, and phospho-MEK and a MEK inhibitor, U0126, were purchased from New England Biolabs (Beverly, MA). Mouse monoclonal anti-hemaggulutinin (HA) antibody was from Covance (Berkeley, CA). Cy3-labeled anti-rabbit Ig antibody, horseradish peroxidase-conjugated rabbit anti-mouse IgG, and donkey anti-rabbit IgG were from Amersham Biosciences. Anti-human IL-2 antibodies were from R & D Systems (Minneapolis, MN). Recombinant glutathione S-transferase (GST)-MEK was prepared as reported (32).

The pcDNA3 expression vectors with HA-tagged wild-type and dominant negative mutant B-Raf cDNAs were provided by Dr. K. L. Guan (33). The RasN17 expression vector was a gift from Dr. T. Kinashi (5). The luciferase reporter construct for IL-2 promoter and AP-1 binding site were kindly provided by Dr. V. A. Boussiotis (34) and Dr. R. M. Niles (35), respectively. The expression vector for GST-MEK was a gift from Dr. Y. Takai (32). NFAT-green fluorescence protein (GFP) reporter construct, consisting of three tandem NFAT-binding sites followed by a gene encoding GFP, was provided by Dr. T. Saito (36).

Cell Stimulation and Inhibitor Treatment—In experiments for stimulation with soluble anti-CD3 antibody for cross-linking, Jurkat cells were incubated on ice for 20 min and then incubated with anti-CD3 antibody (0.25 µg/ml) for 10 min followed by the addition of anti-mouse IgG antibody (1 µg/ml) for 5 min. After the indicated times of incubation at 37 °C, cells were harvested and lysed with lysis buffer (see below). For the analysis of promoter activity and IL-2 production, Jurkat cells (1 × 10⁶/well) were stimulated with immobilized anti-CD3 and CD28 antibodies (5 and 10 µg/ml, respectively). For experiments with inhibitor treatment, cells were preincubated for 30 min with the MEK inhibitor U0126 or Me₂SO as a control. In the time course analyses of the effect of ERK activation on IL-2 production by the treatment with U0126, medium containing U0126 or Me₂SO was added at the indicated time points.

Western Blotting, Immunoprecipitation, and in Vitro Kinase Assay—After the indicated times of stimulation, the Jurkat cells were recovered and lysed with lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 0.25% sodium deoxycholate, a protease inhibitor tablet (Roche Applied Science)). SDS-PAGE, Western blotting, and immunoprecipitations from the cell lysates were carried out as described (30). Raf-1 and B-Raf were immunoprecipitated from cell lysates of T cells with the anti-Raf-1 and the anti-B-Raf antibodies, respectively, as described above. The immunoprecipitates were resuspended in 25 mM HEPES (pH 7.5), 10 mM MgCl₂, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 10 µCi of [γ -³²P]ATP (Amersham Biosciences), and 0.8 µg of recombinant GST-MEK protein. Reaction mixtures were incubated at 32 °C for 20 min, and then the reactions were terminated by adding 5× SDS sample buffer, separated on 7% SDS-PAGE under the reducing condition, transferred to nitrocellulose membrane, and exposed to x-ray film. Relative amounts of MEK or ERK phosphorylation were calculated based on the ratio of the intensities of phospho-MEK or phospho-ERK bands to those of the whole MEK or ERK bands in whole cell lysates at each time point. Signal intensities of the bands were quantified by densitometric analysis using NIH Image 6.2 software. Nuclear

extracts were prepared from B-Raf mutant- or mock-transfected TAg Jurkat cells for nuclear translocation analysis of NFAT using nuclear/cytosol fractionation kits (BioVision).

Flow Cytometric Analysis and Cytokine Measurement—For the NFAT-GFP reporter assay, the TAg-Jurkat cells expressing B-Raf AA or mock vector were transfected with NFAT-GFP reporter construct and stimulated for 9 h with immobilized anti-CD3 and anti-CD28 antibodies. The expression of GFP was analyzed with a flow cytometer and CellQuest software (BD Biosciences). For intracellular staining, cells were fixed and permeabilized with IntraPrep (Immunotech, Marseille, France) and then stained with appropriate fluorescence-labeled antibodies. IL-2 concentrations in supernatants of T cell culture after 48 h of stimulation were measured in an enzyme-linked immunosorbent assay using anti-human IL-2 antibodies.

Reverse Transcription-PCR—Total RNA extraction and first-strand cDNA synthesis from T cells were done as described (37). The cDNA was subjected to PCR amplification using a set of primers specific for human B-Raf: 5'-ACAACAGTTATTGGAATCTCTGG-3' and 5'-AAATGCTAA-GGTAAAAACG-3'.

Luciferase Assay—Reporter constructs were transfected into the Jurkat cells expressing wild-type or mutant B-Raf. A β-galactosidase expression plasmid was co-transfected to normalize the variations in transfection efficiency. After 12 h of transfection, cells were harvested and stimulated. Luciferase assay was carried out according to the protocol in the Pica Gene kit (Toyo Ink, Tokyo, Japan). β-Galactosidase expression was assessed using the Luminescent β-galactosidase detection kit II (Clontech) according to the manufacturer's instructions.

RESULTS

Expression of B-Raf Proteins in Both Human and Mouse T Cells—We investigated the expression of B-Raf in human and mouse T cells using Western blotting (Fig. 1A). Rat PC12 cells, as a positive control gave a 95-kDa band corresponding to B-Raf (*lane 1*), whereas B-Raf expression was negligible in NIH3T3 cells, as reported (*lane 2*) (38). B-Raf was detected in a human CD4⁺ T cell clone, YN5–32 (*lane 3*) (29, 30), a human T cell line, Jurkat (*lane 4*), and mouse CD4⁺ T cells isolated from spleens (*lanes 5*). Furthermore, the expression of human B-Raf mRNA was assessed by reverse transcription-PCR using RNAs isolated from the YN5–32 T cell clone and from Jurkat T cells (data not shown). Intracellular staining and flow cytometric analysis also confirmed the B-Raf expression in human CD3-positive peripheral T cells (Fig. 1B) and mouse TCR-β chain-positive splenic T cells (Fig. 1B, b). Taken together, we conclude that B-Raf is expressed in both human and mouse T cells, allowing us to examine B-Raf functions in TCR-mediated T cell activation.

TCR Ligation Induces Both Raf-1 and B-Raf Activation—Cross-linking of TCRs with soluble anti-CD3 antibody, which mimics the engagement of TCR with the agonistic peptide-MHC complex, induced ERK and MEK phosphorylation within 1 min, reaching a maximal level at ~1–3 min in Jurkat cells (Fig. 2A). The ERK/MEK phosphorylations displayed similar kinetics and were prolonged for up to 60 min. Next, we performed *in vitro* kinase assays for Raf-1 and B-Raf to estimate the strength and kinetics of their kinase activities. Consistent with the previous report (27), Raf-1 was activated at 3 min after TCR ligation and became inactive within 20 min (Fig. 2B). In contrast to the kinetics of Raf-1 activity, there was slight but detectable B-Raf activity even under the basal condition, and B-Raf showed a pronounced increase of its kinase activity at 3 min after TCR stimulation. B-Raf kinase activity was gradually decreased but did last for up to 60 min (Fig. 2B). Raf-1 was inactivated after 20 min of TCR ligation; nevertheless, apparent MEK/ERK activation was still sustained up to 60 min (Fig. 2A). Intriguingly, the kinetics of TCR-mediated B-Raf activation rather than that of Raf-1 activation was similar to that of MEK/ERK activation. The addition of co-stimulation with an anti-CD28 antibody treatment slightly enhanced the B-Raf activity over time compared with that stimulated with an anti-CD3 antibody alone (Fig. 2C).

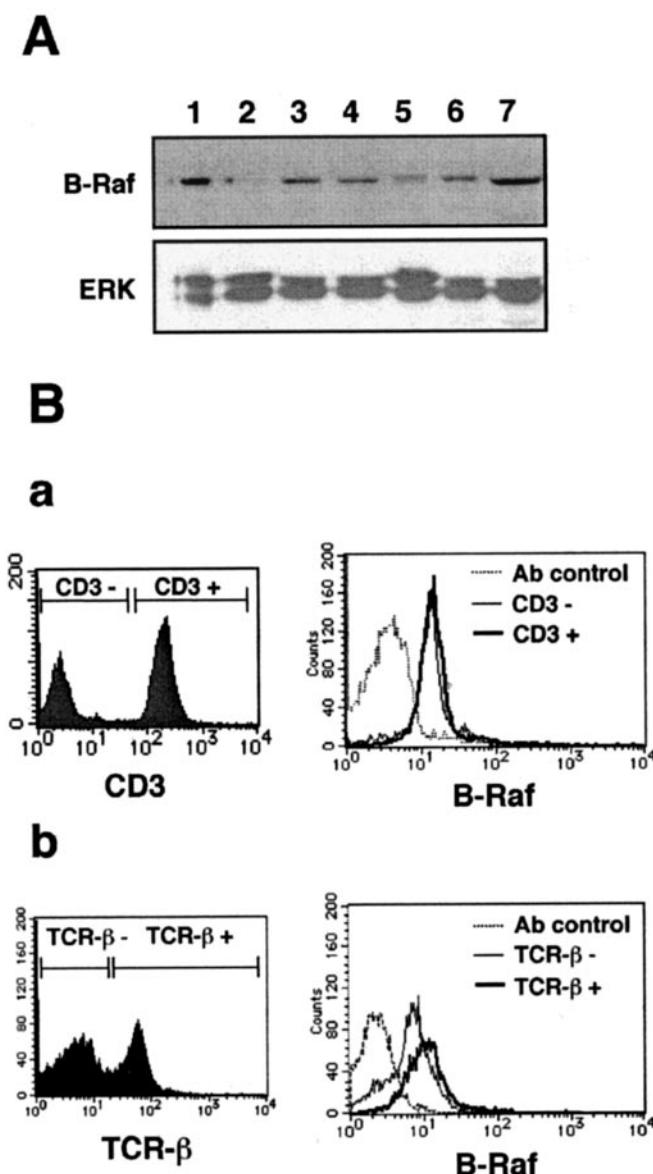


FIG. 1. B-Raf is expressed in both human and mouse T cells. **A**, Western blotting analysis using an anti-B-Raf antibody (top panel). Lane 1, PC12 cells; lane 2, NIH3T3 cells; lane 3, human CD4⁺ T cell clone, YN5-32; lane 4, Jurkat cells; lane 5, mouse CD4⁺ T cells isolated from spleen; lane 6, HeLa cells; lane 7, HeLa cells transfected with B-Raf. ERK blotting indicates comparable protein loading (bottom panel). **B**, flow cytometric analyses of B-Raf expression. Human peripheral blood mononuclear cells were stained with an anti-CD3 antibody (**a**) and murine spleen cells were stained with anti-mouse TCR-β chain antibody (**b**) (left panels). The CD3 or TCR-β chain negative and positive cell populations were gated, and intracellular B-Raf staining was carried out (right panels). An irrelevant rabbit polyclonal antibody was used as negative control for staining. *Ab*, antibody.

Physiological association between Raf family kinases and MEK is necessary for MEK/ERK activation (39); hence, we asked if B-Raf can interact with MEK in T cells in response to TCR stimulation using co-immunoprecipitation methods. For this purpose, wild-type B-Raf tagged with HA was expressed in Jurkat cells and was immunoprecipitated with an anti-HA antibody. The specific association between HA-B-Raf and MEK was achieved at a maximal level at 3 min after TCR ligation, and this interaction lasted for up to 60 min with a slight decrease (Fig. 2D). The intrinsic interaction between B-Raf and MEK was also evaluated by reciprocal immunoprecipitation experiments using an anti-MEK antibody to detect endogenous

B-Raf protein. As shown in Fig. 2E, endogenous B-Raf protein was not detected in immunoprecipitates with the anti-MEK antibody in unstimulated Jurkat cells. Consistent with Fig. 2D, intrinsic B-Raf-MEK complex formation was strongly induced at 3 min after TCR ligation, and then it decreased gradually but remained above the basal level up to 60 min after TCR stimulation *in vivo* (Fig. 2E). The kinetics of B-Raf/MEK interaction paralleled those of B-Raf activation (Fig. 2B). These results strongly suggested that B-Raf was involved in MEK/ERK activation stimulated with TCR ligation, especially in the late phase after Raf-1 had become inactive (Fig. 2B).

TCR-mediated B-Raf Activation Is Partly Dependent on Ras Activity—Previous studies reported that B-Raf activation in fibroblasts was dependent on Ras activation (40, 41). In other cases, Ras activity was not essential for B-Raf activation in PC12 cells (23, 38). In T cells, to determine whether Ras activity is required for the B-Raf activation, TCR-mediated B-Raf activity was measured in TAg-Jurkat expressing the dominant negative Ras mutant RasN17. The RasN17 interfered with endogenous Ras, Raf-1, and MEK/ERK activation until at least 60 min after TCR stimulation (data not shown) (2). As shown in Fig. 3, TCR engagement resulted in a robust activation of B-Raf after stimulation in mock-transfected Jurkat cells. In contrast, RasN17-transfected cells showed decreased B-Raf activation as compared with that observed in the control cells at 3 min after TCR stimulation (75% reduction). Similar inhibitory effects were observed at any given time points. These results indicated that TCR-mediated B-Raf activation is, at least in part, regulated by Ras activation *in vivo*.

B-Raf Contributes to Sustained MEK/ERK Activation—Within the activation segment of B-Raf, there are two sites, Thr⁵⁹⁸ and Ser⁶⁰¹, that can be phosphorylated in response to Ras activation, and the phosphorylation status of these residues is required for the maximal kinase activity of B-Raf (33, 40). Hence, we introduced a dominant negative mutant of B-Raf (B-Raf AA), in which Thr⁵⁹⁸ and Ser⁶⁰¹ were substituted to Ala (33), into T cells to examine the role of B-Raf in TCR-mediated MEK/ERK activation cascade. As shown in Fig. 4A, a Jurkat clone expressing B-Raf AA showed a similar degree of MEK/ERK activation induced by TCR cross-linking with soluble anti-CD3 antibody at 3 min after stimulation in comparison with that of mock-transfected cells. The MEK/ERK activation was effectively sustained for 60 min in the mock transfectants. On the other hand, in the Jurkat clone expressing B-Raf AA, MEK/ERK activation returned to the basal level within 30 min after TCR stimulation, and then it was no longer detected. Densitometric analyses of MEK/ERK activation revealed that the activation kinetic pattern rather than the relative magnitude of MEK/ERK activation was distinct between the mock-transfected clone and the B-Raf AA-expressing clone (Fig. 4B). Since TAg-Jurkat cells transiently transfected with B-Raf AA showed an essentially similar response, the possibility that these results were specific for one particular clone (AA2) was excluded (Fig. 4C). Moreover, these results were not due to the inhibition of Raf-1 activity by B-Raf AA, because the degree of TCR-mediated Raf-1 activation in B-Raf AA-expressing Jurkat cells was indistinguishable from that of mock-transfected Jurkat cells or cells expressing HA-tagged wild-type B-Raf (Fig. 4D and data not shown). In contrast to MEK/ERK activation, no significant differences in phosphorylation of another mitogen-activated protein kinase, p38, were detectable in both mock- and B-Raf AA-transfected TAg-Jurkat cells, suggesting that B-Raf AA did not influence p38 activation (Fig. 4C). The data indicate that B-Raf physiologically and specifically regulated prolonged MEK/ERK activation induced by TCR stimulation in Jurkat cells.

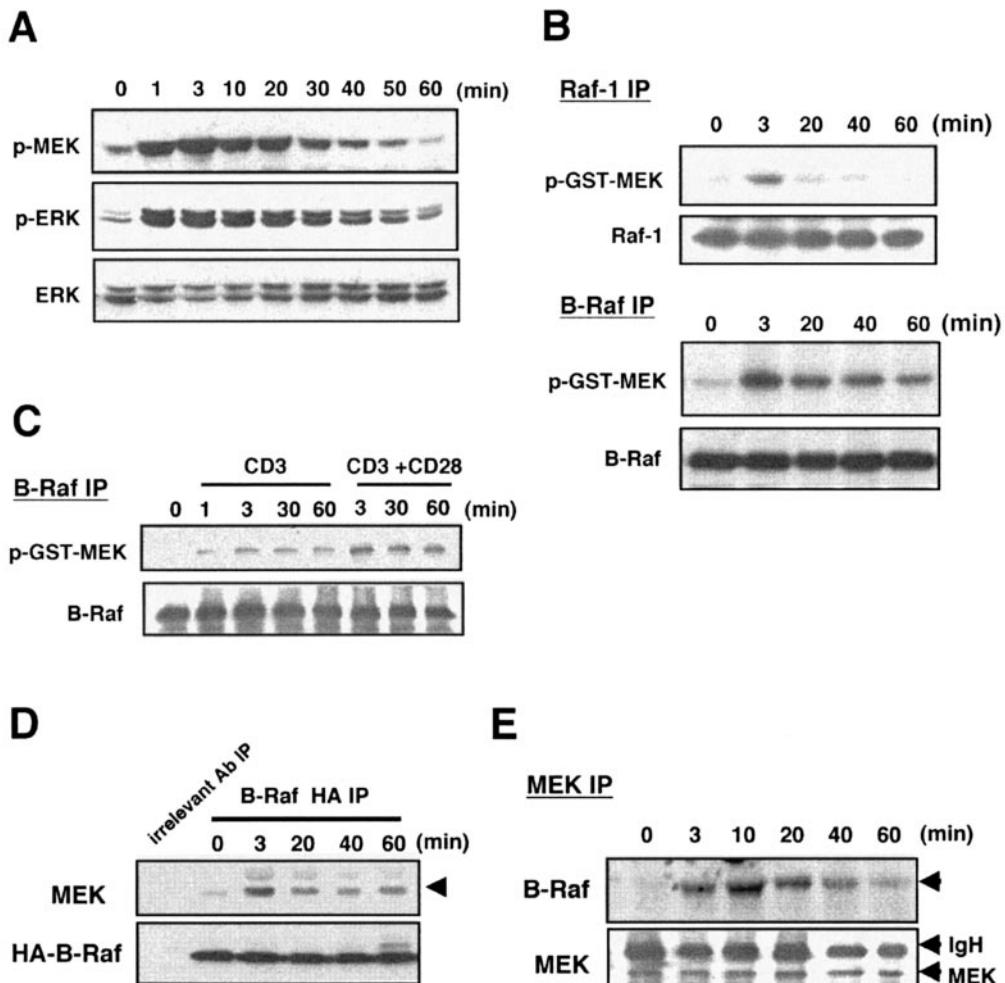


FIG. 2. B-Raf activation and B-Raf/MEK interaction were induced in a TCR stimulation-dependent manner. *A*, Jurkat T cells were incubated with or without (0 min) a soluble anti-CD3 antibody together with a second antibody for the indicated times. The cells were subjected to Western blotting with an anti-phospho-MEK-specific antibody (*top panel*) or an anti-phospho-ERK-specific antibody (*middle panel*). Equal protein loading was confirmed by total ERK blotting (*bottom panel*). *B*, *in vitro* kinase assays for Raf-1 and B-Raf isolated from Jurkat cells stimulated with the anti-CD3 antibody for the indicated times. Recombinant GST-MEK was used as a substrate, and incorporated 32 P radioactivities were visualized by autoradiography. Equal loading of each Raf protein was confirmed by blotting with either an anti-Raf-1 antibody (*upper bottom panel*) or an anti-B-Raf antibody (*lower bottom panel*). *C*, additive anti-CD28 antibody stimulation enhanced the B-Raf kinase activity. Jurkat cells were stimulated with the anti-CD3 antibody alone or the anti-CD3 together with the anti-CD28 antibodies for the indicated times, and an *in vitro* kinase assay was performed. *D*, TAg-Jurkat cells transiently expressing HA-tagged wild-type B-Raf were stimulated with cross-linking of soluble anti-CD3 antibody for the indicated times. Immunoprecipitates with an anti-HA antibody were blotted with an anti-MEK (*upper panel*) or the anti-HA antibodies (*lower panel*). The immunoprecipitates with irrelevant rabbit IgG in Jurkat cells stimulated for 3 min was used as a negative control. *E*, immunoprecipitates with an anti-MEK antibody from Jurkat cells stimulated with the anti-CD3 antibody for the indicated times were blotted with the anti-B-Raf antibody (*upper panel*). The same membrane was reprobed with the anti-MEK antibody (*lower panel*) to monitor equal protein loading. The data are representative of three reproducible experiments in all analyses. *Ab*, antibody; *IP*, immunoprecipitation.

B-Raf Activation and Subsequently Sustained ERK Activation Is Required for Full IL-2 Production—Since IL-2 production is one of the most critical events of ERK-mediated T cell activation, we first utilized the reporter assay controlled by the IL-2 promoter element to investigate the effect of B-Raf activation on IL-2 promoter activity. Whereas TCR stimulation resulted in induction of luciferase, which reflected the IL-2 promoter activity in wild-type B-Raf-transfected clone (WT30), B-Raf AA significantly attenuated the inducible IL-2 promoter activity (Fig. 5*A*). Indeed, as shown in Fig. 5*B*, TCR stimulation induced a marked increase in IL-2 production in mock-transfected Jurkat cells and in wild-type B-Raf-expressing clones (WT30 and WT34), whereas it was substantially reduced in B-Raf AA-expressing clones (AA2 and AA23).

The data described above clearly indicate that T cells expressing B-Raf AA had defects in sustained ERK activation and subsequent full IL-2 production in comparison with the control

cells in response to TCR stimulation. However, whether the sustained ERK activation is directly correlated with the full IL-2 production remained to be solved. To clarify this issue, we investigated the requirement of TCR-mediated sustained ERK activation for the IL-2 production using the pharmacological MEK inhibitor U0126. As shown in Fig. 5*C*, TCR-mediated ERK activation was inhibited by U0126 at the range of 5–10 μ M. In addition to ERK activation, IL-2 production provoked by stimulation with immobilized anti-CD3 and CD28 antibodies was markedly blocked by U0126 at the same range of concentrations.

We also examined the effects of B-Raf AA on the magnitude and period of ERK activation stimulated with immobilized anti-CD3 and CD28 antibodies. It must be noted that, as compared with the stimulation by cross-linking of soluble anti-CD3 antibody with the second antibody (Fig. 2*A*), stimulation with immobilized anti-CD3 and -CD28 antibodies resulted in a re-

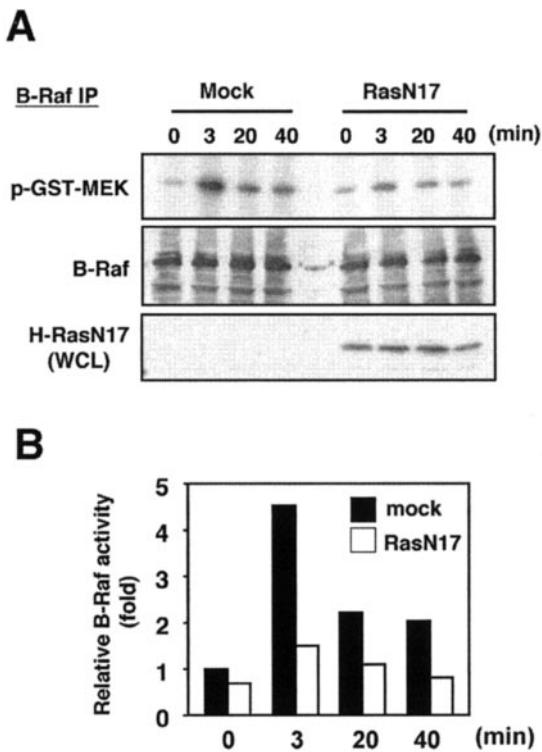


FIG. 3. Ras-regulated B-Raf activation following TCR stimulation in Jurkat cells. *A*, TAg-Jurkat cells were transfected with a mock vector or with the RasN17 expression vector, and then these cells were harvested and stimulated with cross-linking of soluble anti-CD3 antibody for the indicated times. Immunoprecipitates from each cell extract with an anti-B-Raf antibody were mixed with the recombinant GST-MEK as a substrate, and *in vitro* kinase reactions for B-Raf were performed. Blotting with an anti-B-Raf antibody showed equal protein loading (*middle panel*). Whole cell lysates (*WCL*) were blotted with anti-H-Ras antibody to monitor the expression of RasN17 (*lower panel*). *B*, the intensity of the GST-MEK phosphorylation by B-Raf immunoprecipitated from mock-transfected (*black bar*) or RasN17-transfected cells (*white bar*) was quantified by densitometric analysis. The relative B-Raf activity at 0 min in mock-transfected cells was assigned to be 1.0. Essentially similar results were obtained in three independent experiments. *IP*, immunoprecipitation.

tardation of ERK activation and extended ERK activation in mock-transfected cells (Fig. 5*D*). Such temporal differences in ERK activation have been reported, and the authors suggested that this phenomenon was due to the difference in TCR occupancy (42). As shown in Fig. 5*D*, in mock-transfected cells, TCR stimulation induced an accumulation of active ERK within 0.5 h, and this lasted for 6 h, whereas the sustained ERK activation over 2 or 3 h was impaired in cells expressing B-Raf AA. The data also confirmed that B-Raf was required for sustained ERK activation. Based on the results of Fig. 5*C*, 5 μ M U0126 was used to determine whether the sustained ERK activation that can be suppressed by B-Raf AA, as shown in Fig. 5*D*, was required for the maximal IL-2 production. Continuous treatment of T cells with U0126 over the period of TCR stimulation abolished IL-2 production (Fig. 5*E*). Interestingly, the addition of U0126 after 2 or 4 h of TCR stimulation also reduced IL-2 production to a degree comparable with that of cells treated with U0126 from the beginning of stimulation, although the intense ERK activation was induced for up to 2 h after stimulation. The same condition in which B-Raf AA inhibited the sustained ERK activation can be reproduced by treatment of Jurkat cells with U0126 after 2 or 4 h of TCR stimulation. Therefore, not only the intense ERK activation in the early phase but also the sustained ERK activation in the late phase was necessary for maximal IL-2 production. Con-

comitantly, these results suggested that the defect of IL-2 production in Jurkat cells expressing B-Raf AA was due to the lack of potential to maintain the TCR-mediated sustained ERK activation although the transient ERK activation was intact.

AP-1 Activation Induced by TCR Ligation Is Not Impaired in Jurkat Cells Expressing B-Raf AA—To define more precisely the biochemical mechanisms underlying the relationship between B-Raf-dependent ERK activation and IL-2 production, we first investigated the TCR-mediated c-Fos induction, one of the downstream targets of ERK (43). As shown in Fig. 6*A*, the expression of c-Fos was induced within 1 h, and its phosphorylation judged by electrophoretic mobility shift was potentiated by TCR stimulation in cells expressing wild-type B-Raf. There was no significant difference in c-Fos induction between Jurkat clones expressing wild-type B-Raf and B-Raf AA up to 3 h (Fig. 6*A*). Next, to examine whether B-Raf contributed to AP-1 activation, we performed a luciferase assay. Consistent with c-Fos induction, the AP-1 promoter activity in response to TCR stimulation in the Jurkat clone expressing B-Raf AA (AA2) was comparable as compared with that of the control clone (WT30) (Fig. 6*B*). Thereby, TCR-mediated c-Fos induction and AP-1 activation seemed to be less dependent on B-Raf.

B-Raf Activity Is Important for TCR-mediated NFAT Activation—The IL-2 production is regulated by nuclear translocation and activation of the NFAT transcription factor cooperating with the AP-1 components c-Fos and c-Jun (7–9). Thus, NFAT-dependent transcriptional events in T cells require the simultaneous activation of multiple Ras effectors such as the ERK and c-Jun N-terminal kinase pathways (44). We analyzed whether TCR-mediated B-Raf activity would influence NFAT activation, using an NFAT-GFP reporter. GFP expression, which is regulated by a promoter corresponding to the NFAT binding site, is increased in a TCR stimulation-dependent manner in mock-transfected cells (Fig. 7*A*, *a*). Comparable transfection efficiency was monitored by co-transfection of a DsRed expression vector (data not shown). In contrast, GFP expression was significantly suppressed in B-Raf AA-expressing cells, suggesting that B-Raf activity is important for the regulation of TCR-mediated NFAT activity. Given that B-Raf regulated TCR-mediated MEK/ERK activation in late phase, there is a possibility that B-Raf activation couples NFAT activation to MEK/ERK activation. For confirmation, we analyzed whether the inhibition of ERK activity in the late phase blocks NFAT reporter activity. As expected, the TCR stimulation-induced GFP expression was reduced by pretreatment with U0126 (Fig. 7*A*, *b*). Furthermore, similar to IL-2 production, the inhibition of NFAT reporter activity was also observed in the presence of U0126 after 2 h of TCR stimulation, although this suppression was less effective than that observed in simultaneous U0126 treatment at the beginning of the TCR stimulation. These results suggested that not only transient but also sustained ERK activation was necessary for the TCR-mediated NFAT activation.

Upon TCR stimulation, NFAT proteins are dephosphorylated by calcineurin, translocate into the nucleus, and then bind to cognate DNA elements (9). Finally, to dissect the mechanism responsible for the B-Raf mediated induction of NFAT activity, we evaluated the nuclear translocation of NFAT protein induced by TCR stimulation. As shown in Fig. 7*B*, the stimulation of mock-transfected Jurkat cells with TCR ligation drove the translocation of NFAT1 and NFAT2 into nucleus at 3 and 5 h of TCR stimulation. In contrast, the substantial nuclear translocation of NFAT1 and NFAT2 could not be observed in B-Raf AA-expressing Jurkat cells under either nonstimulated or TCR-stimulated conditions. Equal loading of nuclear protein in both cells was estimated by blotting of Lamin B as a nuclear

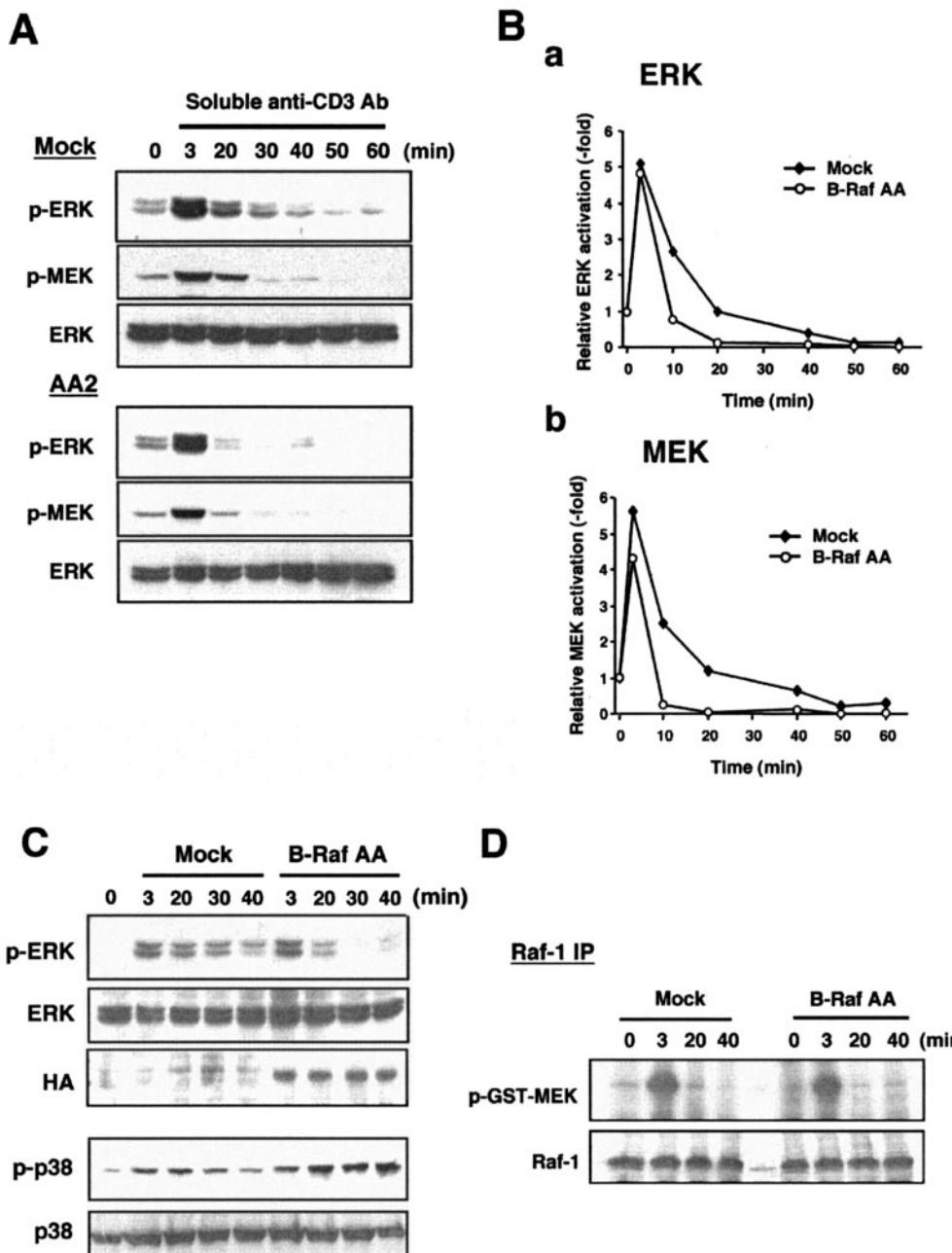


FIG. 4. Dominant negative B-Raf AA prevented T cells from inducing sustained MEK/ERK activation in response to TCR ligation. *A*, phosphorylation kinetics of MEK and ERK in Jurkat clones expressing wild-type B-Raf or B-Raf AA (AA2) induced by TCR cross-linking with soluble anti-CD3 antibody. *B*, kinetics of relative amount of phosphorylated ERK (*a*) and MEK (*b*). The relative value of intensity of phosphoprotein bands divided by that of whole ERK bands at each time point observed in mock- or B-Raf AA (AA2)-expressing clone were plotted. The ratio at 0 min was assigned to be 1.0. *C*, Western blotting analyses were done as described in *A* using whole cell lysates from TAg-Jurkat cells transiently transfected with mock or B-Raf AA expression vector and stimulated for the indicated times. Blotting with anti-phospho-ERK, ERK, HA (B-Raf), phospho-p38, or p38 antibodies are shown. *D*, TAg-Jurkat cells transiently transfected with mock vector or with B-Raf AA expression vector were stimulated with TCR cross-linking for the indicated times. *In vitro* kinase assays for Raf-1 were performed using immunoprecipitates from each cell extract with an anti-Raf-1 antibody (*upper panel*). Blotting with anti-Raf-1 antibody indicated equal protein loading (*lower panel*). Each result from three independent experiments was essentially the same, and one is shown.

marker. It is most likely that the defect of NFAT activity in B-Raf AA expressing cells was due to the aberrant nuclear translocation of NFAT1 and NFAT2. Accordingly, these results suggest that TCR-mediated NFAT activation relies on prolonged B-Raf/MEK/ERK activation and that the attenuation of NFAT activation by B-Raf AA reflects the inhibition of TCR-stimulated IL-2 production.

DISCUSSION

Although it is well known that receptor-mediated signals activate the Raf/MEK/ERK cascade, the precise mechanisms of

how the TCR signal provokes the cellular response through Raf/MEK/ERK activation remain to be investigated. In mouse models, both Raf-1- and B-Raf-deficient mice resulted in embryonic lethality (20, 45), indicating conclusively that the functions of both Raf isoforms for embryogenesis are not completely overlapping. However, it is poorly understood whether the three Raf isoforms have functional redundancy or if the Raf isoforms play a specific role(s) in T cell activation. Until recently, Raf-1 has been considered to be a major signaling mediator for MEK/ERK activation in TCR-stimulated T cells (27, 46). Our observations provided evidence that the functions of

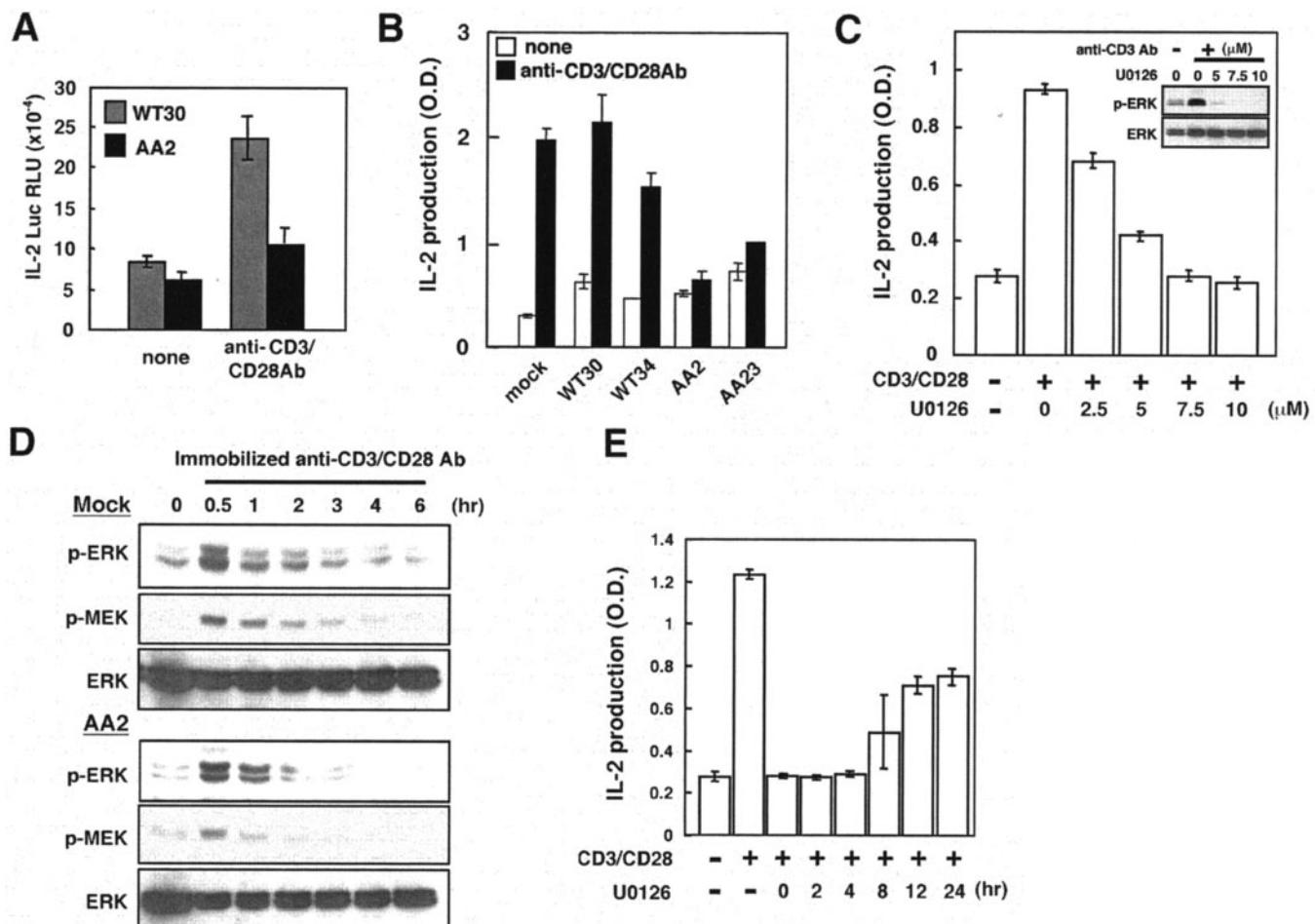


FIG. 5. Sustained ERK activation controlled by B-Raf is critical in TCR-mediated production of IL-2. *A*, luciferase assay for IL-2 promoter activity. Jurkat clone expressing wild-type B-Raf (WT30) or B-Raf AA (AA2) was transfected with an IL-2-luciferase construct and incubated with or without immobilized anti-CD3 and anti-CD28 antibodies for 12 h. Each luciferase activity was evaluated and normalized by the co-transfected β -galactosidase activity. RLU, relative luciferase unit. *B*, Jurkat clones expressing wild-type B-Raf (WT30 and WT34), B-Raf AA (AA2 and AA23), and mock-transfected were stimulated with immobilized anti-CD3 and anti-CD28 antibodies for 48 h. IL-2 in the culture supernatants was measured by enzyme-linked immunosorbent assay. *C*, Jurkat cells were pretreated with MEK inhibitor U0126 at the indicated concentrations for 30 min before stimulation and then were stimulated with an anti-CD3 antibody for 3 min, and phosphorylation of ERK was analyzed with Western blotting (*insets*). For measurement of IL-2, Jurkat cells pretreated with U0126 at the indicated concentrations were stimulated with immobilized anti-CD3 and anti-CD28 antibodies for 48 h. IL-2 in the culture supernatants were measured by enzyme-linked immunosorbent assay. *D*, mock-transfected (*upper panel*) or B-Raf AA expressing Jurkat clone (AA2; *lower panel*) were stimulated with the immobilized anti-CD3 and anti-CD28 antibodies for the indicated times. The whole cell extract from each sample was analyzed by blotting with anti-phospho-ERK (*top panel*), phospho-MEK (*middle panel*), or ERK (*bottom panel*) antibodies, respectively. *E*, vehicle (Me_2SO) or U0126 (5 μM) was added to the culture at the indicated times after the beginning of stimulation of Jurkat cells with immobilized anti-CD3 and anti-CD28 antibodies. After 48 h from the start of stimulation, each culture supernatant was harvested, and the IL-2 concentration was measured, as described in *C*. Typical data from three independent and reproducible experiments are presented here.

B-Raf for TCR-mediated activation do not entirely overlap with those of other Raf proteins. We elucidated that B-Raf activation couples Ras with TCR-mediated MEK/ERK activation and is indispensable for prolongation of substantial MEK/ERK activation *in vivo*.

This sustained MEK/ERK activation correlates with duration and strength of B-Raf activity. We considered that activation thresholds and the mechanisms regulating each Raf activity lead to distinct activation kinetics of these two Raf kinases. In agreement with this interpretation, the following observations were reported. Although the activities of both Raf-1 (47) and B-Raf (Fig. 3) were dependent on Ras activity, in addition to Ras, Src family kinases regulated Raf-1 activity (48). Upon activation, Raf-1 was shown to be phosphorylated on some tyrosine, serine, and threonine residues, which fulfill the regulatory functions, and the phosphorylation status of these sites in Raf-1 is different from that of B-Raf. First, the major target site of Src is Tyr³⁴⁰ in Raf-1; however, B-Raf activity seemed to be less dependent on Src, and Ras activation is sufficient for

B-Raf function because B-Raf lacks the Tyr corresponding to Tyr³⁴⁰ in Raf-1 (41, 49). Thus, B-Raf activation requires Ras but not Src to activate MEK/ERK, whereas Raf-1 activation needs the synergy of Ras and Src tyrosine kinase(s) (41, 49). Second, conserved B-Raf Ser⁴⁴⁵, corresponding to Ser³³⁸ in Raf-1, which is one of the regulatory phosphorylation sites of Raf activity, is constitutively phosphorylated in fibroblasts (49). These results seem to explain the fact that B-Raf exhibits a higher intrinsic kinase activity in a quiescent situation and, once stimulated, a longer activation period than does Raf-1 in our system and other systems.

It should be noted that the dominant negative mutant of B-Raf (B-Raf AA) did not impair the transient MEK/ERK activation but did suppress the sustained MEK/ERK activation although B-Raf was activated and associated with MEK in both the early and the late phase after TCR stimulation. Why was not MEK/ERK activation in the early phase drastically attenuated by B-Raf AA? The most likely explanation is that Raf-1 can compensate for the defects of B-Raf activation due to the

functional redundancy between Raf-1 and B-Raf in early phase. The idea was supported by our observations; the B-Raf AA did not grossly perturb ERK activation when Raf-1 was active in 3–20 min after TCR stimulation (Figs. 2 and 4), suggesting that Raf-1 activity is sufficient to induce ERK activation in the early

phase. On the other hand, ERK activation in the late phase (~20 min) was abrogated by B-Raf AA, because the kinase activity of Raf-1 declined, and Raf-1 could no longer compensate for B-Raf activity. Consequently, although we could not exclude the possibility that Raf-1 activity in early phase modulates the TCR-mediated B-Raf activation, our observations led to the model that Raf-1 activity is responsible and sufficient for the early phase MEK/ERK activation, whereas B-Raf activity is essential for the late phase MEK/ERK activation in TCR-stimulated T cells.

ERK activation is critical for the precise outcome of T cell activation, including IL-2 production (7, 15). The marked decrease in IL-2 production in T cells by the expression of B-Raf AA and by the inhibition of the late phase ERK activation using U0126 leads to the conclusion that ERK activation in the late phase regulated by B-Raf was critical for the full IL-2 production in response to TCR stimulation. In view of no defect of TCR-mediated c-Fos induction and AP-1 activation in Jurkat cells expressing B-Raf AA, it was indicated that these events were less dependent on B-Raf activity. In contrast to AP-1 activation, we found that B-Raf AA inhibited TCR-mediated nuclear translocation of NFAT and NFAT-mediated reporter activation (Figs. 6 and 7). The correlation between B-Raf and these transcriptional factors was also noted by Brummer *et al.* (24), who reported that in B-Raf null chicken B cells, B cell receptor-mediated ERK activation was eliminated in only late phase, whereas c-Fos induction was not abrogated. On the contrary, the loss of B-Raf expression resulted in significant defects in the B cell receptor-mediated activation of NFAT transcription factor, suggesting that NFAT activation is regulated by B-Raf in chicken B cells. The selective role of TCR-mediated B-Raf activation in NFAT regulation was consistent with that observed in B cells, and their regulatory mechanisms may be conserved between immunoreceptor-mediated activation in both B and T cells. These results suggest that NFAT responsible transcriptions and subsequent IL-2 production were dependent on B-Raf and that Raf-1-induced ERK activation in the early phase is not sufficient to provoke these immunoreceptor-mediated activations.

It was expected that the inhibitory effect of B-Raf AA on NFAT activation was due to a defect in sustained ERK activation mediated by B-Raf, because the treatment of Jurkat cells with MEK inhibitor also reduced the NFAT activation. Evi-

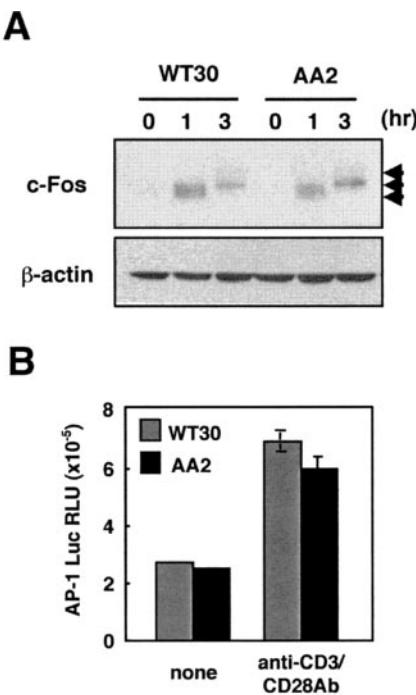


FIG. 6. B-Raf activity does not influence c-Fos induction and AP-1 activation. *A*, Western blotting analysis using an anti-c-Fos antibody (*upper panel*). Jurkat clones expressing wild-type B-Raf (WT30) or B-Raf AA (AA2) were stimulated with immobilized anti-CD3 antibody for the indicated times. Blotting with an anti-β-actin antibody indicated equal loading of proteins (*lower panel*). The arrowheads indicate the phosphorylated forms of c-Fos. *B*, luciferase assay for AP-1 promoter activity. Jurkat clones expressing wild-type B-Raf (WT30) or B-Raf AA (AA2) together with an AP-1-luciferase construct were stimulated with immobilized anti-CD3 and anti-CD28 antibodies for 8 h followed by a 12-h culture. Each luciferase activity was measured and normalized by the co-transfected β-galactosidase activity. RLU, relative luciferase unit. The data are representative of three independent and reproducible experiments. *Ab*, antibody.

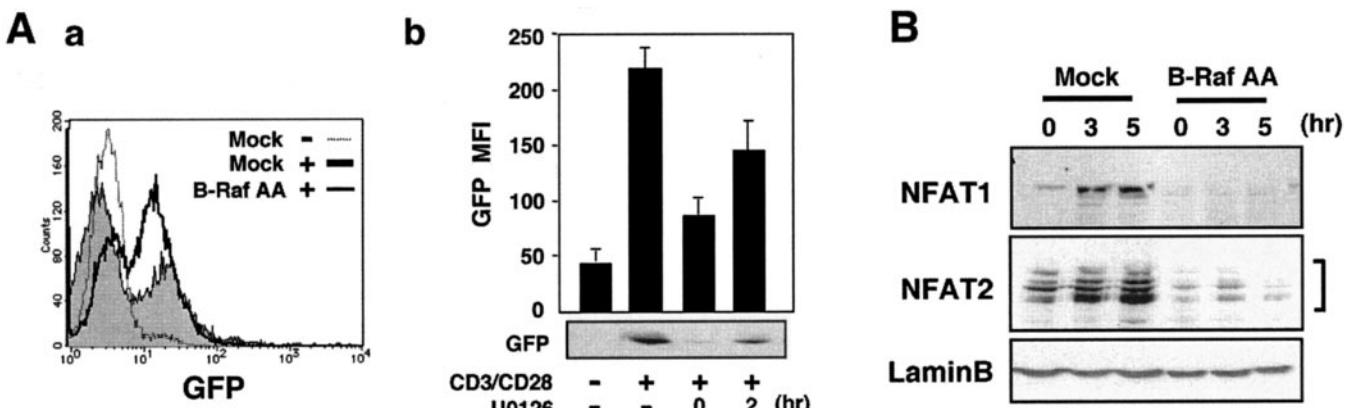


FIG. 7. B-Raf activity is required for NFAT activation. *A*, *a*, TAg-Jurkat cells co-transfected with the NFAT-GFP reporter construct and mock or B-Raf AA expression vector were stimulated with (+) or without (−) immobilized anti-CD3 and anti-CD28 antibodies for 9 h. A representative flow cytometric profile for GFP expression is shown. *b*, NFAT-GFP-transfected Jurkat cells were stimulated, and then GFP expression was examined by blotting with anti-GFP antibody (*lower panel*) and flow cytometry (*upper panel*). The diagram represents the average of GFP mean fluorescence intensity (MFI) obtained from three independent experiments. U0126 (5 μM) was added to the culture at 0 or 2 h after TCR stimulation. *B*, nuclear extracts were isolated from mock- or B-Raf AA-transfected cells stimulated with or without immobilized anti-CD3 and anti-CD28 antibodies for indicated times. Then, the nuclear fractions were separated by SDS-PAGE, and the translocations into nucleus of NFAT1 and NFAT2 were analyzed by Western blotting. Blotting with anti-Lamin B antibody indicated the appropriate nuclear fractionation and protein loading. Essentially similar results were obtained in three independent experiments.

dence has been accumulated that supports the contribution of ERK signaling to NFAT activation. In T cells, the transcriptional activity of NFAT was reported to be regulated by Ras/MEK/ERK acting in synergy with a calcium/calmodulin phosphatase, calcineurin (7, 44). Moreover, the mechanism by which some kinases and phosphatases regulate the NFAT activity implies modulation of nuclear translocation of this factor, its binding to DNA, or transactivation of its target gene expression. Because B-Raf AA abrogated the nuclear localization and transcriptional activity of NFAT, we propose that the model that sustained B-Raf/MEK/ERK activation modulating NFAT-dependent transcription could be achieved by regulation of intrinsic nuclear translocation of NFAT. Supporting this interpretation, it has been reported that ERK1 overexpression augmented the DNA binding activity of NFAT, resulting in NFAT activation in Jurkat cells (50). However, it has been shown that activated ERK binds to and phosphorylates NFAT2, which negatively regulates nuclear translocation and activation of NFAT2 in fibroblasts (51). Conversely, in Jurkat cells, we observed the attenuation of TCR-stimulated nuclear translocation of NFAT by MEK inhibitor.² These seemingly discrepant results might be accounted for by use of different systems and cell types. In any case, the formal demonstration of a role for B-Raf and ERK in the regulation of NFAT activation *in vivo* requires more detailed analysis.

It is noteworthy that temporal difference in the Raf-induced ERK activation signal induces qualitatively different cellular responses. In PC12 cells, epidermal growth factor-driven proliferation was coupled with transient ERK activation. On the contrary, neural growth factor-driven differentiation of PC12 cells into sympathetic neurons was induced by sustained ERK activation (10), which was mediated by B-Raf (23). A similar phenomenon was found in T cells. Mariathasan *et al.* (52) demonstrated that in thymocytes, negatively selecting stimuli by agonistic peptides through TCR induced transient and strong ERK activation, resulting in cell death, whereas positively selecting stimuli by the analogue peptides induced sustained and weak ERK activation, resulting in cell survival. In a very recent study, it has been reported that B-Raf but not Raf-1 was activated with TCR stimulation in CD4⁺CD8⁺ double positive thymocytes (53). These observations and our findings that B-Raf and Raf-1 activities regulated the strength and the duration of TCR-mediated ERK activation prompted us to consider that the Ras/B-Raf/MEK/ERK pathway also could play important roles in determining the cell fate such as thymocyte differentiation regulated by temporally distinct ERK activity.

In summary, our data suggest that Ras/B-Raf/MEK/ERK can serve as a novel component of signaling pathways that regulate the duration of ERK activity in response to TCR stimulation. B-Raf and ERK activation with a proper duration determines biological outcomes such as IL-2 production in human T cells.

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