p38 Mitogen-Activated Protein Kinase Mediates Signal Integration of TCR/CD28 Costimulation in Primary Murine T Cells

Jian Zhang, Konstantin V. Salojin, Jian-Xin Gao, Mark J. Cameron, Isabelle Bergerot and Terry L. Delovitch

*J Immunol* 1999; 162:3819-3829; ;
[http://www.jimmunol.org/content/162/7/3819](http://www.jimmunol.org/content/162/7/3819)
p38 Mitogen-Activated Protein Kinase Mediates Signal Integration of TCR/CD28 Costimulation in Primary Murine T Cells

Jian Zhang, Konstantin V. Salojin, Jian-Xin Gao, Mark J. Cameron, Isabelle Bergerot, and Terry L. Delovitch

Optimal T cell activation requires two signals, one generated by TCR and another by the CD28 costimulatory receptor. In this study, we investigated the regulation of costimulation-induced mitogen-activated protein kinase (MAPK) activation in primary mouse T cells. In contrast to that reported for human Jurkat T cells, we found that p38 MAPK, but not Jun NH2-terminal kinase (JNK), is weakly activated upon stimulation with either anti-CD3 or anti-CD28 in murine thymocytes and splenic T cells. However, p38 MAPK is activated strongly and synergistically by either CD3/CD28 coligation or PMA/Ca2+ ionophore stimulation, which mimics TCR-CD3/CD28-mediated signaling. Activation of p38 MAPK correlates closely with the stimulation of T cell proliferation. In contrast, PMA-induced JNK activation is inhibited by Ca2+ ionophore. T cell proliferation and production of IL-2, IL-4, and IFN-γ induced by both CD3 and CD3/CD28 ligation and the nuclear expression of the c-Jun and ATF-2 proteins are each blocked by the p38 MAPK inhibitor SB203580. Our findings demonstrate that p38 MAPK 1) plays an important role in signal integration during costimulation of primary mouse T cells, 2) may be involved in the induction of c-Jun activation and augmentation of AP-1 transcriptional activity, and 3) regulates whether T cells enter a state of functional unresponsiveness. The Journal of Immunology, 1999, 162: 3819–3829.

The transmission of extracellular signals to intracellular targets in various cell types is mediated by several protein kinases, including the family of mitogen-activated protein kinases (MAPKs) (1–4). MAPKs are serine/threonine kinases that include extracellular signal-regulated kinases (ERKs), Jun NH2-terminal kinases (JNKs), and p38 MAPK (2, 3). MAPKs are activated by dual phosphorylation in a Thr-Xaa-Tyr motif, in which Xaa corresponds to Glu in ERK, Pro in JNK, and Gly in p38 MAPK (2, 4). These dual specificity kinases are relatively specific for each MAPK subgroup, allowing for their independent regulation. Thus, MAPK/ERK kinase-1 (MEK-1) and MEK2 selectively phosphorylate and activate ERKs, whereas MAPK kinase-3 (MKK-3) and MKK6 selectively phosphorylate and activate p38 MAPK. MKK4 does not activate the ERK subgroup, but activates both p38 MAPK and JNK (5–9).

ERKs are activated by agonists for tyrosine-encoded receptors and G protein-coupled receptors that induce mitogenesis or cellular differentiation (2–4). ERKs mediate the effects of these agonists by phosphorylating and regulating the activity of several proteins, including cytoplasmic enzymes and nuclear factors (1, 2). JNKs phosphorylate the NH2-terminal activation domain of c-Jun and activating transcription factor-2 (ATF-2), increasing their transcriptional activity (4, 10). JNKs are activated preferentially by cellular stress and inflammatory cytokines, but also by G protein-coupled receptor agonists, growth factors, and cytoplasmic oncogenes (4, 10–16). Similarly, p38 MAPK is activated by cellular stresses, inflammatory cytokines, LPS (14, 17–19), and G protein-coupled receptors (20), and activated p38 MAPK in turn mediates cytokine production, stress responses, and apoptosis (21–26). The p38 MAPK substrates include MAPK-activating protein kinase-2 (MAPKAP kinase-2), ATF-2 (18, 19, 27, 28), cAMP response element binding protein (CREB), ATF-1 (29), Elk-1 (25), C/EBP-homologous protein (CHOP) (30), and myocyte-enhancer factor 2C (MEF2C) (31).

TCR engagement activates the ERK cascade in T cells (32–34). Analyses of Jurkat human T cells and various activated mouse T cell clones have suggested that JNK activation stimulated by TCR engagement requires CD28 coligation (33, 34). p38 MAPK may be fully activated in mouse T cell clones by signaling via either CD3 or CD28, but CD3/CD28 costimulation does not further enhance the amount of p38 MAPK activation (35). In contrast, stimulation of CD28 fails to activate p38 MAPK, but synergizes with CD3 stimulation to fully activate p38 MAPK in preactivated proliferating T cells (36). These results raise the possibility that p38 MAPK may mediate CD28 costimulation in primary naïve mouse T cells. The latter possibility is supported by reports that, in T cells from MKK-4-deficient mice, CD28-mediated IL-2 production and proliferation are impaired and CD28 costimulation and PMA/Ca2+ ionophore-induced signaling can stimulate proliferation and IL-2.
production independently of JNK activation (37). In addition, in human T cells, the p38 MAPK inhibitor SB203580 blocks CD28-dependent proliferation and IL-2 production (38). Thus, the question of whether p38 MAPK is activated upon either TCR or CD28 stimulation or after TCR/CD28 coligation in primary, unstimulated, naive mouse T cells merits further investigation.

In this study, we determined whether differential regulation of MAPK activation occurs in primary mouse T cells in response to TCR/CD28 or PMA/Ca2+ ionophore costimulation. We show that p38 MAPK activation mediates both TCR- and CD28-induced signaling in primary mouse T cells. Ligation of TCR or CD28 results in only modest p38 MAPK activation, whereas TCR and CD28 synergize upon coligation to elicit enhanced p38 MAPK activation. PMA/Ca2+ ionophore costimulation, which mimics TCR/CD28-mediated signaling, fully activates p38 MAPK in primary mouse T cells. Our results demonstrate that p38 MAPK is involved in both TCR- and CD28-signaling pathways, and that p38 MAPK, but not JNK, is involved in signal integration during costimulation of naive mouse primary T cells.

Materials and Methods

Mice

C57BL/6 (B6) and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME), maintained in the Animal Care Facility of the Faculty of Medicine at the University of Western Ontario (London, ON, Canada), and used at 6–10 wk of age.

Abs, proteins, and reagents

The following reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): rabbit polyclonal Abs against mouse ERK-1, JNK-1/2, and p38 MAPK; mouse mAbs against c-jun, c-Fos, and ATF-2; glutathione S-transferase (GST)-c-Jun (1–79), GST-ATF-2 (1–505), and glutathione-agarose. MBP, IL-2, cycloheximide, and PMA were each obtained from Sigma (St. Louis, MO). Anti-MAPKAP kinase-2 antiserum and recombinant murine heat-shock protein 25 (hsp25) were purchased from Upstate (Burlington, ON, Canada), respectively. The 145-2C11 anti-CD3 and 37.51 anti-CD28 mAbs were purified by protein G affinity chromatography (Pharmacia Biotech, Uppsala, Sweden) of the supernatants of the B cell hybridomas kindly supplied by Dr. J. Bluestone (University of Chicago, Chicago, IL) and Dr. J. Allison (University of California, Berkeley, CA), respectively. The PV-1 antiserum was kindly supplied by Dr. C. June (Naval Medical Research Institute, Bethesda, MD). The p38 MAPK inhibitor 203580 was generously provided by Dr. P. Young (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). Cyclopordin A (CsA) was supplied by Sandoz Canada (Dorval, Quebec, Canada). The mouse anti-human CD3 (OKT3) and CD28 mAbs were obtained from Pharmingen (San Diego, CA). PMA and A23187 was purchased from Calbiochem (San Diego, CA), respectively. IL-2 and cycloheximide were purchased from Sigma.

Cell isolation and stimulation

B6 and BALB/c thymocytes or splenic T cells were purified (purity ≥98%) as determined by FACS analysis of CD3 cell surface expression on T cell enrichment columns (R&D Systems, Minneapolis, MN). Murine T cells were cultured for 5 h at 37°C in complete RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 10 mM HEPES, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 0.05 μM 2-ME, and 2 mM glutamine (all purchased from Life Technologies, Lake Placid, NY) and StressGen Biotechnology (Victoria, Canada), respectively. The 145-2C11 anti-CD3 and 37.51 anti-CD28 mAbs were purified by protein G affinity chromatography (Pharmacia Biotech, Upppsala, Sweden) of the supernatants of the B cell hybridomas kindly supplied by Dr. J. Bluestone (University of Chicago, Chicago, IL) and Dr. J. Allison (University of California, Berkeley, CA), respectively. The PV-1 antiserum was kindly supplied by Dr. C. June (Naval Medical Research Institute, Bethesda, MD). The p38 MAPK inhibitor 203580 was generously provided by Dr. P. Young (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). Cyclopordin A (CsA) was supplied by Sandoz Canada (Dorval, Quebec, Canada). The mouse anti-human CD3 (OKT3) and CD28 mAbs were obtained from Pharmingen (San Diego, CA). PMA and A23187 was purchased from Calbiochem (San Diego, CA), respectively. IL-2 and cycloheximide were purchased from Sigma.

In vitro kinase assays

After stimulation, cells were lysed in ice-cold lysis buffer containing 1% Triton X-100, 10 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EGTA, 50 mM β-glycerophosphate, 2 mM Na3VO4, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4°C, and their protein content was determined by the Bradford assay using BSA as a standard. Lysates were divided into three replicate samples; incubated for 1 h at 4°C with either anti-ERK-1, anti-JNK-1, or anti-p38 MAPK Abs; and further reacted with protein G-agarose (Santa Cruz) or protein A-Sepharose CL-4B (Pharmacia Biotech, Baie d’Urfe, PQ, Canada) for an additional 1 h at 4°C. Immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer (20 mM HEPES (pH 7.5), 20 mM MgCl2, 20 mM MnCl2, 2 mM DTT, 25 mM β-glycerophosphate, and 100 mM Na3VO4). Kinase assays were performed using MBP, GST-c-Jun, and GST-ATF-2 fusion proteins as substrates for ERK-1, JNK-1, and p38 MAPK, respectively. Immunoprecipitates were resuspended in 18 μl kinase buffer containing 5 μg MBP, 1 μg GST-c-Jun, and 1 μg GST-ATF-2 fusion proteins in the presence of 20 μM cold ATP and 20 μCi [γ-32P]ATP (Amersham Life Science, Arlington Heights, IL), and incubated for 30 min at 30°C. Solid-phase JNK assays were performed essentially as previously described (39). Whole cell extracts (2 × 107 cell equivalents) were prepared and reacted for 4 h at 4°C with a GST-c-Jun (1–79) fusion protein bound to glutathione-agarose beads to immobilize JNK. The washed beads were then analyzed for their associated kinase activity by incubation for 30 min at 30°C in kinase buffer containing 20 μCi [γ-32P]ATP. Reactions were terminated by the addition of SDS sample buffer, samples were boiled, and kinase reaction products were resolved by SDS-PAGE. The MAPK kinase assay was performed as described (35), using murine recombinant hsp25 as a substrate. Equal loading of precipitated proteins was confirmed by probing the blots with specific Abs, and phosphorylation of the substrates was quantitated using a Molecular Imager System and Molecular Analyst imaging software (Bio-Rad, Hercules, CA).

T cell proliferation assay

Splenic T cells (106/ml) were resuspended in complete RPMI 1640 medium in the absence or presence of various concentrations of SB203580, and then incubated for 15 min at 37°C. Cells were cultured for 48 h at 37°C in round-bottom 96-well plates (Nunc) precoated with the 145-2C11 anti-CD3 mAb (1 μg/ml) in the presence or absence of the 37.51 anti-CD28 mAb (1 μg/ml). [3H]Thymidine incorporation, which was determined using a Wallac 1450 Microbeta Plus beta counter (Fisher Scientific, Ottawa, ON, Canada). The extent of T cell proliferation was proportional to the amount of [3H]thymidine incorporation, which was determined using a Wallac 1450 Microbeta Plus beta counter (Fisher Scientific, Ottawa, ON, Canada).

Cytokine assays

Splenic T cells (106/ml) were pretreated with different concentrations of SB203580 for 15 min at 37°C, and cultured in round-bottom 96-well plates coated with anti-CD3 (1 μg/ml) in the presence or absence of the 37.51 anti-CD28 mAb (1 μg/ml). Supernatants collected after 48 h were assayed for their cytokine concentrations by ELISA using a double ligand method. IL-2 concentrations were interpolated from a standard curve using murine IL-2-2 captured by the JES6-1A12 mAb and detected by the biotinylated V576-5H4 mAb. IL-4 concentrations were measured using recombinant murine IL-4, the BV4D-1D11 mAb, and biotinylated BV6D-24G2 mAb, while IFN-γ concentrations were detected using recombinant murine IFN-γ, R4-6A2 mAb, and biotinylated XMG1.2 mAb (all obtained from Pharmingen). Briefly, flat-bottom 96-well microtiter plates were coated with 50 μl/well of capture mAb (1 μg/ml) in 0.1 M NaHCO3 overnight at 4°C. Nonspecific binding sites were blocked with 3% BSA for 2 h at 23°C. Standards or samples (50 μl) were added, left overnight at 4°C, and then incubated with 50 μl/well of biotinylated detecting mAb (1 μg/ml) for 45 min at 23°C. Streptavidin-peroxidase conjugates (1 μg/ml; Sigma) in diethanolamine buffer were successively added to develop the reaction at 23°C. Plates were read at 405 nm in an automated microplate reader (Bio-Rad). Cytokine standard curves were linear in the range of 20–20,000 pg/ml.

Cell viability assay

Splenic T cells were stimulated with anti-CD3 mAb as above in the presence or absence of SB203580 (100 μM). Cells were harvested after incubation for 48 h, washed in PBS, stained with propidium iodide, and analyzed by flow cytometry.
Effect of SB203580 on nuclear expression of c-Fos, c-Jun, and ATF-2

Thymocytes (4 × 10^7 /ml) were pretreated for 15 min with 10 μM SB203580, and were then stimulated for 5 or 15 min with anti-CD3 (10 μg/ml), anti-CD28 (5 μg/ml), anti-CD3 plus anti-CD28, or PMA (50 ng/ml). Cell lysates were pre-cleared and immunoprecipitated with anti-ERK-1, anti-JNK-1, or anti-p38 MAPK, respectively. In vitro kinase assays of ERK-1, JNK-1, and p38 MAPK activity associated with the immunoprecipitates were performed using MBP, GST-c-Jun, and GST-ATF-2 as substrates. Lysates were analyzed for protein abundance of ERK-1, JNK-1, and p38 MAPK by immunoblotting (IB).

Results

Coligation of TCR and CD28 synergistically activates p38 MAPK, but neither JNK nor ERK in murine thymocytes and splenic T cells

JNK activation requires colistimulation by either CD28 or Ca^{2+} ionophore in Jurkat T cells, suggesting that JNK is involved in signal integration during T cell colistimulation (33). However, it is not known whether activation of JNK and p38 MAPK occurs in primary naïve T cells that are not further stimulated in vitro. To determine whether CD28 regulates MAPK activation following CD3 ligation, murine thymocytes or purified splenic T cells were stimulated for 15 min with anti-CD3, anti-CD28, or both mAbs. Cells were lysed; ERK-1, JNK-1, and p38 MAPK were immunoprecipitated with specific Abs; and immunoprecipitates were assayed for the activities of associated MAPKs by their ability to phosphorylate the MBP, c-Jun, and ATF-2 substrates, respectively. Anti-CD3 stimulation activated ERK-1 in thymocytes and splenic T cells, and this level of activation of ERK-1 was not increased after CD3/CD28 colistimulation (Fig. 1, A and B, upper panels), as reported (33, 34). More significantly, stimulation by anti-CD3 or anti-CD28 either alone or in combination did not activate JNK (i.e., JNK-1) in murine thymocytes or splenic T cells (Fig. 1, A and B, middle panels). Anti-CD3 or anti-CD28 stimulation induced modest p38 MAPK activity, which was elevated about fivefold after CD3/CD28 colistimulation (Fig. 1, A and B, lower panels). Stimulation of splenic T cells with the Ca^{2+} ionophore A23187 did not activate ERK-1 or JNK-1, but rather stimulated p38 MAPK activity and enhanced the level of p38 MAPK activity induced by PMA alone (Fig. 1B, lower panel). JNK-2 may bind and phosphorylate c-Jun more efficiently than JNK-1 (41, 42). To
FIGURE 2. p38 MAPK, but not ERK-1 or JNK-1, is synergistically activated by CD3/CD28 coligation. A, Thymocytes (2 × 10⁶/ml) were incubated with control normal hamster IgG (NIg, 10 µg/ml) or with anti-CD3 (10 µg/ml) plus incremental concentrations (0–20 µg/ml) of anti-CD28 for 15 min at 37°C. Cell lysates were immunoprecipitated with anti-ERK-1, anti-JNK-1, or anti-p38 MAPK, and kinase activities were assayed as in Fig. 1A. Lysates were analyzed for protein abundance of ERK-1, JNK-1, and p38 MAPK, as above. B, Thymocytes (2 × 10⁶/ml) were stimulated with either NIg (10 µg/ml), PV-1 anti-CD28 (5 µg/ml), anti-CD3 (10 µg/ml) plus incremental concentrations (0–20 µg/ml) of anti-CD3, and assay of ERK-1, JNK-1, and p38 MAPK activities were as in Fig. 1A. Lysates were analyzed for protein abundance of ERK-1, JNK-1, and p38 MAPK, as above. C, Stimulation of thymocytes (2 × 10⁶/ml) with NIg (10 µg/ml) or with anti-CD28 (5 µg/ml) plus incremental concentrations (0–20 µg/ml) of anti-CD3, and assay of ERK-1, JNK-1, and p38 MAPK activities were as in Fig. 1A. Lysates were analyzed for protein abundance of ERK-1, JNK-1, and p38 MAPK, as above. D, Thymocytes (2 × 10⁶/ml) were stimulated with either NIg (10 µg/ml), PV-1 anti-CD28 (5 µg/ml), anti-CD3 (10 µg/ml) plus incremental concentrations (0–20 µg/ml) of anti-CD3, and assay of ERK-1, JNK-1, and p38 MAPK activities were as in Fig. 1A. Lysates were analyzed for protein abundance of ERK-1, JNK-1, and p38 MAPK, as above. 

To further examine the activation of ERK-1, JNK-1, and p38 MAPK induced by stimulation of CD3, CD28, or CD3 plus CD28, thymocytes were pretreated with a constant amount (10 µg/ml) of anti-CD3 together with variable amounts of anti-CD28. With increasing concentrations of anti-CD28 mAb (0.1–20 µg/ml), no further augmentation of ERK-1 activation was observed compared with the amount of ERK-1 activity stimulated by anti-CD3 alone (Fig. 2A, upper panel). A similar result was obtained for JNK-1 activity. However, p38 MAPK was optimally activated by anti-CD28 at concentrations of 1–5 µg/ml (Fig. 2A, lower panel).

To investigate whether the kinetics of ERK-1 and JNK-1 activation differs from that of p38 MAPK, the time courses of activation of ERK-1, JNK-1, and p38 MAPK induced by coligation of CD3 and CD28 were determined. ERK-1 and p38 MAPK activity peaked at 15 and 30 min of stimulation, respectively (Fig. 2B, upper and lower panels), but JNK-1 was not activated throughout the entire time course (Fig. 2B, middle panel). This effect of CD28 costimulation was also analyzed by pretreating thymocytes with an optimum concentration of anti-CD28 mAb (5 µg/ml) plus variable concentrations of anti-CD3 mAb (0.1–20 µg/ml). ERK-1 activation was gradually enhanced by addition of increasing concentrations of anti-CD3, with maximal activation observed at 10 and 20 µg/ml (Fig. 2C, upper panel). Similarly, p38 MAPK activity was elevated by increasing concentrations of anti-CD3 and was evident even at very low concentrations (0.1 µg/ml) of added anti-CD3 (Fig. 2C, lower panel). In contrast, JNK-1 was not activated significantly at any concentration of added anti-CD3 (Fig. 2C, middle panel). The inability to detect JNK-1 activation upon CD3/CD28 coligation in primary mouse T cells may be due to a lack of reactivity of the 37.51 anti-CD28 mAb with the appropriate CD28 epitope required for JNK-1 activation. However, the use of another anti-CD28 mAb, PV-1, which recognizes a different epitope than
In Fig. 1B, we showed that the Ca²⁺ ionophore A23187 (500 ng/ml) activates p38 MAPK, synergizes with PMA to further enhance p38 MAPK activation, and inhibits JNK-1 activity induced by PMA. To analyze the relationship of these findings to the dose of A23187, splenic T cells were stimulated with PMA (50 ng/ml) plus a range of concentrations (15–1000 ng/ml) of A23187. ERK-1 activity was not enhanced at any of the concentrations of A23187 used (Fig. 3A, upper panel). JNK-1 activity was inhibited significantly in a dose-dependent manner when A23187 was added to PMA, and concentrations of ≥125 ng/ml A23187 abolished virtually all detectable JNK-1 activity (Fig. 3A, middle panel). Note that this inhibition of JNK by A23187 was not due to different kinetics of JNK activation induced by PMA/A23187 versus PMA alone. PMA-induced JNK activation was significantly increased in peripheral T cells after stimulation for 15 min, whereas little or no activation of JNK was induced by PMA/A23187 (Fig. 3B). In contrast, p38 MAPK activity was enhanced upon exposure of the T cells to increasing doses of A23187 and reached a maximum at a concentration of 60 ng/ml (Fig. 3A, lower panel). Interestingly, the latter findings on induced p38 MAPK activity correlate closely with our observations on the stimulation of proliferation of splenic T cells by PMA and variable amounts of A23187 (Fig. 3C). The observed inhibition of PMA-induced JNK activation by A23187 suggests that Ca²⁺ ionophore may activate some JNK-specific MAPK phosphatases that inactivate JNK.

To further analyze the effect of Ca²⁺ on p38 MAPK activation, splenic T cells were pretreated with CsA (500 ng/ml), which is believed to inhibit the calcium-dependent phosphatase calcineurin. Consistent with our previous observation in Fig. 1C, stimulation with anti-CD3 or anti-CD28 alone resulted in only modest p38 MAPK activation, and p38 MAPK was activated synergistically by either CD3/CD28 coligation or PMA/A23187 treatment (Fig. 3D, upper panel). These induced p38 MAPK activation were inhibited appreciably by CsA, with the exception that PMA- and CD28-induced p38 MAPK activation was CsA resistant. Interestingly, inhibition of PMA/Ca²⁺ ionophore-induced p38 MAPK activity by CsA pretreatment occurred in a dose-dependent manner (Fig. 3D, lower panel). JNK activity was induced only by PMA, and this PMA-induced JNK activity was inhibited by Ca²⁺ ionophore (Fig. 3D, upper panel). However, CsA did not further inhibit JNK activity induced by PMA/Ca²⁺ ionophore (Fig. 3D, lower panel). Thus, optimal activation of p38 MAPK in primary mouse T cells requires two signals, one provided by the TCR/CD3 complex or PMA and another provided by CD28 or Ca²⁺ ionophore.

Different requirements for JNK-1 and p38 MAPK activation in Jurkat T cells, proliferating T cells, and primary T cells

JNK activation requires two signals generated by the TCR and CD28 or PMA and Ca²⁺ ionophore in Jurkat T cells and mouse T cell clones (33, 34). To determine whether p38 MAPK activation has the same requirements in Jurkat T cells, we compared the activation of ERK-1, JNK-1, and p38 MAPK in response to ligation of CD3, CD28, CD3 plus CD28, PMA, and PMA plus Ca²⁺ ionophore in Jurkat T cells. ERK-1 activation was only seen upon anti-CD3 or PMA stimulation, and no further augmentation was observed upon CD3/CD28 coligation or exposure to PMA and A23187 (Fig. 4A, upper panel). JNK-1 activation required

Synergistic activation of p38 MAPK requires costimulation by Ca²⁺ in primary mouse T cells

FIGURE 3. PMA and Ca²⁺ ionophore synergistically activate p38 MAPK, but not ERK-1 or JNK-1. A. Splenic T cells (10⁶/ml) were incubated with PMA (50 ng/ml) and variable (0–1000 ng/ml) concentrations of A23187 in DMSO for 15 min at 37°C. In vitro kinase activities of ERK-1, JNK-1, and p38 MAPK were detected as in Fig. 1A. lysates were analyzed for protein abundance of ERK-1, JNK-1, and p38 MAPK. B. Splenic T cells (10⁶/ml) were either stimulated with PMA (50 ng/ml) or PMA plus A23187 (500 ng/ml) for 1, 5, 10, and 15 min at 37°C, or left unstimulated. Cells were lysed, and a solid-phase kinase assay of JNK was performed as in Fig. 1C. C. Splenic T cells (10⁶/ml) were cultured in the presence of PMA (50 ng/ml) and incremental concentrations (15–1000 ng/ml) of A23187 for 48 h at 37°C. Cell proliferation was determined by [³H]thymidine incorporation. The results of triplicate cultures are expressed as the mean values ± SD. D. Splenic T cells (10⁶/ml) were pretreated in the absence or presence of CsA (500 ng/ml) for 15 min at 37°C, and were then stimulated with either anti-CD3, anti-CD28, anti-CD3 plus PMA, or PMA. Alternatively, splenic T cells were pretreated with various amounts of CsA (0–500 ng/ml) for 15 min at 37°C, and were then stimulated with PMA plus A23187. p38 MAPK activity was assayed as in Fig. 1A, and a solid-phase JNK assay was performed as in Fig. 1C. Data from one of three reproducible experiments are shown.
CD3/CD28 coligation. PMA induced modest JNK-1 activation, which was significantly enhanced by PMA/A23187 stimulation (Fig. 4A, middle panel). Similarly, anti-CD3 or PMA weakly activated p38 MAPK, but full activation of p38 MAPK required CD3/CD28 coligation or PMA/Ca\(^{2+}\) ionophore costimulation (Fig. 4A, lower panel). Next, we examined the effect of Ca\(^{2+}\)/calcineurin on the activation of ERK-1, JNK-1, and p38 MAPK. To obtain additional evidence that the requirement for MAPK activation differs between T cells at different stages of the cell cycle, we analyzed the activation of ERK-1, JNK-1, and p38 MAPK in response to TCR or CD28 stimulation, or both, in proliferating mouse T cells. B6 splenic T cells were first stimulated with plate-bound anti-CD3 and IL-2 for 48 h and then expanded in medium containing IL-2 for 4 days (36). Interestingly, we noted that, although the cells were proliferating, about 30–40% of the T cells were nonviable at the time of harvest. Viable cells were recovered, stimulated, lysed, and immunoprecipitated. Consistent with previous reports for Jurkat T cells and primary mouse T cells, TCR stimulation induced full ERK-1 activation without the requirement of CD28 costimulation (Fig. 4C, upper panel), whereas optimal p38 MAPK activation required TCR/CD28 costimulation (Fig. 4C, lower panels). In contrast, JNK-1 was modestly activated by anti-CD3 stimulation, but no synergistic activation of JNK-1 occurred upon CD3/CD28 coligation. JNK-1 was fully activated, however, by CD28 ligation (Fig. 4C, middle panel). These data suggest that Jurkat human T cells, proliferating mouse T cells, and primary mouse T cells differ in terms of their requirements for ERK-1, JNK-1, and p38 MAPK activation.

p38 MAPK activity is required for CD3 or CD3/CD28 ligation-induced T cell proliferation

The requirement of p38 MAPK activation for T cell proliferation induced by CD3 or CD3/CD28 ligation was investigated. Splenic T cells were preincubated with different concentrations of

![FIGURE 4](http://www.jimmunol.org/). Different requirements for MAPK activation in Jurkat T cells. A, Jurkat T cells (2 × 10\(^7\)/ml) were stimulated with anti-CD3 (10 μg/ml), anti-CD28 (2 μg/ml), anti-CD3 plus anti-CD28, PMA (50 ng/ml), and PMA (50 ng/ml) plus A23187 (1 μg/ml) for 15 min at 37°C. Cell lysates were immunoprecipitated with anti-ERK-1, anti-JNK-1, and anti-p38 MAPK polyclonal Abs, respectively. In vitro kinase assays of ERK-1, JNK-1, and p38 MAPK were performed as in Fig. 1A. B, Jurkat T cells were stimulated as in A, but were either pretreated or not with CsA (500 ng/ml) for 15 min at 37°C before stimulation. C, Proliferating T cells were stimulated with Nlg, anti-CD3 (10 μg/ml), anti-CD28 (5 μg/ml), and anti-CD3/CD28 for 15 min at 37°C. ERK-1, JNK-1, and p38 MAPK activities were assayed as in Fig. 1A. Lysates were analyzed for protein abundance of ERK-1, JNK-1, and p38 MAPK. Data from one of three reproducible experiments are shown.
SB203580, a specific inhibitor of p38 MAPK activity, and were then cultured in complete RPMI 1640 medium with plate-bound anti-CD3 mAb in the presence or absence of anti-CD28 (1 μg/ml). Cell proliferation was determined by [3H]thymidine incorporation. The results of triplicate cultures are expressed as the mean values ± SD. B. Splenic T cells (10⁶/ml) were pretreated with either SB203580 (100 μM) in DMSO or an equivalent amount of DMSO, and then stimulated with anti-CD3 (1 μg/ml). Cell death was detected by propidium iodide staining and flow-cytometric analysis. C. Splenic T cells (10⁶/ml) were preincubated with SB203580 (1–100 μM) for 15 min at 37°C, and then stimulated with anti-CD3 or anti-CD3 plus anti-CD28 for an additional 15 min. p38 MAPK activity was detected as in Fig. 1A. MAPKAP kinase-2 activity was performed using recombinant murine hsp25 as a substrate. Note that SB203580 was present during the immunoprecipitation and kinase reactions. D. Splenic T cells (10⁷/ml) were preincubated with SB203580 (1–100 μM) for 15 min at 37°C, and then stimulated with anti-CD3 or anti-CD3 plus anti-CD28, or alternatively with PMA for an additional 15 min. ERK-1 activity was assayed as in Fig. 1A, and a solid JNK assay was performed as in Fig. 1C. ERK-1 abundance was detected as in Fig. 1A. Data from one of two reproducible experiments are shown.

At concentrations of ≥10 μM, the SB203580 inhibitor may lose its selectivity and also block JNK2β1 and JNK2β2 (44). To confirm the specificity of this inhibitor, purified splenic T cells were pretreated with various concentrations of SB203580 (0–100 μM), then stimulated with either anti-CD3, anti-CD3 plus anti-CD28, or PMA, and lysed. Cell lysates were either reacted with GST-c-Jun precoupled to glutathione-agarose beads or immunoprecipitated with an anti-anti-ERK-1 Ab in the presence of SB203580. The immobilized kinases were then incubated with substrates and SB203580 (0–100 μM) for 30 min at 30°C. ERK-1 and JNK activities remained unaffected in the presence of increasing concentrations of SB203580 (Fig. 5D).
treatment with SB203580 induces T cells to become functionally unresponsive.

SB203580 represses the nuclear expression of c-Jun and ATF-2

Our data suggest that p38 MAPK, but not JNK, is involved in signal integration of TCR- and CD28-mediated signaling pathways in primary mouse T cells. However, JNK is a major kinase responsible for c-Jun phosphorylation and p38 MAPK cannot phosphorylate c-Jun (28, 31). Indeed, using in vitro kinase assays, we failed to detect c-Jun phosphorylation by p38 MAPK (data not shown). Since phosphorylation of c-Jun and/or ATF-2 is required for c-Jun gene induction, we analyzed whether blockade of p38 MAPK activity results in the reduced phosphorylation of c-Jun and ATF-2. Thymocytes were preincubated for 15 min at 37°C with 10 μM SB203580 or an equal amount of DMSO as control, and were then either stimulated with anti-CD3 plus anti-CD28 for 5 and 15 min, or left unstimulated. Nuclear extracts were blotted with anti-c-Jun and anti-ATF-2 mAbs, respectively. Phosphorylation of ATF-2, as revealed by its reduced mobility, was evident after 5 and 15 min of stimulation, and these mobility shifts were significantly inhibited by SB203580 (Fig. 7A, upper panel). In contrast, no c-Jun phosphorylation was observed (Fig. 7A, lower panel), consistent with our in vitro data that JNK is not activated upon CD3 or CD3/CD28 ligation. Note that the failure to detect c-Jun phosphorylation cannot be attributed to the anti-c-Jun mAb used because it clearly recognized the phosphorylated form of c-Jun induced by PMA stimulation, as reported (45). SB203580 can block the induction of the c-Fos and c-Jun genes by diverse stimuli (45, 46), indicating that p38 MAPK activity may play a role in the induction of c-Jun gene expression. This may explain our finding of the nuclear expression of the c-Jun and ATF-2 proteins at later time points following CD3/CD28 coligation. Interestingly, while SB203580 pretreatment did not alter the nuclear expression of c-Fos after CD3 or CD3/CD28 stimulation (Fig. 7B, upper panel), SB203580 pretreatment significantly reduced the nuclear expression of c-Jun and ATF-2 induced by CD3 or CD3/CD28 ligation (Fig. 7B, middle and lower panels). The nuclear expression of c-Jun and ATF-2 was enhanced more upon...

FIGURE 6. SB203580 inhibits cytokine production induced by CD3 or CD3/CD28 ligation in primary mouse T cells. Splenic T cells (10⁷/ml) were preincubated with different concentrations (0–50 μM) of SB203580 for 15 min at 37°C, and then stimulated with anti-CD3 (1 μg/ml) in the presence or absence of the 37.51 anti-CD28 mAb (1 μg/ml). Supernatants were collected after 48 h, and were assayed for their concentrations of IL-2, IL-4, and IFN-γ by ELISA using a double ligand method. The results of triplicate cultures are expressed as the mean values ± SD. Data from one of three reproducible experiments are shown.

FIGURE 7. SB203580 inhibits the nuclear expression of the c-Jun and ATF-2, but not c-Fos proteins in primary mouse T cells. A, Thymocytes (4 x 10⁷/ml) were incubated in the presence (+) or absence (−) of SB203580, and were either left unstimulated or stimulated for 5 or 15 min at 37°C with anti-CD3 plus anti-CD28. B, Thymocytes were preincubated with or without SB203580 and then stimulated for 4 h at 37°C with anti-CD3, anti-CD3 plus anti-CD28, or PMA. C, Thymocytes were either left untreated or pretreated for 15 min with 20 μM cycloheximide (CHX), and were then stimulated for 4 h at 37°C with anti-CD3 or anti-CD3 plus anti-CD28. Nuclear extracts of cell lysates were analyzed by SDS-PAGE. Immunoblotting was conducted using anti-c-Fos, anti-c-Jun, and anti-ATF-2 mAbs, respectively, and the c-Fos, c-Jun, and ATF-2 proteins were detected by chemoluminescence. Data from one of three reproducible experiments are shown.
CD3/CD28 coligation than ligation of CD3 alone. The nuclear expression of c-Fos, c-Jun, and ATF-2 stimulated by PMA was not inhibited by SB203580.

To confirm that the increase of c-Jun expression in the nucleus was due to enhancement of de novo c-Jun protein synthesis, the sensitivity of c-Jun nuclear expression to cycloheximide, a protein synthesis inhibitor, was determined. Although cycloheximide may stimulate JNK activity, pretreatment of thymocytes with 20 μM cycloheximide virtually abolished the increased abundance of c-Jun induced by CD3 and CD3/CD28 ligation (Fig. 7C). The blockade of c-Jun expression in the nucleus by SB203580 suggests that p38 MAPK activity plays a role in the induction of expression of the c-Jun protein.

Discussion

Previous studies of MAPK activation in the Jurkat human T cell line and mouse T cell clones in long-term culture suggested that JNK activation requires costimulatory signals via CD28 or Ca2+ ionophore to synergize with TCR- or PMA-driven signals (33, 34). However, the pathways of activation of JNK and p38 MAPK in primary T cells were not studied extensively and are less well understood. Accordingly, we investigated the regulation of ERK-1, JNK-1, and p38 MAPK activation stimulated by TCR/CD28 or PMA/Ca2+ ionophore-mediated signaling in naive mouse thymocytes and splenic T cells. Our main findings are: 1) CD3 or CD28 stimulation of primary naive thymocytes and T cells activates p38 MAPK; 2) CD3/CD28 coligation synergistically activates p38 MAPK, but not JNK; 3) activation of p38 MAPK is Ca2+ dependent; and 4) the p38 MAPK inhibitor, SB203580, blocks CD3 and CD3/CD28 ligation-induced T cell proliferation as well as production of IL-2, IL-4, and IFN-γ. These results suggest that p38 MAPK, but not JNK, is involved in signal integration during CD28 costimulation of murine primary T cells and may also play an important role in the induction of T cell anergy.

Recently, it has been shown that CD28 and PMA/Ca2+ ionophore-triggered signaling stimulate thymocytes and lymph node T cells to proliferate and produce IL-2 independently of JNK activation in MKK-4-deficient mice (37), suggesting that other MAPK(s) might be involved in primary T cell costimulation. More importantly, impaired CD28-mediated IL-2 production and proliferation by T cells were observed in these mice (37). Since MKK-4 activates both JNK and p38 MAPK (5–9), this impairment might be attributed to defective p38 MAPK activation in MKK-4-deficient mice. However, p38 MAPK activity was not analyzed in the latter report. Nonetheless, deficient expression of MKK4 does not affect p38 MAPK activation in embryonic stem cells (47), and SB203580 inhibits CD28-dependent proliferation and IL-2 production in human T cells (38). These two sets of evidence suggest a role for p38 MAPK in the costimulation of primary mouse T cells. Although T cell proliferation in response to Con A and PMA/ionomycin stimulation is normal in dominant-negative p38 transgenic mice, TCR/CD28-mediated T cell proliferation and p38 MAPK activity in response to different stimuli were not investigated in this study (48). Additional studies conducted primarily with previously activated T cells have shown that p38 MAPK activation is inducible by CD3, CD28, or CD3/CD28 stimulation (35, 36). Interestingly, however, we observed that MAPK activation differs between primary naive T cells and proliferating preactivated T cells. In proliferating T cells, while ERK-1 and p38 MAPK activity are similar to that in primary naive T cells, JNK-1 is weakly activated by CD3 stimulation and is fully activated only upon CD28 stimulation. It is important to note that a high percentage of proliferating T cells become apoptotic, perhaps due to the continuous exposure to IL-2 (49). Thus, p38 MAPK and JNK-1 activation in proliferating T cells may result in part from activation-induced apoptosis.

The requirements for p38 MAPK and JNK-1 activation in murine primary T cells were found to differ from those in Jurkat cells, a human T cell line frequently used to identify pathways of T cell signaling. In Jurkat cells, optimal activation of both p38 MAPK and JNK-1 requires TCR/CD28 coligation or PMA/Ca2+ ionophore costimulation. In primary mouse T cells, however, stimulation by either CD28 or Ca2+ ionophore activates p38 MAPK, and p38 MAPK can be activated synergistically by CD3/CD28 coligation or PMA/Ca2+ ionophore stimulation. Whereas high concentrations of Ca2+ ionophore inhibit JNK-1 activity in primary T cells, these same concentrations of Ca2+ ionophore augment p38 MAPK activity. This enhancement of p38 MAPK activity correlates closely with T cell proliferation induced by optimal concentrations of PMA and Ca2+ ionophore. Furthermore, CsA inhibited p38 MAPK activation by anti-CD3, anti-CD3 plus CD28, or PMA plus Ca2+ ionophore. Although the levels of activation of ERK-1 and p38 MAPK in CD3- or CD3/CD28-stimulated proliferating and naive primary mouse T cells are similar, JNK-1 is fully activated by CD28 ligation alone in proliferating T cells. These data demonstrate that the requirements for full activation of JNK-1 and p38 MAPK differ among primary T cells, Jurkat T cells, and proliferating T cells. Such differences may arise from the stage of activation of the T cell. Jurkat T cells are transformed activated cells in continuous growth in culture, and these cells appear to differ somewhat in their signaling pathways from fresh isolated primary T cells that presumably consist mainly of T cells in a resting state.

As JNK-1 activation was not detected after CD3/CD28 costimulation in primary T cells, we further analyzed the reactivity and epitope specificity of the 37.51 anti-CD28 mAb used for T cell stimulation. Costimulation by a wide concentration range of this mAb plus a single dose of anti-CD3 did not induce JNK-1 activation. In contrast, optimum conditions of CD3/CD28 costimulation elicited the full activation of p38 MAPK. While recent studies indicate that JNK-1 may be activated by CD3/CD28 coligation in mouse lymph node T cells, this was achievable only after preactivation of the T cells in the presence of plate-bound anti-CD3 and anti-CD28 mAbs for 40 h (50). Replacement of the 37.51 anti-CD28 mAb with the PV-1 anti-CD28 mAb, which differs in its CD28 epitope specificity from the 37.51 mAb, also did not stimulate JNK-1 activation. Furthermore, the 37.51 anti-CD28 mAb elicited full JNK-1 activation in preactivated proliferating mouse T cells. Importantly, the results obtained from a solid-phase JNK assay were similar to those observed using in vitro kinase assays of JNK-1 and JNK-2. This suggests that the failure to detect JNK activation in response to CD3 and CD28 ligation is not due to the inability of the anti-JNK Abs used to immunoprecipitate all isoforms of JNK. Note that the gel loading of equivalent amounts of JNK proteins was confirmed by anti-JNK immunoblotting, which excludes the possibility that these observations result from unequal amounts of kinase proteins loaded. It is evident therefore that the failure to detect JNK activation in response to CD3 and CD28 ligation in primary mouse T cells is not due to the use of an inappropriate anti-CD28 mAb. Thus, JNK does not appear to mediate CD28 costimulatory signaling in primary mouse T cells.

Additional supportive evidence for a role for p38 MAPK in T cell costimulation was derived from the use of SB203580, a highly specific pyridyl imidazole inhibitor of p38 MAPK (17). SB203580 effectively blocked T cell proliferation, cytokine (IL-2, IL-4, and IFN-γ) production, and p38 MAPK activation following CD3 or CD3/CD28 stimulation. These results lend credence to the
idea that p38 MAPK plays an important role in TCR/CD28 costimulation of proliferation and cytokine production in primary T cells. Since inhibition of T cell proliferation and cytokine secretion by SB203580 does not result from T cell death or T cell toxicity, SB203580 treatment appears to induce the functional unresponsiveness of T cells. The latter observation supports the notion that p38 MAPK may regulate the entry of T cells into a state of anergy. Consistent with this observation, SB203580 has been shown to inhibit anti-CD3-induced deletion of CD4⁺CD8⁻ thymocytes in fetal thymic organ culture (51).

The protein complex that binds to the AP-1 transcriptional activation site of the IL-2 gene promoter region may comprise either a c-Jun-c-Jun homodimer or c-Jun-Fos heterodimer, and formation of the AP-1 complex is critical for the regulation of IL-2 gene expression (33). Decreased binding of the AP-1 protein complex to the IL-2 promoter has been implicated in the molecular basis of T cell anergy (52). Phosphorylation of c-Jun at Ser⁶³⁷ and Ser⁶⁷³ activates c-Jun gene transcription (10, 53), and this phosphorylation is mediated by JNK, but not p38 MAPK (28). We found that CD3/CD28 or PMA/Ca²⁺ ionophore costimulation did not result in appreciable JNK-1 or JNK-2 activation in primary mouse T cells; rather, PMA/Ca²⁺ ionophore costimulation inhibited JNK-1 activity in these T cells. Although we did not detect any c-Jun phosphorylation by p38 MAPK in CD3/CD28-costimulated mouse thymocytes, c-Jun protein synthesis induced by CD3 or CD3/CD28 ligation was completely inhibited by SB203580. Consistent with our findings, SB203580 is known to block the induction of the c-Fos and c-Jun genes by various stimuli despite the lack of an effect of SB203580 on c-Jun and ATF-2 phosphorylation (45, 46).

This raises the possibility that the phosphorylation of other transcription factors by p38 MAPK may be essential for c-Jun gene activation. We have shown that ATF-2 can be phosphorylated by p38 MAPK in vitro, and phosphorylation of ATF-2 in the nucleus is inhibited by SB203580. ATF-2 may therefore mediate the induction of c-Jun gene activation. Furthermore, in monocytes that migrate to a site of inflammation, phosphorylation of MEF2C by p38 MAPK in vitro, and phosphorylation of ATF-2 in the nucleus of the JNK cascade through multiple growth factor and cytokine receptors. Sci- ence 274:1194.


