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*J Immunol* 2001; 167:827-835;
doi: 10.4049/jimmunol.167.2.827
http://www.jimmunol.org/content/167/2/827

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CD28 Signaling Augments Elk-1-Dependent Transcription at the c-fos Gene During Antigen Stimulation

Wei Li,* Carmella D. Whaley,* Jody L. Bonnevier,* Anna Mondino,† Molly E. Martin,* Kjersti M. Aagaard-Tillery,* and Daniel L. Mueller2*

Untransformed CD4+ Th1 cells stimulated with Ag and APC demonstrated a dependence on B7- and CD28-mediated costimulatory signals for the expression and function of AP-1 proteins. The induction of transactivation by the c-fos gene regulator Elk-1 mirrored this requirement for TCR and CD28 signal integration. c-Jun N-terminal kinase (JNK) (but not extracellular signal-regulated kinase or p38) protein kinase activity was similarly inhibited by neutralizing anti-B7 mAbs. Blockade of JNK protein kinase activity with SB 202190 prevented both Elk-1 transactivation and c-Fos induction. These results identify a unique role for B7 costimulatory molecules and CD28 in the activation of JNK during Ag stimulation in Th1 cells, and suggest that JNK regulates Elk-1 transactivation at the c-fos gene to promote the formation of AP-1 complexes important to IL-2 gene expression. The Journal of Immunology, 2001, 167: 827–835.

Cell growth factor production and proliferation in response to Ag stimulation is dependent on the simultaneous recognition of costimulatory ligands also expressed by the APC (1). The biochemical nature of those signal transduction pathways used by T cells to carry such costimulatory information to the nucleus before the induction of growth factor gene expression remains poorly understood. Our own previous investigations of costimulatory signal-transducing pathways in untransformed cloned CD4+ Th1 cells using potent peptide Ag or mitogens together with live APC or anti-CD3 plus anti-CD28 stimulatory mAbs have indicated that CD28 signals are not entirely necessary for 1) TCR γ- and ζ-chain phosphorylation, 2) the hydrolysis of phosphatidylinositol 4,5-bisphosphate and production of free inositol phosphates and diacylglycerol, 3) the mobilization of calcium free ions, or 4) the dephosphorylation and nuclear translocation of NFAT (2–4). Thus, intense TCR/CD3 stimulation in Th1 cells may alleviate any requirement for B7/CD28 costimulation in the TCR-dependent activation of select proximal and distal signaling pathways. Nevertheless, IL-2 gene expression (both steady-state mRNA levels and protein synthesis) as well as AP-1-dependent transactivation in Th1 cells have been found to be entirely suboptimal in the absence of costimulatory signals.

Like the IL-2 gene itself, the activation of c-Jun N-terminal kinase (JNK) (but not extracellular signal-regulated kinase, ERK) in murine Th1 cells was found to be greatly augmented by Ab-mediated costimulation through the CD28 molecule (4). This finding is consistent with data originally obtained using the Jurkat T-leukemia cell line, where anti-CD28 mAb was shown to augment CD3-induced AP-1 transactivation through JNK-dependent phosphorylation of the c-Jun transactivation domain (5–9). Serine phosphorylation of c-Jun on residues 63 and 73 by JNK has previously been shown to increase its capacity to transactivate at enhancer DNA (10). However, our result was somewhat paradoxical in that Th1 cells do not express detectable c-Jun protein early during activation when JNK activity is mostly up-regulated by CD3 and CD28 signals, and the AP-1 complexes isolated from Th1 cells do not contain c-Jun at a time when peak IL-2 transcription is observed (3, 11). Therefore, experiments shown here have examined the mechanism by which B7/CD28-mediated costimulatory signals and Ag-receptor occupancy integrate to regulate the expression and function of AP-1 proteins important to IL-2 gene transactivation in normal Th1 cells. Our results suggest that B7 costimulatory ligands on APC enhance JNK protein kinase activity leading to more vigorous Elk-1 transactivation at the c-fos gene and the increased expression of AP-1 proteins during Ag stimulation.

Materials and Methods

T cells, mice, and reagents

The AE.7 and 16B.2 pigeon cytochrome c (PCC)-specific and I-Ed-restricted murine Th1 cells used in this study, and their cultivation, have been previously described (12, 13). The DO11 chicken OVA-specific and I-Ak-restricted murine Th1 cells were derived from CD4+ lymph node cells of DO11.10 TCR-transgenic mice (14) and maintained as short-term lines by intermittent stimulation with Ag and 3000 rad-irradiated BALB/c spleen cells. These DO11 T cells were used after 2–5 rounds of in vitro expansion with Ag and IL-2, and were rested 14 days before each experiment. The human T-leukemia cell line Jurkat (American Type Culture Collection (ATCC), Manassas, VA) was also examined in some experiments, for comparison with the normal Th1 cells. For Ab stimulations, Th1 cells (2×106 cells) were incubated with polystyrene-immobilized anti-CD3 (mAb 145-2C11) (15) and/or anti-CD28 (mAb 37.51) (16) as previously described (4). In some experiments, a goat anti-hamster IgG was used as a first-step reagent followed by immobilization of the anti-CD3 and/or -CD28 mAbs, with similar results. The calcineurin inhibitor cyclosporin A (CSA), the mitogen-activated protein kinase (MAPK)/ERK kinase 1 (MEK1) inhibitor PD 098059, and the stress-activated protein kinase (SAPK) inhibitor SB 202190 were obtained from Calbiochem (La Jolla, CA). CSA was dissolved in ethanol (final concentration 0.1%), whereas PD...
Ag-presenting cells

Animal use has been approved by the University of Minnesota Institutional Animal Use and Care Committee and is in accordance with National Institutes of Health guidelines. For the production of APC, spleens were harvested from B10.A or BALB/c mice. Leukocytes were incubated in culture dishes in complete medium for 2–3 h at 37°C after which nonadherent cells were removed by vigorous rinsing with PBS. The adherent spleen cells were treated with 10 μg/ml LPS (Sigma, St. Louis, MO) at 37°C for 16 h in complete medium and then removed from the plate using a cell scraper. Accessory molecule expression on recovered, activated spleen cells was determined by FACScan flow cytometry using CellQuest software (BD Biosciences, Mountain View, CA). For Ag stimulations, LPS-activated spleen APC were exposed to the Ag (either OVAp323–339 or PCsP21–107) at 37°C for 2 h before mixing with the responding Th1 cells (3 × 10^5) at an APC/T-cell ratio ranging from 1:2 to 1:2.5. To neutralize potential costimulatory molecules on the APC, hybridomas producing hamster IgG anti-murine B-71 mAb 16-10A1 (provided by H. Reiser, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT) (17), rat IgG2a anti-murine B-72 mAb GL1 (ATCC) (18), and rat IgG2a anti-murine ICAM-1 (ATCC) (19) were obtained and their mAb purified using standard protein A chromatography techniques. Purified mAbs were used at a final concentration of 5–20 μg/ml as indicated. The combination of anti-B7-1 and -B7-2 mAbs was routinely capable of inhibiting IL-2 production (assay as previously described in Ref. 20) by >90% in Th1 cells responding to Ag and these APCs (see Fig. 1A). Hamster IgG and rat IgG2a served as irrelevant Ab controls (Pierce, Rockford, IL).

Stable transfectant Th1 cells

The pNFATZH, pSXNeo/AP-1, and p15MT-II plasmids were provided by G.R. Crabtree (Stanford University, Palo Alto, CA). pNFATZH contains three tandem repeats of the human IL-2 NFAT binding sequence (−286 to −257 relative to the transcriptional start site of the IL-2 gene) within the XhoI site of a minimal IL-2 promoter (bp −319 to +47 of the IL-2 gene containing an internal deletion and replacement of bp −296 to −72 by XhoI linker DNA) fused to the lacZ gene. Plasmid pAP-1Zf contains five tandem repeats of the sheep metallothionine MT-II gene-1 site (TGACTCA) within the minimal IL-2 promoter and was constructed as previously described (3). Plasmid pIl-2ZfH was constructed similarly by inserting the entire human S′ IL-2 enhancer DNA sequence (−326 to +47) contained within the XmnI-HindIII fragment of pl15MT-II/120 to a pNFATZH vector that had its corresponding XmnI-HindIII fragment deleted. The resulting pl2ZfH plasmid was identical with pNFATZH and pSXNeo except that 10 μg/ml DNA contained the intact 5′ IL-2 gene sequence replaced the 3′ NFAT/LIL-2 minimal promoter or 5xAP-1/LIL-2 minimal promoter sequences, respectively. The generation of the NFATZH and AP-1Zf stable transfectants of cloned 16B.2 Th1 cells was previously described (3), and these transfectants have been maintained as pools of cells to eliminate any effects of site of integration. IL-2ZfH 16B.2 cells (stably transplanted with pIl-2ZfH) were produced and maintained by the same method. Lysis of activated cells and assay for β-galactosidase activity was performed as previously described (3).

EMSAs of DNA-binding complexes

EMSAs was performed on nuclear extracts proteins as previously described (3) using the following T4 kinase-mediated [γ^2P]ATP-dependent double-stranded oligonucleotides: mouse NFAT, 5′-TGGAGAAGGAAAAATTTGTTTCATACAGAAGGCG-3′; mouse AP-1, 5′-TGGAGAAATTTCCAGACAGTACGAGAAAGA-3′.

Western blot detection of Fos and Jun proteins

c-Fos, FosB, and JunB protein expression were determined by Western blot as previously described (3). T cells (1–1.5 × 10^6) were solubilized in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM NaVO_3, 2 mM sodium pyrophosphate, 1 mM PMSF, and 10 μg/ml leupeptin for 30 min. Insoluble material was removed by high-speed microcentrifugation at the cold, and samples were diluted 2:1 in 3× Laemmli sample buffer. Following separation by SDS-PAGE, proteins were transferred to nitrocellulose. Equal protein loading was confirmed at that time by staining of membranes with Ponceau S (data not shown). Membranes were then probed with either a pan anti-Fos Ab (peptide 128–152) or anti-JunB Ab (peptide 45–61) obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Detection was performed using a goat anti-rabbit Ig-HRP conjugate (Bio-Rad, Hercules, CA), and ECL chemiluminescence reagents (Amersham, Arlington Heights, IL).

In vitro kinase assays and ex vivo detection of phosphoproteins

The solid-phase JNK protein kinase assay is analogous to that by Hibi et al. (21) and was previously described (4). In this assay, JNK is first immobilized on a GST-c-Jun fusion protein bound to glutathione Sepharose 4B beads (Amersham Pharmacia Biotech, Piscataway, NJ), and then an in vitro kinase reaction is conducted using the GST-c-Jun as substrate. The ERK immune complex kinase assay was also performed according to the method of Hibi et al. (21) and was conducted as previously described using anti-ERK2 polyclonal antisera (Santa Cruz Biotechnology) and myelin basic protein (Upstate Biotechnology, Lake Placid, NY) as substrate (4). Alternatively, ERK activity was assayed by solid-phase in vitro kinase reaction using GST-Erk1-2 as both the ERK immobilizer and substrate. This GST-Erk1-2 fusion protein contains aa 307–428 based on the human Erk1-2 complementarity DNA (plasmid encoding GST-Erk1-2 provided by R.A. Hipskind, Institut de Geneique Moleculaire de Montpellier, Montpellier, France) and was expressed in Escherichia coli strain HB101. We have found that this GST-Erk1-2 fusion protein binds only activated (dual phosphorylated) ERK, and does not bind either resting or activated JNK (data not shown). Incorporation of 32P into all substrates was found to be linear with time over the course of the in vitro kinase reactions and sensitive to differences in enzyme activity, as established by SDS-PAGE and autoradiography. Dual-specific phosphorylation of p38 indicative of its activation was detected using phospho-p38 MAPK (Thr180/Tyr182) Ab obtained from New England Biolabs (Beverly, MA) to probe a Western blot. The statistical comparisons indicated relied on Student’s paired t test.

Construction of expression plasmids and reporter genes for transient transfection

Plasmid pEF-Gal4 was prepared by first excising a 1.7-kb PvuII-Sall fragment containing the EF-1α promoter from the mammalian expression vector pEF (provided by G. Koretzky, University of Iowa, Iowa City, IA) and then inserting it into the polylinker of the pSP72 shuttle vector (Promega, Madison, WI). A 1.7-kb PvuII-BamHI fragment was then excised from this EF-1α shuttle vector and used to replace a 0.4-kb PvuII-Bcl/II fragment containing the SV40 promoter of the mammalian expression vector pBG1 (provided by L.M. Staudt, National Institutes of Health, Bethesda, MD) to create the plasmid pEF-Gal4. This pEF-Gal4 vector contains an EF-1α promoter followed by a cDNA encoding the Gal4 DNA-binding domain with a downstream polylinker and poly(A) tail. To create pEF-Gal4-Elk1-1, a fragment encoding the Elk1-1 transcriptional domain (aa 307–428) was generated by PCR using a plasmid containing the human Elk1-1 complementary DNA (ATCC). The forward primer sequence (A) 5′-GGTGTAGCGTCCTCTCCAGACAG-3′ and the reverse primer (B) 5′-TGAAGGCTTCGGGCCTCTGGGGCTTGG-3′ contained a BamHI restriction site, and the reverse primer (B) 5′-TGAAGGCTTCGGGCCTCTGGGGCTTGG-3′ contained a BglII site that facilitated cloning. Following amplification, the Elk1-1 PCR product was cut with BamHI and BglII and inserted into the BamHI site of pEF-Gal4, maintaining the Elk1-1 sequences in-frame with Gal4. To make plasmid pEF-Gal4-Elk1-1(m) encoding a mutant Gal4-Elk1 fusion protein with alanine substitutions at serines 383 and 389, PCR mutagenesis was performed by Xu et al. (22). First, a mutated PCR fragment encoding aa 383–428 was generated from the Elk1-1 complementary DNA using a 5′-phosphorylated forward primer 5′-GTCCTCCATTGCCGCCCTGTCCTC-3′ (where the alanine substitutions at residues 383 and 389 are indicated in bold) and reverse primer (B). Second, a fragment encoding Elk1-1 residues 307–382 was generated using forward and a 5′-phosphorylated reverse primer 5′-CAAGTCATGCAAGGTAAGTG-3′. These two PCR products were then directly ligated, restricted with BamHI and BglI, and then cloned into the BamHI site of pEF-Gal4 as before.

To make the 5xGal4Luc reporter gene called p5GT109Luc, a 200-bp BamHI-Sall fragment from pSG5EC (provided by L. M. Staudt), containing five tandem repeats of the Gal4 DNA binding sequence and linker DNA, was cloned upstream of the thymidine kinase promoter at identical restriction sites within the pTi109Luc plasmid (ATCC). Unlike pEF-Gal4-Elk1-1, cotransfection of pEF-Gal4-Elk1-1(m) with pGT109Luc did not result in luciferase reporter gene induction, demonstrating the requirement for serines 383 and 389 for Gal4-Elk1-1 transcriptional activity (data not shown). pTi109Luc itself served as a control luciferase reporter gene in these experiments. (A) 5′-CACATTGCCGCCCTGTCCTC-3′ was cloned upstream of the thymidine kinase promoter at identical restriction sites within the pTi109Luc plasmid. The statistical comparisons indicated relied on Student’s paired t test.

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Consistent with this, further experimentation with stably transduced Th1 cells confirmed that in addition to AP-1 elements, CD3 and CD28 mAbs also synergistically induce transactivation at NFAT DNA response elements, as well as at the 5' IL-2 gene enhancer itself (data not shown).

Based on these results, we investigated whether physiological interactions between B7 costimulatory molecules and CD28 play an important role in the up-regulation of AP-1-dependent transactivation in Th1 cells responding to Ag and APC. Adherent spleen cells activated with LPS served as the APC in these experiments. Use of such a heterogeneous population of cells (containing activated dendritic cells, macrophages, and some B cells) was intentional, and ensured the presence of multiple potential costimulatory ligands including B7-1 (CD80), B7-2 (CD86), and ICAM-1 (CD54) on the APC (data not shown). These APC were found to be potent stimulators of IL-2 synthesis in Th1 cells when Ag was present, and their expression of B7 molecules proved essential to this response because the combination of anti-B7-1 and B7-2 mAbs prevented the secretion of IL-2 (Fig. 1A). Using these Ag-loaded APC as stimulator cells, the pair of anti-B7 mAbs was observed to significantly reduce the activation of the AP-1 and NFAT reporter genes in Th1 cells (Fig. 1B). Transactivation of the 5' IL-2 gene enhancer reporter was also significantly inhibited by the addition of anti-B7 mAbs, and this effect appeared specific because the addition of an irrelevant IgG failed to affect the response (Fig. 1, B and C). Interestingly, an anti-ICAM-1 mAb demonstrated significantly less inhibitory capacity than the B7 mAb pair. Thus, Ag- and APC-stimulated 5' IL-2 gene enhancer activation in Th1 cells was found to be highly dependent on a synergy between the TCR and a unique set of signals arising from B7-ligated CD28 molecules. Because the activation of each of these three reporter genes can be expected to rely on the binding of an AP-1 transcription factor complex to cis-acting DNA elements, AP-1 proteins appeared to be the targets of these synergistic signals in Th1 cells.

**Transient cotransfection reporter system**

Transient cotransfection of the Th1 cells was carried out by electroporation using a GenePulser (Bio-Rad) set to 960 μF and 340 V. Before transfection, Th1 cells (up to 8 × 10^5 cells in 0.4 ml of OPTI-MEM I medium per cuvette) were incubated at room temperature for 10 min with plasmids: 5 μg pGreen Lantern-1 (Life Technologies, Gaithersburg, MD); 10 μg of control luciferase reporter gene pT109muC, pSGT109muC, or pIL-2-luc; 30 μg of control vector pEF-Ga4, pEF-Ga4Elk-1, or pEF-Ga4Elk-1(m). Cells were then harvested for 15 min at room temperature in the cuvettes after electroporation, and then incubated in complete medium for 2 days before stimulation. Luciferase activity was assayed in cell lysates using Reporter Lysis Buffer and Luciferase Assay System (Promega) according to the manufacturer’s instructions. Relative light units shown have been corrected for protein concentration in each lysate. The transfection efficiency for each electroporated sample was determined using the FACScan (FL1 detector) to detect pGreen Lantern-1 (green fluorescent protein) expression.

**Results**

**Transactivation at the IL-2 gene by NFAT and AP-1 DNA elements is costimulated by B7/CD28 interactions in Th1 cells**

Using immobilized anti-CD3 mAb as a mimic of TCR/CD3 ligation in the absence of either Ag or APC, we had previously determined that normal Th1 cells require CD28 costimulation to maximally induce the transcription of a stably transfected AP-1 reporter gene (4), thus predicting a capacity for the CD28 molecule to regulate AP-1-dependent transactivation at a number of cis-acting DNA elements within the 5' IL-2 gene enhancer.

**FIGURE 1.** Unique role for B7 costimulatory signals in Ag-induced AP-1-dependent IL-2 gene transactivation. A, Ag- and APC-induced IL-2 production either in the absence or presence of a combination of anti-B7-1 plus anti-B7-2 mAbs (10 μg/ml each). The numbers of 16B.2 Th1 cells and APC were held constant in each sample at a ratio of 5:1, and the PCPC<sub>81–104</sub> loading concentration was varied as indicated. B, Pools of 16B.2 Th1 cells stably transduced with β-galactosidase reporter gene constructs containing a 5x multimer of a consensus AP-1 binding site (AP-1Z), a 3x multimer of the human NFAT binding site (NFATZ), or a single copy of the 5' IL-2 gene enhancer (IL-2Z) were stimulated for 3 h with Ag and APC either in the absence or presence of the pair of anti-B7 mAbs (10 μg/ml each). β-Galactosidase activity was calculated as milliunits of activity per milligram of protein from whole cell extract and reported as fold increase above unstimulated controls. Bars represent the mean ± SEM of at least five independent experiments for each reporter construct. C, Stimulation of IL-2Z Th1 cells with Ag/APC as in B, but percent inhibition observed with the addition of an irrelevant hamster IgG (10 μg/ml), the combination of anti-B7-1 and -B7-2 mAbs (10 μg/ml each), or an anti-ICAM-1 mAb (10 μg/ml) is reported. Bars represent the mean ± SEM inhibition for n = 2 (hamster IgG), n = 10 (anti-B7 mAbs), and n = 2 (anti-ICAM mAb) independent experiments.
Induction of AP-1 complex formation during Ag presentation to Th1 cells depends on specific signals arising from the B7/CD28 interaction

The requirement for B7/CD28 interactions for the up-regulation of AP-1-dependent transcription during Ag stimulation is seemingly consistent with previous demonstrations of B7 and anti-CD28 mAb costimulation of NFAT activity in T cells, and with a presumed role for JNK in the regulation of transactivation by Jun-containing AP-1 complexes (4, 5, 23, 24). However, at closer inspection these observations are more difficult to reconcile, as only a minority of AP-1 complexes in Th1 cells contain a c-Jun or JunD protein capable of regulation by direct JNK-dependent phosphorylation (3). Therefore, we examined whether physiological interactions between B7 costimulatory molecules and CD28 are important in the up-regulation of AP-1 complex formation in Th1 cells responding to Ag and APC. Using Ag-loaded APC to stimulate the activation of Th1 cells, T cell nuclear extracts were examined for the presence of AP-1 transcription factors using NFAT and AP-1 double-stranded oligonucleotide probes and an EMSA system. We and others have previously determined that the NFAT DNA-binding activity in Th1 cells consists of a trimeric protein complex containing both NFATp as well as a Fos and Jun (AP-1) heterodimer (3); therefore, any requirement for B7 costimulatory signals during the induction of NFAT DNA-binding activity could be expected to be at the level of AP-1 complex assembly or its association with NFAT protein.

Th1 cells stimulated with Ag and APC up-regulated both NFAT and AP-1 DNA-binding activities (Fig. 2). Similar to the IL-2 production and transactivation experiments described above, the addition of a combination of neutralizing anti-B7-1 and -B7-2 mAbs (10 μg/ml each), or anti-ICAM-1 mAb (10 μg/ml), and then nuclear extracts were assayed for the presence of NFAT (A) or AP-1 (B) DNA-binding complexes by EMSA. Results are representative of three experiments.

**FIGURE 2.** Induction AP-1 DNA-binding activity in Th1 cells following stimulation with Ag and APC requires costimulation by B7. Th1 cells were stimulated for 3 h with Ag PCCp and APC as indicated in the absence or presence of a combination of anti-B7-1 and -B7-2 mAbs (10 μg/ml each), or anti-ICAM-1 mAb (10 μg/ml), and then nuclear extracts were assayed for the presence of NFAT (A) or AP-1 (B) DNA-binding complexes by EMSA. Results are representative of three experiments.

The induction of AP-1 complex formation during Ag presentation to Th1 cells depends on specific signals arising from the up-regulation of AP-1-dependent transcription during Ag stimulation for the dephosphorylation of NFATp that leads to its nuclear translocation (3); therefore, any requirement for B7 costimulatory signals during the Ag-stimulation of NFAT DNA-binding activity could be expected to be at the level of AP-1 complex assembly or its association with NFAT protein.

**FIGURE 3.** B7- and CD28-costimulated Fos protein expression in Th1 cells. A, Stimulation of Th1 cells with Ag OVAp-loaded LPS-treated adherent splenic APC for 4 h in the presence of either anti-B7-1 plus -B7-2 mAbs or isotype-matched irrelevant rat and hamster control Abs (20 μg/ml each). B, Th1 cells were stimulated for 3 h with immobilized anti-CD3 mAb at the indicated concentration, either alone or in the presence of anti-CD28 mAb (10 μg/ml). C, A 4-h stimulation of Th1 cells with immobilized CD3 and CD28 mAbs (10 μg/ml each) either in the presence or absence of SB 202190 (4 or 40 μM) or PD 098059 (50 μM) as indicated. Fos protein expression was detected in a Western blot analysis using a pan-Fos rabbit Ab. The c-Fos and FosB identifications noted at 62 and 46 kDa, respectively, are based on previous results using c-Fos- and FosB-specific Ab probes (3). All results shown are representative of at least three independent experiments.

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Activation of JNK during Ag/APC stimulation relies on the recognition of B7 costimulatory ligands

This requirement for B7/CD28 costimulation in the up-regulation of AP-1 protein expression following exposure of Th1 cells to Ag and APC was reminiscent of the CD28 dependence of JNK activation previously demonstrated by us and others (4, 5). We also observed that the calcineurin inhibitor CSA could blunt the induction of Fos protein in Th1 cells stimulated with either Ag plus APC or with the combination of anti-CD3 and -CD28 mAbs (data not shown), consistent with a role for a SAPK such as JNK or p38 in the regulation of fos gene regulation in these cells (7, 8, 27). Therefore, we hypothesized that a CD28-dependent MAPK mediates the costimulatory effects of B7/CD28 interactions on AP-1 protein synthesis in Th1 cells.

To address this issue, we examined whether the physiological activation of ERK, JNK, or p38 could depend on the ligation of CD28 by B7 molecules. Whole cell extracts from Th1 cells stimulated with Ag and APC either in the presence or absence of anti-B7 mAbs were assayed for ERK and JNK protein kinase activities, and for activating threonine-180 and tyrosine-182 phosphorylations of p38. Stimulation of helper T cells with Ag rapidly induced both JNK and ERK protein kinase activities, and p38 became phosphorylated consistently with its activation. Interestingly, the addition of anti-B7 mAbs did significantly limit the activation of JNK (58 ± 19% inhibition, p ≤ 0.01, n = 7) (Fig. 4). In contrast, ERK activation was little affected by the addition of anti-B7 mAbs (5 ± 14% inhibition, p ≤ 0.89, n = 6). Similarly, Ag- plus APC-induced phosphorylation and activation of p38 demonstrated no significant inhibition following the addition of the anti-B7 mAbs (9 ± 26% inhibition, p ≤ 0.96, n = 5). The ERK and p38 activation events were also independent of ICAM-1/LFA-1 interactions, given the lack of inhibition observed following the addition of anti-ICAM-1 together with the anti-B7 mAbs. These data implied that neither ERK nor p38 could mediate the unique costimulatory effects of B7 molecules binding CD28 receptors that occurs during the response to Ag-bearing APC. In contrast, the activation of JNK correlated well with the B7-dependent expression of Fos protein.

Elk-1 transactivation during stimulation with Ag depends on B7 costimulation

This direct correlation observed between JNK activation and the induction of AP-1 protein expression during Ag and APC stimulation in Th1 cells suggested that JNK may play an important role as a downstream mediator of B7/CD28 signals to the fos and junB genes. Consistent with this notion, CSA proved incapable of inhibiting the activation of either ERK or p38 during the response to Ag and APC, whereas JNK activation (like Fos expression) was sensitive to the effects of CSA (data not shown). c-fos gene transcriptional regulation has been well studied in a number of cell types, and is thought to depend mainly on the phosphorylation of the c-fos gene regulator Elk-1 by a MAPK such as ERK (28). It has generally been assumed that the activation of ERK in response to TCR ligation would be sufficient to maximally induce Elk-1-dependent c-fos gene expression in T cells (29). Less is known about the regulation of FosB or JunB expression; however, Ets-like transcription factors with sequence homology to Elk-1 and similar regulation by MAPK such as ERK have been implicated in the control of fosB and junB gene transcription (30–32). Perhaps consistent with these published data, we found that Ag-stimulated Fos protein expression in Th1 cells could not be induced under conditions where ERK activation was blocked with the MEK1 inhibitor PD 098059 (Fig. 3C). But based on this simple paradigm, one should have expected little costimulatory signal dependence of AP-1 protein induction and no sensitivity to CSA. Our results instead suggested a more complicated regulation of c-fos, fosB, and junB gene transcription that integrates signals from both the TCR and CD28.

The Elk-1 transcription factor is known to be a target of phosphorylation by each of the MAPK (33–37), and we found using an in-gel kinase reaction that the transactivation domain of Elk-1 can indeed be phosphorylated by ERK, JNK, and p38 extracted from activated Th1 cells (W. Li, unpublished observation). Based on this, we undertook an investigation of the hypothesis that the Elk-1 transactivation domain consists of a recombinant Gal4-Elk-1 fusion protein containing a Gal4 DNA-binding domain together with the Elk-1 transcription domain, and a 5xGal4 luciferase reporter gene containing five tandem repeats of the Gal4 DNA-binding sequence was constructed for these experiments. Transient coexpression of Gal4-Elk-1 together with the 5xGal4 luc reporter gene led to the accumulation of luciferase activity when the transfected Th1 cells were activated with a combination of CD3 plus CD28 mAbs, or with Ag plus APC (Fig. 5). The induction of the c-Fos protein, Elk-1 transactivation was observed to be suboptimal in response to CD3 ligation alone; however, the addition of immobilized anti-CD28 mAb led to an improved response. Finally, Ag-induced Gal4-Elk-1 transactivation was found to be signficantly reduced when B7 molecules on the APC were neutralized with Abs, consistent with the requirement for CD28 ligation by B7 that leads to increased JNK signaling.

MEK1 protein kinase activity is necessary, albeit insufficient, to activate Elk-1 transactivation

Similar to its inhibitory effects on Fos protein expression and on 5′ IL-2 gene enhancer/promoter activity itself (Figs. 3C and 5C), blockade of MEK1 protein kinase activity in the Th1 cells with PD 098059 effectively prevented transactivation by Elk-1 in this reporter gene system (Fig. 5C). Despite this apparent requirement for ERK activation in the elicitation of Elk-1-dependent c-fos gene transcription, the intense activation of ERK that accompanies stimulation with CD3 mAb alone or Ag stimulation in the presence of anti-B7 mAbs was clearly insufficient to maximally induce Elk-1 activity (Figs. 4A and 5, A and B; data not shown). The activation of calcineurin also appeared necessary to stimulate maximal Elk-1 transactivation, based on its sensitivity to inhibition with CSA (data not shown). Because CSA has no capacity to inhibit the
activation of ERK in Th1 cells (data not shown), pathways downstream of CD28 and calcineurin and independent of ERK activation also appeared necessary to fully activate this c-fos gene transcriptional regulator.

The induction of Elk-1 transactivation and c-fos gene transcription in Th1 cells depends on the protein kinase activity of JNK

Based on the similarities observed between JNK activation and Elk-1 transcriptional regulation in Th1 cells, we hypothesized that JNK protein kinase activity mediates the B7- and CD28-dependent induction of Elk-1 transactivation in cooperation with TCR-induced ERK activity. To directly address these issues, we took advantage of the SAPK inhibitor SB 202190 (38). Using SB 202190 at a low dose capable of interrupting the kinase activity of only p38 (4 μM) (data not shown), we found that the drug only ineffectively limits the up-regulation of c-Fos and FosB protein expression (Fig. 3C). In contrast, at a higher dose (40 μM) capable of interfering with the kinase activity of both p38 and JNK (data not shown), SB 202190 proved to be a potent inhibitor of both c-Fos and FosB protein expression (Fig. 3C). Consistent with such a role for JNK in the transcriptional regulation of c-fos gene expression, Elk-1 function and IL-2 gene transactivation were similarly blocked in the presence of SB 202190 at concentrations approaching 40 μM (Fig. 5D). SB 202190 (40 μM) appeared to be neither toxic to Th1 cells nor nonspecific in its actions, as this dose of drug did not interfere with the activation of ERK (data not shown).

Discussion

The activation of the SAPK JNK is shown here to be dependent on B7-induced costimulatory signals that arise during the Ag stimulation of Th1 cells. The finding that anti-B7 mAbs also significantly inhibited c-Fos protein expression supports the hypothesis that JNK-dependent phosphorylation events mediate the costimulation of AP-1 complex formation and IL-2 production in Th1 cells. Consistent with this, the SAPK inhibitor SB 202190 prevented Elk-1-dependent transactivation, c-Fos expression, and 5′ IL-2 gene enhancer activation when present at sufficient concentration (40 μM) to block both p38 and JNK function, whereas a concentration sufficient to inhibit only p38 (4 μM) failed to prevent these responses.

There is currently considerable controversy surrounding the role of JNK in the regulation of IL-2 secretion and T cell proliferation. In naive CD4+ T cells, JNK is unlikely to mediate
such a B7 costimulatory effect on AP-1-dependent IL-2 gene regulation. JNK levels are relatively low in these cells (39). Furthermore, our own investigation of JNK activation and c-Jun phosphorylation in naive CD4+ T cells stimulated with Ag in vivo indicates that JNK activation can occur even in the absence of CD28 signaling. A careful analysis of fresh splenic T cells in JNK2-deficient mice did in fact reveal a requirement for JNK2 signaling to achieve effective IL-2 production and proliferation in response to CD3 plus CD28 cross-linking (40); however, other independently derived JNK2-deficient mice demonstrated no apparent defect (41). Interestingly, naive CD4+ T cells developing in JNK1 and JNK2 double knockout mice, or in mice homozygous mutated for the JNK activator gene MAPK kinase 7 (MKK7), also demonstrated no defect in either IL-2 production or proliferation (42), implying that in naive T cells parallel signaling pathways exist to transmit costimulatory signals to the IL-2 gene. IL-2 production has not been examined specifically in untransformed Th1 cells genetically deficient in both JNK1 and JNK2 activity because differentiation to the Th1 phenotype is inhibited in the absence of JNK1 (43). Previous experiments using transformed Jurkat cells and transfection of a GST-c-Jun fusion protein to antagonize endogenous JNK function have consistently indicated that JNK protein kinase activity is essential for AP-1- and NFAT-dependent transactivation (5, 44). Furthermore, overexpression of dominant negative mutants of MKK4 or MKK7 (potential activators of JNK) leads to reductions in NFAT and 5' IL-2 gene enhancer DNA-dependent reporter gene activation in Jurkat T cells (7, 45). Data shown here strengthen the hypothesis that JNK is an important regulator of IL-2 production in Th1 cells, through its ability to sense the ligation of CD28 and as a result of its capacity to control Elk-1 transactivation and AP-1 complex assembly.

Although this study provides evidence that JNK mediates the unique B7/CD28-dependent costimulatory signal that promotes AP-1 complex formation during Ag stimulation by increasing the activity of the Elk-1 transcription factor, this activation of Elk-1 nevertheless also depends on MEK1. This indicates that Elk-1 may be an important point of signal integration between pathways highly sensitive to TCR signals (e.g., ERK activation) and pathways downstream of CD28 that promote the activation of JNK. Consistent with this, 5' IL-2 gene enhancer-driven transactivation, IL-2 mRNA accumulation, and IL-2 secretion have been shown to be consistently inhibited by the addition of the MEK1 inhibitor PD 098059 by overexpression of dominant negative mutants of MEK1 or by overexpression of the MAPK-specific phosphatase (MKP-1) (45–47).

We note that this signal integration observed in Th1 cells could not have been predicted based on results obtained using the Jurkat T-leukemia cell line alone, where significant Elk-1-dependent transactivation has been shown to be elicited with a PKC-activating phorbol ester alone (29). We have confirmed this Jurkat result; nevertheless, treatment of Th1 cells with PMA was insufficient to activate transcription in our Gal4-Elk-1 reporter gene system or to induce high levels of c-Fos protein expression (data not shown). These differences may relate to the differentiation state of the two T cell types, or alternatively may reflect the effects of cell transformation on signal transduction in the Jurkat T-leukemia line. Regardless, the costimulatory signal-dependent activation of Elk-1 in Th1 cells observed here appears to be essential for the up-regulation of immediate-early genes such as c-fos, the accumulation of AP-1 complexes at the 5' IL-2 gene enhancer, and the induction of IL-2 transcription in response to Ag stimulation.

The biochemical nature of the cooperativity that exists between JNK and MEK1 (or perhaps ERK) at the level of Elk-1 function remains unknown. Elk-1 is known to constitutively associate with serum response factor (SRF) homodimers at the serum response element (SRE) of the 5' c-fos gene DNA promoter/enhancer (reviewed by Cahill et al. in Ref. 28). Elk-1-dependent transactivation is regulated by phosphorylation at serine-383 and -389, and each of the MAPKs has been shown to be competent to phosphorylate this nuclear factor at these residues in vitro (33–37). Perhaps the in vivo pattern of phosphorylation of Elk-1 in Th1 cells following activation of both JNK plus ERK is unique and different from that achieved by either kinase alone. Subtle differences in phosphorylation specificity have been observed with the various MAPKs, although the significance of these differences is not well understood. Unique phosphorylations might affect the ability of Elk-1 to transactivate, or to interact with the SRF dimer or some other transcriptional regulator.

Alternatively, an individual MAPK may have the capacity to directly regulate the function of some other signaling molecule that interacts with Elk-1 and influence its transactivation. The kinase suppressor of Ras (KSR), the adaptor protein Gab2, and calcineurin have each been identified as signaling proteins capable of interfering with the function of Elk-1 (48–51). Perhaps these negative regulators are themselves turned off by costimulatory signals as a result of their phosphorylation by JNK. In contrast, the transcriptional coactivator CREB-binding protein (CBP) has been shown to physically interact with Elk-1, is capable of augmenting Elk-1 transactivation, and is itself a phosphorylation target for MAPK (52). CBP (as well as the related protein p300) acts as a bridge between the inducible transactivation factors and the basal transcription machinery at the promoter, and also regulates local chromatin structure through its histone acetyltransferase activity (53–55). Several groups have recently determined in T cells that CBP binds to NFAT and integrates signals from both Raf and the Rac1 GTPase to enhance NFAT-dependent transactivation (56, 57). Because Rac1 can also mediate the CD28-dependent activation of JNK and AP-1 in Jurkat T cells (7, 8, 29, 58), we speculate that TCR and CD28 signal integration at the c-fos gene in Th1 cells results from JNK and ERK cooperatively activating Elk-1/CBP complexes at the SRE.

Finally, the discovery of unique B7/CD28 costimulatory signals integrating with TCR signals at the level of Elk-1 transactivation does not preclude the possibility of other costimulatory signals also acting elsewhere within the c-fos and IL-2 genes to influence transcription. The p38 downstream effector molecule MAPK-activated protein kinase-2 is responsive to CD28 signals in T cells and has been shown to be a SRF kinase (59, 60). In addition, the c-fos gene enhancer contains a cis-acting aCAMP response element just downstream of the SRE, and transactivation by phosphorylated CREB appears to be sensitive to costimulatory signals in T cells (61). Consistent with this, CREB phosphorylation can be induced by the p38 (and ERK) downstream effector molecule mitogen- and stress-activated protein kinase-1 (62). Of note, the CBP/p300 coactivators bind both CREB and SRF, in addition to Elk-1, NFAT, Rel, and AP-1 proteins (52–57, 63–66). It remains to be determined whether costimulatory signals play a general role in the regulation of CBP-mediated transcriptional coactivation at immediate-early genes as well as at the 5' IL-2 gene enhancer itself.

Acknowledgments

We thank E. V. Wattenberg for help in developing our p38 assay systems.
M. Mescher and S. Jameson for critical reading of the manuscript, and
M. K. Jenkins for continued helpful discussions.

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