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CD28 costimulation amplifies TCR-dependent signaling in activated T cells, however, the biochemical mechanism(s) by which this occurs is not precisely understood. The small GTPase Rac-1 controls the catalytic activity of the mitogen-activated protein kinases (MAPKs) and cell cycle progression through G₁. Rac-1 activation requires the hydrophospho-tyrosine (p-Tyr)-dependent recruitment of the Vav GDP releasing factor (GRF) to the plasma membrane and assembly of GTPase/GRF complexes, an event critical for Ag receptor-triggered T cell activation. Here, we show that TCR/CD28 costimulation synergistically induces Rac-1 GDP/GTP exchange. Our findings, obtained by using ZAP-70-negative Jurkat T cells, indicate that CD28 costimulation augments TCR-mediated T cell activation by increasing the ZAP-70-mediated Tyr phosphorylation of Vav. This event regulates the Rac-1-associated GTP/GDP exchange activity of Vav and downstream pathway(s) leading to PAK-1 and p38 MAPK activation. CD28 amplifies TCR-induced ZAP-70 activity and association of Vav with ZAP-70 and linker for activation of T cells (LAT). These results favor a model in which ZAP-70 regulates the intersection of the TCR and CD28 signaling pathways, which elicits the coupling of TCR and CD28 to the Rac-1, PAK-1, and p38 MAPK effector molecules. The Journal of Immunology, 1999, 163: 844–853.

E ngagement of the TCR by MHC-bound antigenic peptide ligands (“signal 1”) and the binding of the CD28 costimulatory receptor to its ligands, B7-1 (CD80) and B7-2 (CD86) (“signal 2”), trigger different types of signals required for full T cell activation and IL-2 production during an immune response (1, 2). The small GTPase Ras plays an important role in T cell activation (1), as the active GTP-bound form of Ras controls the catalytic activity of multiple downstream effectors, including the mitogen-activated protein kinases (MAPKs). Whereas Ras induces MAPK activation via the Ras-Raf-MEK-MAPK cascade, other small GTP-binding proteins, such as Rac-1, Cdc42, and Rho, activate the c-Jun N-terminal kinases (JNKs) and p38 MAPKs (3–5). p38 MAPK phosphorylates MAPK-activated protein kinase 2 and activates and mediates the transcription of activating transcription factor (ATF)-2 (2, 5). JNK-induced phosphorylation is an critical event in activation of c-Jun, a component of the transcription factor AP-1 known to bind to the IL-2 promoter (2, 6). Since CD3 and CD28 stimulation activates the JNK and p38 MAPK pathways in T cells (6, 7), it is possible that the JNK and p38 MAPKs mediate a critical role for Rhos and Cdc42 in cell cycle progression through G₁ and IL-2 transcription (2, 8, 9).

How do upstream TCR- and CD28-proximal signaling events regulate downstream signaling along the extracellular signal-regulated kinase (ERK)-, JNK-, and p38 MAPK-mediated pathways in T cells? An essential step in the activation of small GTPases is the hydrophospho-tyrosine (p-Tyr)-dependent recruitment of GDP releasing factors (GRFs) to the membrane and the assembly of GTPase/GRF complexes. GRFs activate small GTPases by promoting the conversion of GDP-bound GTPases to the active GTP-bound state. Vav, a GRF expressed exclusively in cells of the hematopoietic lineage, is critical for Ag receptor-triggered T and B cell activation and thymocyte development (10, 11). In the absence of Vav, IL-2 production by T cells is reduced considerably, possibly due to a disruption of either the TCR or CD28 signaling pathways (11). Indeed, Vav is Tyr phosphorylated in response to TCR and CD28 triggering (12). When Vav is overexpressed in Jurkat T cells, Vav elicits the activation of nuclear factors that control IL-2 expression, including NF-AT (13). Vav may also function as a GRF for Rhos and Cdc42, and as a Rac-1 effector (14), and in this Vav Tyr phosphorylation links some receptors (e.g., FceRI) to activation of the Rac-1-JNK pathway (15). Moreover, the binding of Vav to the TCR ζ-chain-associated PTK, ZAP-70, via residue Tyr315, plays a critical role in Ag receptor-mediated signal transduction (17, 18). These observations prompted us to investigate whether TCR and CD28 signaling are linked to MAPK pathways by Vav. Our objectives were: 1) to analyze whether signaling pathways activated by TCR and CD28 converge at the level of ZAP-70 and/or Vav/Rac-1 interactions, and 2) to determine whether ZAP-70 controls the catalytic activity of multiple downstream effectors of Rac-1 involved in TCR and/or CD28 stimulation.

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Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; ATF, activating transcription factor; CHO, Chinese hamster ovary; oligo-DN, oligodeoxynucleotide; ERK, extracellular signal-regulated kinase; LAT, linker for activation of T cells; GRF, GDP releasing factor; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein; p-Tyr, phosphotyrosine; PTK, protein tyrosine kinase; SOS, son of sevenless.
Materials and Methods

Mice

BALB/cJ mice were purchased from Taconic (Germantown, NY), and were used at 6–8 wk of age.

Reagents

The mAbs used were: biotin-conjugated anti-mouse TCRβ (H57-597), CD4 (L3T4), CD28 (37.51), anti-human CD28 (CD28.2) (all from Phar- mingen, San Diego, CA); CD3ε (OKT3); American Type Culture Collection (ATCC), Manassas, VA; anti-v-H-Ras (Oncogene Research Products, Cambridge, MA); anti- Vav, anti-Rac-1 and 4G10 anti-p-Tyr (Upstate Bio- technology, Lake Placid, NY); anti-ZAP-70 and anti-Grb2 (Transduction Laboratories, Lexington, KY). The following polyclonal rabbit Abs were supplied by Santa Cruz Biotechnology (Santa Cruz, CA): anti-Vav, anti- Rac-1, anti-SLP-76, anti-TCRγ, anti-JNK, anti-ERK-1, anti-ERK-2, anti-p38 MAPK, and anti-PAK-1. Purified GST-Rac-1 was obtained from Up- state Biotechnology. Rabbit polyclonal anti-ALP (linker for activation of T cells) serum was kindly provided by Dr. L. Samelson (National Institutes of Health, Bethesda, MD). The expression plasmid for the Rac-1 GST- fusion protein was provided by Dr. M. J. Hart (Onyx Pharmaceuticals, Richmond, CA), and this GST-fusion protein was expressed in Escherichia coli and purified by glutathione-agarose affinity chromatography (4).

Cell lines

The Lck-negative JCaM1.6 and parental human Jurkat leukaemic cell line, E6.1, were obtained from ATCC, and have been described previously (19). The Lck-ZAP-70-deficient T cell line, a variant of the Jurkat E6.1, T cell line (20), was kindly provided by Dr. R. T. Abraham (Department of Immuno- pathology, Mayo Clinic, Rochester, MN). The level of CD28 and TCR surface expression is very similar in all of the Jurkat cell lines studied (data not shown). The EL4 mouse T cell line was purchased from ATCC. Chinese hamster ovary (CHO) cells, which were transfected with and express B7-2 on their surface (21), were a generous gift from Dr. T. Watts (Department of Immunology, University of Toronto, Toronto, ON, Canada). Cells were maintained in RPMI 1640 (Life Technologies, Burlington, ON) medium supplemented with 10% heat-inactivated FCS (Sigma, St. Louis, MO).

Cell activation and lysis

BALB/cJ peripheral splenic T cells were purified on T cell-enrichment columns (R&D Systems, Minneapolis, MN) (purity >95%). T cells were washed and incubated in RPMI 1640 medium supplemented with 2 mM HEPES before stimulation. If not otherwise indicated, T cells were stimulated at 37°C with 1 μg/105 cells of the biotin-conjugated anti-TCR or anti-CD3ε mAb either alone or together with the biotin-conjugated anti-CD4 mAb or anti-CD28 mAb. Cross-linking of mAbs was accomplished using strepta- vidin (Sigma) for various times at a 4:1 w/w ratio. The anti-human CD28 mAb either alone or together with the biotin-conjugated anti-CD4 mAb or anti-p38 MAPK or anti-PAK-1. Purified GST-Rac-1 was obtained from Upstate Biotechnology. Rabbit polyclonal anti-ALP (linker for activation of T cells) serum was kindly provided by Dr. L. Samelson (National Institutes of Health, Bethesda, MD). The expression plasmid for the Rac-1 GST-fusion protein was provided by Dr. M. J. Hart (Onyx Pharmaceuticals, Richmond, CA), and this GST-fusion protein was expressed in Escherichia coli and purified by glutathione-agarose affinity chromatography (4).

Guanine nucleotide exchange assay

GST-Rac-1 fusion protein (5–10 pmol/time point) was loaded (60 min, 32°C) with [8-32P]GDP (25 pmol, 13.3 Ci/mmole; Amersham, Arlington Heights, IL) in 40 μl exchange buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM MgCl2, 1 mM DTT, and 50 μg/ml BSA). Vav immune complexes immunoprecipitated by rabbit anti-Vav Abs were washed five times with exchange buffer. Reactions (performed in duplicate) were started by addition of 8-32P-GDP-loaded GST-Rac-1 in exchange buffer containing nonradioactive GDP (400 μM). At various times (0, 10, and 30 min), aliquots (40 μl) of reaction mix were removed, centrifuged, and GST- Rac-1 was immobilized on a solid support by using glutathione Sepharose, as described (14, 23). Bound radioactivity was quantified by liquid scintillation.

Incorporation of [32P]-labeled guanine nucleotides by Rac-1

Cells (2 × 106) were cultured for 4 h in phosphate-free RPMI medium containing diazylol 10% heat-inactivated FCS and labeled with 32P-orthophosphate (0.2 mCi/ml) for 4 h at 37°C in phosphate-free RPMI/ HEPES (2 mM). Mouse primary T cells were permeabilized by addition of 0.4 U/ml of streptolysin O (Wellcome Diagnostics, Greenville, NC) before labeling, as described (24). Cells were left unstimulated (0 min) or were treated with anti-TCRα–anti-CD28 for 15 min. Precleared postnuclear lysates were immunoprecipitated by protein G agarose precomplexed to either with an anti- Rac-1 (6 μg) or anti-Ras (15 μg) Ab in 50 mM Tris-HCl (pH 7.5), lysis buffer containing 20 μM MgCl2, 350 mM NaCl, 1.5% Nonidet P-40, 0.01% SDS, 1 mM PMSF, 10 μl/ml pepstatin, 10 μl/ml aprotinin, 10 μl/ml leupeptin, 1 mM NaF, and 1 mM Na3V. [32P]-labeled guanine nucleotides bound to Rac-1 and Ras were eluted by heating in 20 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GDP, and 0.5 mM GTP, and fractionated using polyethyleneimine TLC plates (J. T. Baker, Phillipsburg, NJ). Positions of the [32P]-labeled guanine nucleotides were determined according to the mobility of the unlabelled GDP and GTP markers visualized under ultra- violet light.

Anti-sense oligodeoxynucleotide (oligo-DN) treatment

The following oligo-DNs were used to block Vav expression in T cells: (5′-AGGGACAGGGACAGGGCA-3′)-anti-sense and (5′-AGGCTGAAA- GACAGGGCA-3′)- control (scrambled) oligo-DN. The sequences of these oligo-DNs are based on the human proto-Vav cDNA sequence (25). T cells were cultured in the presence of 20 μg/ml oligo-DNs for 48 h.

Kinase assays

Jurkat or EL4 T cells were incubated at 37°C for 3–4 h in FCS-free RPMI 1640 medium before stimulation to reduce background kinase activities to workable levels. PKA-1 kinase activity in Jurkat T cells was assayed after an overnight incubation in serum-free medium. Primary T cells were used without additional preincubation in serum-free medium. T cells were washed twice in RPMI 1640 10 mM HEPES before stimulation. Proteins immunoprecipitated from precleared postnuclear lysates were assayed for association in vitro kinase activity after washing the beads in kinase buffer (25 mM HEPES (pH 7.4), 5 mM MnCl2) by incubating (30 min, 30°C) with [γ-32P]ATP (15 μCi); New England Nuclear, Boston, MA) in 25 mM kinase buffer containing 1.5 μg GST-c-Jun (Santa Cruz Biotechnology; JNK kinase assay), 1.5 μg of the cAMP response element binding protein, ATF-2 (Santa Cruz Biotechnol- ogy; p38 MAPK assay), 3 μg of myelin basic protein (MBP; Upstate Bio- technology; ERK-1, ERK-2, and PKA-1 kinase assays) or 0.6 μg of the cytoplasmic fragment of human erythrocyte band 3 (cfb3; kindly provided by Dr. A. Veillette, McGill University, Montreal, Canada) as substrates. Reactions were stopped by boiling with gel sample buffer. GST-c-Jun, ATF-2 MBP, and cfb3 were resolved by SDS-PAGE and their phosphor- ylation was visualized using a phosphoimager (Bio-Rad). Immunoblotting showed that equal amounts of the JNK, p38, ERK-1, ERK-2, and PKA-1 proteins were precipitated before and after stimulation of all Jurkat T cell variants.

Results

TCR and CD28 signaling pathways converge at the level of the Tyr phosphorylation-dependent Vav-mediated activation of Rac-1

TCR triggering increases the Tyr phosphorylation of Vav (12), which may be critical in the activation of nuclear transcription factors that control the expression of IL-2, including NF-AT (13),
Anti-p-Tyr Western blots of Vav immunoprecipitates showed that, while only weak basal Tyr phosphorylation of Vav occurs in unstimulated BALB/c T cells (Fig. 1A), Vav Tyr phosphorylation is increased substantially following stimulation (5 min) by anti-TCR, anti-TCR plus anti-CD4, anti-CD28, and anti-TCR plus anti-CD28 mAbs, respectively. Vav Tyr phosphorylation was rapidly increased within 1.5 min after TCR, CD28, or TCR/CD28 cross-linking, and this increase is sustained at 20 min after TCR and 60 min after CD28 or TCR/CD28 stimulation, respectively (Fig. 1B). Similarly, Vav Tyr phosphorylation was induced after stimulation of T cells with CHO cells expressing surface B7-2, as previously reported (12). Note that both TCR and CD28 signaling induce similar amounts of Vav Tyr phosphorylation. Moreover, TCR/CD28 coligation significantly increases the levels of Vav Tyr phosphorylation relative to that of T cells stimulated with TCR or CD28 alone (Figs. 1 A–C). Thus, the costimulatory effects of CD28 may result from an additive and sustained increase of Vav Tyr phosphorylation leading to the activation of Vav downstream effectors.

Vav is a GRF for Rho-related proteins, including Rac-1 (14), Cdc42, and RhoA (15). Previously, we confirmed that Vav functions as a GRF for Rac-1 in vitro in a phospho-Tyr-dependent manner and that Vav GRF activity may be regulated by both TCR and CD28 signaling (our unpublished observations). These findings prompted us to investigate whether TCR- and CD28-mediated signals promote Rac-1 nucleotide exchange in vivo. Analysis of the [32P]orthophosphate-labeled guanine nucleotides bound to Rac-1, as determined by polyethyleneimine TLC of Rac-1 immunoprecipitates, revealed that upon TCR and CD28 stimulation of Jurkat T cells (Fig. 2A) and primary mouse T cells (Fig. 2B), the majority of Rac-1 exists in the GTP-bound form. This result supports the notion that the high intrinsic GTPase activity of Rho family proteins precludes the detection of GTP incorporated by small GTPases (26). Under these conditions, the level of [32P]-labeled GDP bound to Rac-1 is the only indicator of Rac-1 nucleotide exchange. The absence of commounprecipitation of other small GTPases with Rac-1 by the anti-Rac-1 mAb used confirmed the specificity of this mAb (Fig. 2C). Neither Ras nor Rho or Cdc42 were detected in Rac-1 immuncprecipitates (data not shown). Essentially no labeled GDP bound to Rac-1 was observed in either control precipitates formed using isotype-matched preimmune Ig or unstimulated T cells, suggesting that, under these experimental conditions, Rac undergoes minimal nucleotide exchange (Figs. 2, A and B). In contrast, TCR, CD28, and TCR/CD28 cross-linking yielded ~5-, 4-, and 10-fold increases, respectively, in the amounts of GDP-bound Rac-1 in Jurkat T cells. Similarly, an acceleration of Rac-1 GDP/GTP exchange was evident in primi- mouse T cells after TCR, CD28, and TCR/CD28 stimulation (5-, 3-, and 7.5-fold increases, respectively) (Fig. 2B). Neither TCR nor CD28 cross-linking affected the levels of 32P-labeled GTP incorporated by Rac-1, confirming the high intrinsic GTPase activity of Rac-1. Moreover, CD28 triggering by B7-2 accelerated Rac-1 GDP/GTP exchange in T cells (Fig. 2A).

Ras and Rac GTPase signaling pathways may be coupled through the Ras GRF son of sevenless (SOS) (23). In addition, activation of CD28 by an anti-CD28 mAb, but not by the B7-1 ligand, activates Ras in Jurkat T cells (12). Nonetheless, we observed only relatively little, if any, effect of anti-CD28 costimulation on TCR-induced Ras GDP/GTP exchange in vivo in T cells (Fig. 2D). This finding was confirmed by analysis of the kinetics of Ras GDP/GTP exchange in T cells at different time points of CD28 stimulation or costimulation (data not shown). Moreover, TCR-stimulated ERK-1 and ERK-2 activity did not increase further following CD28 co-cross-linking, suggesting that ERK-1/2 do not require a costimulatory signal for maximal activation (Fig. 2E). Therefore, the observed small increase in Ras GDP/GTP exchange that occurs in CD28-stimulated T cells is insufficient to elicit ERK-1 and ERK-2 activation. These results reveal the differential activation of SOS-Ras-ERK- and Vav-Rac-1-mediated signaling in TCR- and CD28-stimulated T cells, respectively, and suggest that coupling of the CD28 and Rac signaling pathways does not result from Ras activation by SOS.

**FIGURE 1.** Tyr phosphorylation of Vav in BALB/c splenic T cells following TCR stimulation and CD4 or CD28 costimulation (A), and time course of Vav Tyr phosphorylation in TCR- and CD28-stimulated BALB/c T cells (B). Immunoblotting (IB) of the Vav immunoprecipitates (IP) was performed with an anti-p-Tyr mAb (upper panels). The blot was then stripped and reprobed with an anti-Vav mAb (lower panels). Specificity of the Vav band was confirmed using control isotype-matched preimmune Ig (C-Ig) for immunoprecipitation. C, Inducible Vav Tyr phosphorylation following stimulation of BALB/c splenic T cells with TCR and/or B7-2. BALB/c splenic T cells were activated for 15 min with wild-type CHO cells (CHO) or B7-2-transfected CHO cells (B7-2) in the absence or presence of anti-TCR. Analysis of Vav Tyr phosphorylation was performed as described in A above. Data are expressed as the relative fold change for the relative signal intensities calculated as $a/b$, where $a$ is the specific signal intensity [(density × area)/lane] obtained after TCR, CD28 (CHO-B7-2), or TCR/CD28 (TCR/CHO-B7-2) stimulation, and $b$ is the basal signal intensity (T cells without stimulation, CHO).
The most representative time (15 min) are shown. Data are expressed quantitatively as in Fig. 1C and Ras were fractionated by polyethyleneimine TLC, as indicated in Materials and Methods. Several times of stimulation were analyzed, and results from the most representative time (15 min) are shown. Data are expressed quantitatively as in Fig. 1C. The anti-Rac-1 mAb and isotype-matched control Ig (C-Ig) were used for immunoprecipitation and immunoblotting to confirm the specificity of the anti-Rac-1 mAb. The positions of migration of Rac-1 and IgH chains are shown. E, TCR but not CD28 stimulation activates ERK-1 and ERK-2 in Jurkat T cells. In vitro kinase assays of ERK-1 and ERK-2 activity in T cells that were either unstimulated or stimulated for 10 min with anti-TCR, anti-CD28, or anti-TCR + anti-CD28 were performed as indicated in Materials and Methods using MBP as substrate. Representative results of three independent reproducible experiments of each type are shown.

Effects of TCR and/or CD28 cross-linking on Rac-1

**FIGURE 2.** Effects of TCR and/or CD28 cross-linking on Rac-1 (A and B) and Ras (D) GDP/GTP loading in vivo. Jurkat T cells (A) or BALB/c splenic T cells (B) labeled with [32P]orthophosphate (200 μCi/ml) for 4 h were left unstimulated (none) or were stimulated for 15 min by anti-TCR, anti-CD28, anti-TCR + anti-CD28 (TCR/CD28), B7-2 transfected CHO cells (B7-2), or wild-type CHO (CHO) cells. [32P]-labeled guanine nucleotides bound to Rac-1 and Ras were fractionated by polyethyleneimine TLC, as indicated in Materials and Methods. Several times of stimulation were analyzed, and results from the most representative time (15 min) are shown. Data are expressed quantitatively as in Fig. 1C, C. The anti-Rac-1 mAb and isotype-matched control Ig C-Ig were used for immunoprecipitation and immunoblotting to confirm the specificity of the anti-Rac-1 mAb. The positions of migration of Rac-1 and IgH chains are shown. E, TCR but not CD28 stimulation activates ERK-1 and ERK-2 in Jurkat T cells. In vitro kinase assays of ERK-1 and ERK-2 activity in T cells that were either unstimulated or stimulated for 10 min with anti-TCR, anti-CD28, or anti-TCR + anti-CD28 were performed as indicated in Materials and Methods using MBP as substrate. Representative results of three independent reproducible experiments of each type are shown.

basal and TCR/CD28-induced Tyr phosphorylation of Vav were markedly increased in parental Jurkat T cells relative to that of the P116 ZAP-70− T cells (Fig. 3A). In contrast to the enhanced TCR/CD28-induced GTP/GDP exchange activity of Vav in parental Jurkat T cells, significantly lower TCR/CD28-induced increases in Vav GRF activity were detected in ZAP-70-deficient P116 T cells (Fig. 3B). Moreover, TCR and/or CD28 costimulation did not significantly increase Rac-1 GDP/GTP exchange in ZAP-70-deficient Jurkat T cells in vivo, as analyzed by polyethyleneimine TLC of Rac-1 immunoprecipitates (our unpublished data). Thus, ZAP-70 appears to control Vav-mediated guanine nucleotide exchange on Rac-1 by means of Vav Tyr phosphorylation stimulated by TCR and/or CD28 ligation.

Anti-sense oligo-DN-mediated inhibition of Vav expression down-regulates TCR- and CD28-induced JNK activity in T cells

Based on the ability of TCR/CD28 costimulation to synergistically activate the Vav-Rac-1 and JNK pathways in T cells (6, 9), we analyzed the effect of anti-sense oligo-DN-mediated inhibition of Vav expression on TCR- and CD28-induced JNK activity in T cells. Pretreatment of Jurkat T cells with Vav anti-sense oligo-DN significantly, albeit not completely, inhibited the TCR/CD28 stimulation of JNK activity for its c-Jun substrate in an in vitro kinase assay. As shown in Fig. 3C, the anti-sense treatment caused a 2.5-fold inhibition of the TCR/CD28-induced JNK activity. The level of JNK expression was not significantly reduced in Jurkat T cells following the Vav anti-sense oligo-DN treatment. These results suggest that, in T cells, 1) TCR and CD28 regulate downstream signaling along the JNK pathways by increasing Vav-dependent Rac GDP/GTP exchange, and 2) JNK may be activated by a Vav-independent pathway(s).

Requirements for TCR- and CD28-induced synergistic activation of PAK-1, JNK, and p38 MAPK: critical role of ZAP-70

Considering that the induction of Tyr phosphorylation and GRF activity of Vav both require ZAP-70, we determined whether ZAP-70 controls the catalytic activity of downstream effectors of Rac-1 involved in TCR and CD28 costimulation. The activities of JNK and p38 MAPK were assayed, as they mediate an important role for Rho-family GTPases in cell cycle progression through G1, and IL-2 transcription stimulated by TCR and CD28 ligation (2, 6–9). In wild-type Jurkat T cells, JNK activity for c-Jun was significantly enhanced upon TCR or CD28 stimulation both before (Fig. 4A) and after (Fig. 4B) cross-linking of these receptors. An ~4-fold increase in the JNK activity was observed upon TCR ligation, and JNK activity was not further enhanced (3.4-fold stimulation) after TCR cross-linking. As expected, coligation of TCR and CD28 synergistically activated JNK (22-fold enhancement). Interestingly, a 4.5-fold increase in JNK activity was obtained upon CD28 activation, and this activity was further enhanced upon CD28 cross-linking (8.5-fold increase) (Fig. 4, A and B). These findings agree closely with previous reports that a TCR signal alone stimulates JNK activity and that a CD28 signal synergistically amplifies TCR signaling (6, 7, 9, 27). Further, these results demonstrate that the strength of CD28 signaling, as reflected by the stimulation of JNK activity, can be further increased by CD28 cross-linking.

To investigate whether similar increases in CD28-induced JNK activity are elicited by CD28/B7-2 interactions, we quantitated the amounts of c-Jun phosphorylation mediated by JNK precipitated by guest on November 7, 2017 http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/
Both basal and TCR-, CD28-, and TCR/CD28-induced JNK and p38 MAPK activities were reduced substantially in P116 ZAP-70-deficient Jurkat T cells relative to that of wild-type Jurkat T cells, particularly if total kinase activity is taken into account (Fig. 4, A–C). The absence of ZAP-70 therefore affects TCR- and CD28-mediated signals and results in the down-regulation of both the JNK and p38 MAPK pathways. However, we noted a reduction in basal JNK activity in P116 T cells when compared with wild-type Jurkat T cells, and therefore analyzed JNK activation relative to the basal JNK activity in each cell type. In contrast to the 4.3-, 4.5-, and 21.8-fold increases of JNK activity seen in wild-type Jurkat T cells, TCR-, CD28-, and CD28/TCR-ligation resulted in 2.5-, 3.2-, and 4-fold increases (quantitation not shown) of JNK activity in ZAP-70-deficient P116 Jurkat T cells. Thus, despite the reduced total JNK activity in ZAP-70-deficient T cells, these cells retained the ability to up-regulate JNK activity following CD28 stimulation (3.2-fold increase in P116 Jurkat T cells vs a 4.5-fold increase in wild-type Jurkat T cells).

Accordingly, the regulation of CD28-mediated JNK activity cannot be ascribed solely to the ZAP-70-dependent pathway and another pathway may control the amplitude of CD28-induced JNK kinase activity. Consistent with the partial dependence of JNK activity upon ZAP-70, secondary CD28 cross-linking yielded an ~2-fold difference in stimulation of JNK activity between wild-type (8.5-fold increase) and ZAP-70-deficient (4.2-fold increase) Jurkat T cells. Conversely, the differences in JNK activity between ZAP-70-deficient and wild-type Jurkat T cells were evident upon TCR stimulation (4.3-fold vs 2.5-fold) and were significantly more pronounced upon TCR/CD28 ligation (21.8-fold vs 4-fold). It is

from T cells stimulated with untransfected or B7-2-transfected CHO cells. Fig. 4D shows that no JNK activity was observed in either unactivated T cells or in T cells stimulated by control untransfected CHO cells. CD28 stimulation by B7-2 also did not activate p38 MAPK (Fig. 4E). The greatest elevation (~4-fold) of p38 MAPK activity was observed after TCR/CD28 coligation, using either anti-CD28 (Fig. 4C) or B7-2 (Fig. 4E) to engage CD28. Thus, despite the inability of CD28 stimulation to activate p38 MAPK, a CD28-dependent pathway enhances TCR-mediated activation of p38 MAPK. Moreover, B7-2- and anti-TCR/B7-2-mediated increase of JNK and p38 MAPK activities could be detected over an extended period of time, since these activities persisted even after 30 min of stimulation (Fig. 4, D and E). In contrast, TCR-induced kinase activities of JNK and p38 were more transient with a tendency to decline somewhat after 20 min of TCR ligation. Thus, the signals generated by CD28 costimulation translate into a longer duration of both Tyr phosphorylation of Vav and downstream signaling events, including the activation of JNK and p38.
evident, therefore, that ZAP-70 plays an important role in the TCR/CD28 costimulation-induced amplification of JNK activity. To further examine whether a CD28 costimulatory signal ("signal 2") requires an intact TCR signal ("signal 1"), we bypassed the requirement for ZAP-70-dependent events by substituting the Ras-dependent "signal 1" with the mitogenic tumor promoter PMA, which activates Ras via the protein kinase C pathway (1, 6). Phorbol esters are capable of sensitizing JNK to other signals (6), and combined stimulation of T cells with PMA plus CD28 mimics the TCR/CD28 synergistic effect on JNK activation in Jurkat T cells (6, 9). Fig. 5A shows that PMA/CD28 costimulation elevates JNK activity; 3-fold less in ZAP-70-deficient Jurkat T cells than in wild-type Jurkat T cells. Moreover, PMA/CD28 costimulation did not bypass the requirement of ZAP-70 for the stimulation of p38 MAPK activity (Fig. 5B). Our results demonstrate that ZAP-70 is indeed required for the TCR/CD28 costimulation-induced synergistic activation of JNK and p38 MAPK, and that PMA can substitute only partially for TCR in this type of costimulation.

Further support for the notion that ZAP-70 is necessary for optimum TCR/CD28 costimulation was obtained by assaying the activity of PAK-1, which is enhanced by either CD28 or TCR/CD28 stimulation in Jurkat T cells (28) and is controlled by Rac- and Cdc42-mediated signaling (29, 30). Both TCR and CD28 stimulation induced elevated PAK-1 kinase activity in Jurkat T cells (Fig. 5C) and primary mouse T cells, including thymocytes and peripheral splenic T cells (Fig. 5D). The highest stimulation of PAK-1 kinase activity was observed after TCR/CD28 coligation (Fig. 5C). Interestingly, in contrast to the 2.9- to 9.4-fold increases seen in wild-type Jurkat T cells, neither TCR, CD28, nor TCR/CD28 ligation elevated PAK-1 activity in ZAP-70-deficient P116 Jurkat T cells (Fig. 5C). These results confirm that PAK-1 is activated upon TCR, CD28, or TCR/CD28 stimulation, demonstrate the activation of PAK-1 in primary T cells, and, more significantly, illustrate that ZAP-70 regulates both TCR- and CD28-induced activation of PAK-1.

CD28 amplifies TCR-induced ZAP-70 activity and association of Vav with ZAP-70 and LAT

We have shown that ZAP-70 activity is required for the Tyr-phosphorylation of Vav following TCR and CD28 ligation. To investigate whether TCR and CD28 signals converge at the level of association of ZAP-70 with Vav, we quantitated the amount of

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**FIGURE 4.** Coupling of TCR and CD28 signaling and activation of JNK and p38 MAPK via ZAP-70 in T cells. Parental Jurkat and ZAP-70-deficient P116 Jurkat T cells were stimulated for 10 min by anti-TCR, anti-CD28, or anti-TCR + anti-CD28 (TCR/CD28) in the absence (A and C) or presence (B) of cross-linking by a secondary anti-mouse Ab. JNK (A and B) and p38 MAPK (C) kinase assays were performed as indicated in Materials and Methods using GST-c-Jun and ATF-2 as substrates, respectively. Data are expressed as the fold change relative to the basal signal intensity in unstimulated wild-type Jurkat T cells. Time course of JNK (D) and p38 MAPK (E) kinase activities in B7-2-, TCR-, and TCR/B7-2-stimulated T cells. EL4 T cells were activated for the indicated times with either wild-type control CHO cells alone (CHO) or in combination with anti-TCR (CHO/TCR) or B7-2-transfected CHO cells alone (CHO-B7-2) or in combination with anti-TCR (CHO-B7-2/TCR). JNK (D) and p38 MAPK (E) immunoprecipitates were assayed for associated kinase activities as above. The results shown are representative of one of three similar experiments.
ZAP-70 in Vav immunoprecipitates of unstimulated, TCR- and/or CD28-stimulated Jurkat T cell lysates. At 7 min after stimulation, TCR and CD28 ligation each induced an ~5-fold increase in the amount of ZAP-70 bound to Vav, while the amount of Vav-associated ZAP-70 was increased by ~16-fold after TCR/CD28 stimulation (Fig. 6A). ZAP-70 was also induced to associate with Vav upon B7-2 stimulation of CD28 in the presence or absence of TCR costimulation (Fig. 6B). TCR/CD28 costimulation, but not stimulation by TCR or CD28 alone, induced the association of Vav with TCRz and LAT, a pp36 Tyr phosphoepitope of Vav (Fig. 6C).

Additional analyses revealed that Grb2, an adaptor protein implicated in Vav Tyr phosphorylation and JNK activation in T cells (27), constitutively associates with Vav, and this binding is enhanced upon TCR/CD28 costimulation in Jurkat cells (Fig. 6A). In marked contrast, TCR and/or CD28 stimulation of ZAP-70-deficient Jurkat T cells failed to induce association of Grb2, LAT, and TCRz with Vav (data not shown).

While TCR and TCR/CD28 ligation elicited similar levels of ZAP-70 Tyr phosphorylation, CD28 stimulation did not significantly increase ZAP-70 Tyr phosphorylation (Fig. 6C). Tyr phosphorylation of ZAP-70 occurs at both positive and negative regulatory sites of activation. The Tyr-292-negative regulatory phosphorylation site of ZAP-70 binds to the Cbl phospho-Tyr-binding domain (PTB-domain) following T cell activation, which may result in the negative regulation of ZAP-70 (32). Since ZAP-70 activity is increased 3- to 4-fold by TCR stimulation (17), we analyzed whether CD28 stimulation modulates ZAP-70 kinase activity following TCR/CD28 coligation (Fig. 6D). Anti-Syk immunoblotting confirmed that phosphorylation of erythrocyte band 3 in this assay was mediated by ZAP-70 and not by the Syk PTK (data not shown), which is also capable of phosphorylating this substrate.

The relevance of the results obtained on ZAP-70 kinase activity in Fig. 6E was confirmed by examining the ZAP-70-mediated Tyr phosphorylation of SLP-76, a natural in vivo substrate of ZAP-70 (34), which is involved in the regulation of Vav-mediated Pak-1 activation and cytoskeleton organization (35). We observed a significant increase in Tyr phosphorylation of SLP-76 following TCR/CD28 coligation in splenic T cells (Fig. 6F).

Discussion

The results presented here demonstrate that a signaling pathway leading from TCR and CD28 through Rac-1 to Pak-1, JNK, and p38 MAPK requires the activity of ZAP-70, which is indispensable for the phosphorylation-dependent increase of Vav GRF activity. These observations implicate a ZAP-70/Vav complex as a point of convergence for the TCR and CD28 signaling pathways. This possibility is supported by the report that ZAP-70-deficient Jurkat T cells fail to up-regulate the Tyr phosphorylation and GRF activity of Vav and exhibit multiple defects in TCR-mediated signaling, including impaired Tyr phosphorylation of PLC-γ1 and IL-2 promoter-driven and NFAT-dependent transcription (20).

Based on our results, we propose that ZAP-70 regulates the TCR/CD28-induced transcriptional activation of ATF-2 and c-Jun via the enhancement of Vav GRF activity. Our observations favor a model in which: 1) TCR/CD28-mediated signaling is coupled via ZAP-70 to the activation of Pak-1, JNK, and p38 MAPK by interactions between components of the CD28 and TCR signaling pathways; and 2) replacement of TCR proximal signals (“signal 1”) by stimulation of protein kinase C by PMA in ZAP-70-deficient T cells does not fully restore the costimulatory potential of
CD28 triggering ("signal 2") with respect to the activation of JNK and p38 MAPK. These data substantiate a recent report that CD28 potentiates the response to PMA more weakly in Lck-deficient than in wild-type Jurkat T cells (9).

This model provides a basis for the ability of CD28/B7-2-mediated costimulation to prevent the induction of anergy. Indeed, ligation of CD28 by B7-2 leading to productive immunity induces the Tyr phosphorylation of TCRζ and CD3 chains and facilitates the association of TCRζ with Lck following the recruitment of ZAP-70 to this complex (36). The ability of B7-2-mediated costimulation to elevate ZAP-70 activity, as well as the association of Vav with ZAP-70, TCRζ, and Tyr-phosphorylated LAT, may be important for efficient signaling along the Vav/Rac-1-regulated PAK-1 and p38 MAPK pathways required for T cell proliferation and IL-2 production (6, 8, 9–11). The fact that this Vav/ZAP-70/TCRζ complex contains LAT, which is localized mainly to

FIGURE 6. A, Analysis of TCR- and/or CD28-induced Vav-containing signaling complexes in Jurkat T cells. Jurkat T cells (3 × 10⁷ T cells/sample) were stimulated (7 min, 37°C) by cross-linking with anti-TCR, anti-CD28, or anti-TCR + anti-CD28 (TCR/CD28) mAbs (3 μg/10⁷ cells). Vav immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with an anti-p-Tyr mAb (upper panel) or anti-ZAP-70, anti-LAT, anti-CD3e, anti-TCRζ, anti-Grb2, and anti-Vav mAbs. Molecular weight markers and the position of migration of IgL chains are indicated. B, EL4 T cells (3 × 10⁷ cells/lane) were left unstimulated (none) or activated for 15 min with wild-type control CHO cells (CHO), anti-TCR (TCR), or B7-2-transfected CHO cells (BT-2), either alone or in combination with anti-TCR (TCR/CHO and TCR/BT-2). Vav immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with an anti-ZAP-70 or anti-Vav mAb. Data are expressed as in Fig. 1C. C, EL4 T cells were activated with wild-type CHO cells (CHO) or B7-2-transfected CHO cells (B7-2) in the absence or presence of anti-TCR. Immunoblotting of the ZAP-70 immunoprecipitates was performed with an anti-p-Tyr mAb. D, ZAP-70 activity was analyzed in immune complex kinase reactions by measuring its ability to phosphorylate erythrocyte band 3 (cbd3) (upper panel). ZAP-70 immunoprecipitates were immunoblotted with the anti-ZAP-70 mAb to confirm equal loading (lower panel). E, BALB/c thymocytes or splenic T cells were activated with wild-type CHO cells (CHO) or B7-2-transfected CHO cells (B7-2) in the absence or presence of anti-TCR. ZAP-70 activity was analyzed in immune complex kinase reactions as in D. Anti-ZAP-70 immunoblotting confirmed equal amounts of ZAP-70 in each lane (data not shown). F, Tyr phosphorylation of SLP-76 in BALB/c T cells following TCR stimulation and CD28 costimulation. SLP-76 immunoprecipitates (IP) were immunoblotted (IB) with an anti-p-Tyr mAb (upper panel). The blot was then stripped and reprobed with an anti-SLP-76 Ab (lower panel).
sphingolipid-cholesterol-rich microdomains that cluster critical signaling molecules (e.g., Ras- and Rho-like GTPases) in the plasma membrane of activated T cells (31, 37), suggests that ZAP-70-dependent phosphorylation of LAT plays a critical role in the assembly of Vav-LAT signaling complexes and its translocation in the vicinity of Vav downstream effectors, including Rac-1. Our observations demonstrate that CD28 signaling may potentiate ZAP-70 kinase activation induced by TCR stimulation, and support the model proposed above in which Tyr phosphorylation of SLP-76 bridges the TCR-associated ZAP-70 with downstream effectors, such as PAK-1, p38 MAPK, and JNK. The Cbl phosphotyrosine-binding domain selects a D(N/D)XpY motif and binds to CD28 and its role in co-signaling. J. Biol. Chem. 272:7776.

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References