Tyrosine-phosphorylated Vav1 as a Point of Integration for T-cell Receptor- and CD28-mediated Activation of JNK, p38, and Interleukin-2 Transcription*§

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In this study we identified tyrosine-phosphorylated Vav1 as an early point of integration between the signaling routes triggered by the T-cell receptor and CD28 in human T-cell leukemia cells. Costimulation resulted in a prolonged and sustained phosphorylation and membrane localization of Vav1 in comparison to T-cell receptor activation alone. T-cell stimulation induced the recruitment of Vav1 to an inducible multiprotein T-cell activation signaling complex at the plasma membrane. Vav1 activated the mitogen-activated protein kinases JNK and p38. The Vav1-mediated activation of JNK employed a pathway involving Rac, HPK1, MLK3, and MKK7. The costimulation-induced activation of p38 was inhibited by dominant negative forms of Vav1, Rac, and MKK6. Here we show that Vav1 also induces transcription factors that bind to the CD28RE/AP element contained in the interleukin-2 promoter. A detailed mutational analysis of Vav1 revealed a series of constitutively active and nonfunctional forms of Vav1. Almost all inactive versions were mutated in their DBL homology domain and behaved as dominant negative mutants that impaired costimulation-induced activation of JNK, p38, and CD28RE/AP-dependent transcription. In contrast to NF-AT-dependent transcription, Vav1-mediated transcriptional induction of the CD28RE/AP element in the interleukin-2 promoter could only partially be inhibited by cyclosporin A, suggesting a dual role of Vav1 for controlling Ca2+-dependent and -independent events.

Solely triggering the T-cell receptor (TCR)† without stimulation of accessory receptors leads to a state of unresponsiveness termed anergy (1). Full activation of T-lymphocytes necessarily requires two signals (2). The first signal is provided by the interaction of major histocompatibility complex molecules loaded with processed foreign antigens on antigen-presenting cells with the specific TCR-CD3-CD4 complex. The second signal is mediated by the occupancy of auxiliary receptors such as CD28. The two signaling pathways derived either from TCR or CD28 merge and synergistically stimulate the activity of JNK, NF-κB, and the expression of various target genes including IL-2, which promotes the proliferation and differentiation of T-cells and is therefore required for the full immune response (3). Costimulation also induces an actin/myosin-dependent, directional transport of proteins including the TCR and lipid domains to a cap structure, termed the immunological synapse (4).

One of the earliest events in TCR signaling is the activation of cytoplasmic protein tyrosine kinases, including members of the Src (Lck and Fyn) and Syk (ZAP70 and Syk) families (5). Induced tyrosine phosphorylation of target proteins allows the formation of multiprotein complexes at the inner leaflet of the cell membrane. Membrane anchorage is mediated by transmembrane adaptor proteins including linker for activation of T-cells (LAT) (6), which serve as docking ports for the formation of multiprotein complexes. This complex, which we refer to as T-cell activation signaling complex (TASC), contains other crucial signaling molecules such as PLCγ, phosphatidylinositol 3-kinase, and the adaptor protein SLP76 (7). The TASC propagates the signals and links them to multiple downstream signaling pathways. Tyrosine-phosphorylated LAT binds to PLCγ which then cleaves phosphatidylinositol diphosphate thus generating inositol 1,4,5-trisphosphate and diacylglycerol. Whereas diacylglycerol mediates activation of protein kinase C family members (8), inositol 1,4,5-trisphosphate mobilizes Ca2+ from intracellular stores. The Ca2+-mediated activation of the serine phosphatase calcineurin stimulates the nuclear entry of transcription factor NF-ATc (9).

Among the substrates for protein tyrosine kinases is also the Vav1 protein, which is exclusively expressed in hematopoietic cells. The 95-kDa product of the Vav1 proto-oncogene displays a unique arrangement of signaling motifs including a calponin homology domain, an acidic domain, a DBL homology (DH) domain, a pleckstrin homology (PH) domain, a cysteine-rich domain (CR), a SH2 domain flanked by two proline-binding SH3 domains, and a bipartite putative nuclear localization signal (10). In vitro experiments show that Vav1, once activated by phosphatidylinositol-3,4,5-trisphosphate binding and Lck phosphorylation, stimulates the GDP/GTP exchange activity of Rac (11, 12). Therefore, Vav1 is a guanine nucleotide exchange factor (GEF) with selectivity for the Rho family of GTPases such as Rac. Gene disruption experiments reveal the importance of Vav1 for receptor-mediated proliferation, cy-
toskeletal reorganization, and thymic selection (13–18). The Vav1-induced IL-2 promoter- and NF-AT-dependent transcription (19) can be further augmented by overexpression of Vav1 together with SLP76 (20). However, the underlying biochemical mechanisms for these functions and the signaling routes employed by Vav1 are not fully understood (10).

The molecular events mediating the cooperation between TCR- and CD28-induced signaling are incompletely known. The synergistic activation appears to be unique for JNK and NF-κB (21), since neither ERK nor NF-AT requires coreceptor-derived signals. The signals generated at the cell surface are then transmitted downstream to the cell nucleus on different routes. Small GTP-binding proteins employ the widely used mitogen-activated protein kinase (MAPK) pathways that lead to the activation of p38 and JNK. The different cytoplasmic signals are finally integrated by multiple recognition motifs contained within the promoter of the IL-2 gene (22). This promoter harbors binding sites for numerous transcription factors including NF-AT, NF-κB, AP-1, and the CD28RE/AP composite element. The latter is contacted by various proteins including members of the NF-κB/Rel and AP-1 families of transcription factors and so far only partially characterized proteins comprised in the so-called CD28 response complex (23).

The aim of this study was to study and characterize the early molecular mechanisms underlying the synergism between TCR- and CD28-mediated signaling. We identify tyrosine-phosphorylated Vav1 as an early point of signal integration upstream from Rac. Vav1 shows synergistic and sustained phosphorylation, membrane localization, and assembly into a multiprotein TASC in response to CD28 costimulation. Vav1 synergizes with TCR/CD28- and also CD28-derived signals to activate the MAPK family members JNK and p38. Vav1 strongly activates transcription from the CD28RE/AP element contained within the IL-2 promoter. The effects of various inactive and constitutively active Vav1 mutants reveal Vav1 as an important integrator for signals provided by the TCR and CD28.

**Experimental Procedures**

**Cell Culture, Transfections, and Stimulations**—Jurkat T-leukemia cells expressing the large T antigen were grown at 37 °C in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, 10 mm Hepes, 1% (v/v) penicillin/streptomycin (all from Life Technologies, Inc.), 2 mm glutamine, and 2 mg/ml G418. Jurkat cells were transfected by electroporation using a gene pulser (Bio-Rad) at 250 V/800 microfarads. Stimulation of Jurkat cells was performed in a final volume of 500 μl by adding cOD3 (final concentration 10 μg/ml, clone OKT3) and/or cOD28 (final concentration 10 μg/ml, clone 9.3). Cell lysates were prepared and cleared from the stimulating antibodies with protein A/G-Sepharose prior to further analysis.

**Antiserum and Reagents**—OFLAG-M2 antibody was purchased from Sigma, and α-HA antibody (12CA5) was from Roche Molecular Biochemicals; α-phosphotyrosine (4G10), α-Myc (9E10), and αVav1 monoclonal antibodies were from Upstate Biotechnology, Inc.; α-phospho-p38 was from New England Biolabs. αTCR(CD3) (OKT3), cOD28, and iso-type-matched control antibodies (IgG2) were kindly provided by Dr. R. Breitkreuz. Antibodies against LAT, SLF76, Lck, PLCγ, p85, and Lck were purchased by Santa Cruz Biotechnology. All other reagents were from Sigma or Roche Molecular Biochemicals.

**DNA-binding Assays**—Electrophoretic mobility shift assays (EMSA) were performed essentially as described by preparing nuclear extracts (24). Equal amounts of nuclear protein were tested for DNA binding to the following CD28RE/AP oligonucleotides: 5′-TCTGGTTTAA-GAAAGATCCTAAAAAGGTACATCG-3′ and 3′-CAAAATCTTTTTAGG-TTCTAGATGTCT-5′.

The supershift experiments were performed by preincubating the extracts with 2 μg of the respective antibodies (α-Rel, gift from Dr. N. Rice; αp65 and αp50, Santa Cruz Biotechnology) for 15 min at 4 °C.

**Coprecipitation Experiments and Immunoblotting**—Cells were washed with phosphate-buffered saline, and the pellets were resuspended on ice for 30 min in 250 μl of Nonidet P-40 lysis buffer (50 mm Tris/HCl, pH 7.5, 150 mm NaCl, 1 mm phenylmethylsulfonyl fluoride, 10 mm NaF, 0.5 mm sodium vanadate, leupeptin (10 μg/ml), 1% (v/v) Nonidet P-40, and 10% (v/v) glycerol). The cell debris was pelleted upon centrifugation with 14,000 rpm at 4 °C for 10 min. Extracts from antibody-stimulated cells were pre cleared with protein A/G-Sepharose. Equal amounts of protein contained in the supernatants were mixed with 1–2 μg of antibody and 15 μl of protein A/G-Sepharose. After rotation for 4 h on a spinning wheel at 4 °C, the immunoprecipitates were washed 5× in lysis buffer. Immunoprecipitates were boiled in 1× SDS sample buffer and separated by SDS-PAGE prior to immunoblotting. The proteins were detected after extensive washing with a horse radish peroxidase-coupled secondary antibody using the ECL system (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. Western blotting was quantitated using the Lumi-Image er™ from Roche Molecular Biochemicals.

**Subcellular Fractionation**—3× 10⁷ Jurkat cells were stimulated with αTCR(CD3)/αCD28 antibodies as specified in the figure legends. Cells were collected by centrifugation and washed in phosphate-buffered saline. The cell pellet was then resuspended in 250 μl of Buffer S1 (10 mm Hepes/KOH, pH 7.4, 38 mm NaCl, 1 mm phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 μm NaF, 1 μm vanadate) and subjected to four freeze-thaw cycles. Subsequently nuclei were pulletted by centrifugation. Membrane fractions were obtained after a 100,000 × g centrifugation step of cytosolic extracts. The supernatant of this step represents the S100 fraction. The membrane pellets were air-dried and resuspended in 6 μl urea prior to the addition of SDS sample buffer (fraction “membrane”).

**JNK Assays**—Cells were lysed in Nonidet P-40 lysis buffer 1 day after transfection. The JNK protein contained in the cell lysate was precipitated by the addition of 1 μg of αHA antibody (12CA5) and 25 μl of protein A/G-Sepharose. The precipitate was washed three times in lysis buffer and two times in kinase buffer (20 mm Hepes/KOH, pH 7.4, 25 mm β-glyceroophosphate, 2 mm dithiothreitol, 20 mm MgCl₂). The kinase assay was performed in a final volume of 20 μl of kinase buffer containing 2 μg of GST-c-Jun, 20 μM ATP, and 5 μM γ[32P]ATP. After incubation for 20 min at 30 °C, the reaction was stopped by the addition of 5× SDS loading buffer. After separation by SDS-PAGE the gel was fixed, dried, and quantitated using a PhosphorImager.

**Expression Vectors and Reporters**—Vav1 deletion mutants were constructed by polymerase chain reaction and inserted into pEF-BOS-derived vectors containing either a FLAG or Myc epitope tag. Site-directed mutagenesis was done using the Quickchange Kit (Stratagene) according to the manufacturer’s instructions. All point mutants were sequenced, and Vav1 inserts were subcloned into the original vectors to avoid point mutations in the vector backbone. pRK5-Vav1 was provided by Dr. A. Ullrich (25), and SLF76 expression vectors were provided by Dr. G. Koretzky (26). HA-JNK was a gift from Dr. M. Karin (27), and Rac cDNAs provided by Dr. S. Gutkind (28) were Myc-tagged and inserted into pEF-BOS-derived vectors. pEF-Vav1-Myc and 4xRE/AP-Luc constructs were generous gifts from Dr. A. Weiss (24). The IL-2-promoter luciferase construct was obtained from Dr. W. Kolanus, and the NF-κB-dependent reporter was from Dr. L. Samelson (6). Lck expression vectors were obtained from Dr. C. Micelli. MEKK constructs were gifts from Drs. T. Maniatis and M. Karin. HPK1 was provided by Dr. N. Iscoe (29), and MLK3 was from Dr. N. Lassam (30). MKK7 vectors were a gift from Dr. E. Nishida (31). MKKK4, p38 expression constructs were provided by Dr. R. Davis.

**Luciferase Assays**—Cells were harvested by centrifugation and washed twice with cold phosphate-buffered saline buffer and lysed in reporter lysis buffer (25 mm trisphosphate, 2 mm dithiothreitol, 2 mm EDTA, 10 mm β-glycerophosphate, 2 mm dithiothreitol, 2 mm MgCl₂, 10% (v/v) Triton X-100). The luminometer (Duol Lumat LB 9507, Berthold) was programmed to inject 50 μl of assay buffer (40 mm Tricine, 2.14 mm (MgCO₃)₄Mg(OH)₂ × 5 H₂O, 5.34 mm MgSO₄, 0.2 mm EDTA, 66.6 mm dithiothreitol, 540 μM CoA, 940 μM luciferin, 1.06 mm ATP) and to measure light emission for 10 s after injection.

**RESULTS**

Sustained and Enhanced Tyrosine Phosphorylation and Membrane Recruitment of Vav1 in Response to Costimulation—The most proximal events in T-cell signaling occur at the plasma membrane. Here, not only the receptors but, in addition, also many consecutive downstream effectors such as Src family kinases or PLCγ are in proximity to the membrane. Therefore, we investigated the subcellular localization of the Vav1 protein in response to T-cell activation. Exposure of hu-
man T-cell lymphoma Jurkat cells tested for high level expression of both surface molecules (data not shown) to a combination of soluble agonistic αTCR(CD3) and αCD28 antibodies, followed by subcellular fractionation and immunoblotting, revealed an immediate and nearly complete recruitment of Vav1 to the plasma membrane (Fig. 1A). Relocation to the cytosol was detectable approximately 30 min after stimulation. Subsequently, we compared the membrane localization of Vav1 in response to TCR(CD3)/CD28 costimulation with the stimulation of both receptors alone (Fig. 1B). TCR(CD3)-stimulated T-cells showed a rapid and almost complete membrane recruitment of Vav1, whereas CD28 stimulation failed to induce membrane localization of Vav1. The TCR(CD3)/CD28-costimulated T-cells showed no significant difference in respect to the amount of membrane-recruited Vav1, but the kinetics of Vav1 relocation to the cytoplasm was significantly slower in the costimulated T-cells, when compared with T-cells that received their signals only from the TCR.

Previous studies identified Vav1 as one of the earliest targets for protein tyrosine kinases following a wide array of different stimuli (32). Vav1 is not only phosphorylated upon TCR cross-linking but also in response to solely triggering the CD28 receptor by its physiologic ligand B7.1 or by agonistic antibodies (33). To investigate whether Vav1 receives signals from both receptors, we compared the tyrosine phosphorylation of Vav1 in response to stimulation of TCR(CD3) or CD28 alone versus simultaneous stimulation of both receptors. Human T-cell lymphoma Jurkat cells were treated with αTCR(CD3) and/or αCD28 antibodies as indicated (Fig. 1C). The tyrosine phosphorylation status of the immunoprecipitated Vav1 protein was analyzed by immunoblotting with a monoclonal α-phosphotyrosine (αPY) antibody. The induced phosphorylation of Vav1 occurred 1 min after stimulation of TCR(CD3) and reached a maximum after 5 min. Albeit less intense, the CD28-triggered Vav1 phosphorylation was significantly more durable and stable in comparison to TCR(CD3) activation. The coligation of both receptors resulted in a stronger and prolonged tyrosine phosphorylation of Vav1, showing that the cooperative effects between TCR and CD28 are already manifested on the level of Vav1 tyrosine phosphorylation. Vav1 phosphorylation was accompanied by the increased appearance of two coprecipitating tyrosine-phosphorylated proteins, which were identified as SLP76 and the regulatory p85 subunit of phosphatidylinositol 3-kinase by immunoblotting (Fig. 1C).

**Fig. 1. TCR coligation synergistically stimulates phosphorylation and membrane-recruitment of Vav1.** A, Jurkat T-cells were costimulated for the indicated periods with agonistic αTCR(CD3) and αCD28 antibodies. Equal amounts of proteins contained in the membrane fraction and the cytosolic S100 extract were analyzed by Western blotting (WB) for the occurrence of Vav1. Unstim., unstimulated. B, Jurkat cells were stimulated with the indicated combinations of αTCR(CD3) and αCD28 antibodies for the given periods. The S100 and membrane fractions were analyzed by immunoblotting for Vav1. C, Jurkat T-cells were stimulated with αTCR(CD3) and/or αCD28 antibodies for the indicated periods. Vav1 was immunoprecipitated (IP) from cell lysates, and the immunoprecipitated proteins were resolved by SDS-PAGE. After Western blotting, tyrosine-phosphorylated proteins were detected with a monoclonal α-phosphotyrosine (αPY) antibody. The stripped membrane was reincubated with antibodies recognizing p85 and SLP76. Representative experiments out of three are shown.

**Costimulation-induced Formation of a Multiprotein TASC—**

We next asked whether the costimulation-induced increase in the coprecipitating phosphoproteins reflects an increased tyrosine phosphorylation or enhanced protein/protein interactions.
Jurkat cells were left untreated or stimulated with various combinations of a TCR(CD3) and a CD28 antibodies. Total cell extracts were prepared and endogenous Vav1 protein was isolated by immunoprecipitation. Tyrosine-phosphorylated coprecipitating proteins were detected by immunoblotting (Fig. 2A, upper). These experiments revealed several proteins that were inducibly phosphorylated by costimulation. Western blot experiments revealed the prominent phosphotyrosine protein of 95 kDa as Vav1 (data not shown). Some of the Vav1-associated coprecipitating proteins were identified by Western blotting (Fig. 2A, lower). Triggering of the costimulatory CD28 receptor alone induced the association of Vav1 with PLCγ, p85, SLP76, Lck, and LAT. Upon ligation of both receptors, a synergistic association was evident for PLCγ, p85, SLP76, and LAT, whereas the association with Lck was only additive (Fig. 2A, lower). Remarkably, these differences became only evident following a stimulation period of 15 min but were hardly detectable in response to receptor activation for less than 10 min. In summary, these experiments suggest that the signals emanating from both receptors merge at an early stage of signal transduction and affect Vav1 phosphorylation and the inducible TASC formation.

The proteins constituting the TASC are not completely identified, and their stoichiometry and sequential order of binding is not fully understood. The coprecipitation between Vav1 and LAT (see Fig. 2A) raises the possibility that the membrane attachment of Vav1 is mediated via LAT. We therefore addressed the question whether Vav1 directly binds to LAT or whether this contact requires intermediate proteins such as SLP76, which is known to bind both proteins (7). Jurkat cells were left untreated or stimulated with various combinations of a TCR(CD3) and a CD28 antibodies. Cell lysates were prepared and divided into several pools that were immunodepleted with antibodies recognizing either Vav1, SLP76, or LAT (Fig. 2B). SLP76 and LAT were not detectable in Vav1-depleted extracts that were prepared from TCR(CD3)- or TCR(CD3)/CD28-stimulated cells. In contrast, Vav1-depleted extracts from unstimulated or CD28-treated cells still contained SLP76 and LAT, indicating that binding of Vav1 with SLP76 and LAT is inducible by TCR(CD3) or TCR(CD3)/CD28 stimulation. In a complementary experimental approach, SLP76-depleted extracts were tested for the occurrence of Vav1 and LAT by immunoblotting. Ligation of TCR(CD3) and costimulation triggered association of both proteins, whereas extracts from untreated and CD28-stimulated cells still contained Vav1 and LAT. LAT-depleted extracts showed TCR(CD3)- and TCR(CD3)/CD28-induced binding to Vav1 and SLP76, revealing that all three proteins are mutually binding in an inducible manner. These results show that the entire pool of Vav can be incorporated into a LAT-containing protein complex, which may mediate the membrane recruitment of Vav1.

Tyrosine-phosphorylated Vav1 Synergizes with Lck and Rac to Activate JNK after TCR(CD3)/CD28 Stimulation—Activat-
tion of JNK necessarily requires at least two different inputs, either from CD3 plus CD28 or each of these stimuli in combination with phorbol esters such as PMA (34). An early signal integrating role of Vav1 raises the possibility that it also controls downstream signaling events such as the activation of JNK. This possibility was tested by transient transfection of Jurkat T-cells with a constant amount of an expression vector encoding HA-tagged Vav1 together with 5 μg of HA-tagged JNK. After CD3/CD28 stimulation, JNK was immunoprecipitated from cell lysates, and its activity was determined by immune complex kinase assays using recombinant GST-c-Jun(5–89) as substrate. A sample of each lysate was analyzed by immunoblotting for protein expression of Vav1 (middle) and JNK (lower). B, the experiment was performed as described in A with the exception that cells were stimulated with αCD28 and PMA (10 ng/ml). C, expression vectors for JNK (5 μg), Vav1 (2 μg), and Lck at the indicated combinations were transfected into Jurkat cells. The cells were stimulated as shown, and JNK activity was determined by immune complex assays. Aliquots from each lysate were tested for expression of Lck (upper), Vav1 (middle), and JNK (lower). D, Jurkat cells received a constant amount of expression vectors encoding Vav1 (5 μg) and JNK (5 μg) together with increasing concentrations of dominant negative Lck (LckR273Y505F). The cells were stimulated with αTCR/CD3/αCD28 antibodies as shown, and JNK activity was determined (upper). Control lysates analyzed by immunoblotting display the expression of Lck (endogenous and overexpressed), Vav1, and JNK. E, Jurkat cells were transfected with expression vectors for JNK (5 μg), Vav1 (2 μg), and Rac WT at the indicated combinations. The experimental procedures and presentation are as described in A. F, constant amounts of expression vectors encoding Vav1 (5 μg) and JNK (5 μg), together with increasing concentrations of Asn-17 Rac were transfected into Jurkat cells. These were stimulated as shown, and JNK activity was determined (upper). Control experiments showing the relative expression levels of Rac, Vav1, and JNK in cell lysates are shown in the lower part. G, Jurkat cells were transfected with expression vectors for JNK (5 μg) and Vav1 (5 μg) together with 10 μg of plasmids encoding the transdominant negative forms of the indicated signaling proteins. Subsequently the cells were stimulated as shown, and JNK was immunoprecipitated and used for an immune complex kinase assay which is shown in the upper part. Aliquots of the cell lysates were analyzed for the expression of tagged proteins (lower). All experiments were repeated at least three times with comparable results. Representative Western blots and autoradiograms from reducing SDS gels and quantitative evaluations obtained by phosphorimaging are shown.

FIG. 3. Tyrosine-phosphorylated Vav1 activates JNK on a pathway employing Rac, HPK1, MLK3, and MKK4/7 in T-cells. A, Jurkat cells were transiently transfected with increasing amounts of an expression vector encoding Myc-tagged Vav1 together with 5 μg of HA-tagged JNK. After CD3/CD28 stimulation, JNK was immunoprecipitated from cell lysates, and its activity was determined by immune complex kinase assays using recombinant GST-c-Jun(5–89) as substrate. A sample of each lysate was analyzed by immunoblotting for protein expression of Vav1 (middle) and JNK (lower). B, the experiment was performed as described in A with the exception that cells were stimulated with αCD28 and PMA (10 ng/ml). C, expression vectors for JNK (5 μg), Vav1 (2 μg), and Lck at the indicated combinations were transfected into Jurkat cells. The cells were stimulated as shown, and JNK activity was determined by immune complex assays. Aliquots from each lysate were tested for expression of Lck (upper), Vav1 (middle), and JNK (lower). D, Jurkat cells received a constant amount of expression vectors encoding Vav1 (5 μg) and JNK (5 μg) together with increasing concentrations of dominant negative Lck (LckR273Y505F). The cells were stimulated with αTCR/CD3/αCD28 antibodies as shown, and JNK activity was determined (upper). Control lysates analyzed by immunoblotting display the expression of Lck (endogenous and overexpressed), Vav1, and JNK. E, Jurkat cells were transfected with expression vectors for JNK (5 μg), Vav1 (2 μg), and Rac WT at the indicated combinations. The experimental procedures and presentation are as described in A. F, constant amounts of expression vectors encoding Vav1 (5 μg) and JNK (5 μg), together with increasing concentrations of Asn-17 Rac were transfected into Jurkat cells. These were stimulated as shown, and JNK activity was determined (upper). Control experiments showing the relative expression levels of Rac, Vav1, and JNK in cell lysates are shown in the lower part. G, Jurkat cells were transfected with expression vectors for JNK (5 μg) and Vav1 (5 μg) together with 10 μg of plasmids encoding the transdominant negative forms of the indicated signaling proteins. Subsequently the cells were stimulated as shown, and JNK was immunoprecipitated and used for an immune complex kinase assay which is shown in the upper part. Aliquots of the cell lysates were analyzed for the expression of tagged proteins (lower). All experiments were repeated at least three times with comparable results. Representative Western blots and autoradiograms from reducing SDS gels and quantitative evaluations obtained by phosphorimaging are shown.
Vav1 with increasing amounts of wild type (WT) Lck. Cells were costimulated with αTCR/CD3/αCD28 antibodies as indicated, and JNK activity was determined (Fig. 3C). In contrast to wild type Lck alone that was ineffective in JNK activation (data not shown), the coexpression of this kinase together with Vav1 resulted in a strong increase of induced JNK activity. The same set of experiments was performed using a dominant negative (DN) form of Lck, which contains a point mutation in the ATP binding domain (LckR273Y505F). Coexpression of increasing amounts of LckR273Y505F efficiently reduced TCR/CD3/CD28-induced activation of JNK (Fig. 3D), indicating the importance of Lck for the activation of Vav1. Jacinto and colleagues (35) also described the participation of Rac for the synergistic activation of JNK. Therefore, we tested the impact of simultaneous Rac and Vav1 coexpression on the costimulation-induced JNK activity. In the presence of constant amounts of Vav1, the coexpression of increasing amounts of WT Rac strongly stimulated the receptor-initiated JNK activation (Fig. 3E), whereas basal activities were only moderately changed by the wild type form of Rac (data not shown). Coexpression of a dominant negative form of Rac (Asn-17 Rac) dose-dependently decreased the TCR/CD3/CD28-induced activation of JNK (Fig. 3F), showing that Rac is an important member of the JNK activation cascade triggered by Vav1.

Vav1-mediated JNK Activation in T-cells Requires HPK1, MLK3, and MKK7—The understanding of signaling pathways and the identification of kinases participating in JNK activation is constantly progressing (36), but relatively little is known about the JNK-activating kinases in T-lymphocytes. In order to assess systematically the role of various kinases that are putative downstream targets of Vav1 in the TCR/CD28 signal transduction pathway, Jurkat cells were transfected with expression vectors encoding Vav1 together with dominant negative forms of HPK1 (29), MLK3 (30), MEKK1, MEKK4, MKK4, and MKK7 (31). The cells were stimulated with αTCR/CD3/αCD28 antibodies as indicated, and JNK activity was determined (Fig. 3G). Coexpression of DN MEKK1Δ and MKK4 only moderately reduced JNK activity, and the dominant negative form of MEKK4 (MEKK4DN) displayed no inhibitory activities. All other transdominant negative proteins (HPK1, MLK3, and MKK7) efficiently impaired JNK activation, thus revealing their importance for JNK signaling in T-lymphocytes. Similar results were obtained when the TCR/CD3/CD28 stimulus was replaced by the expression of a constitutively active form of Rac (RacQL) (data not shown). In summary, these experiments suggest the existence of multiple pathways downstream of or in parallel with Rac. These data show that the Vav1/Rac-controlled HPK1-MLK3-MKK7 pathway is relevant for the activation of JNK in T-cells.

Dominant Negative and Constitutively Active Mutants of Vav1 Reveal Its Necessity for JNK Activation—It was then mandatory to determine the role of Vav1 for these signaling pathways by testing the functional behavior of active and inactive forms of this protein. On the basis of the structure and sequence alignments with other DH/PH domain-containing proteins (37), we constructed a series of point and deletion mutants as schematically displayed in Fig. 4A. The biological activities of the indicated Vav1 variants were tested by transfecting Jurkat T-cells with an empty expression vector or with one of the respective Vav1 mutants. The cells were left untreated or TCR/CD3/CD28-stimulated, and the activity of coexpressed HA-JNK was determined by immune complex kinase assays (Fig. 4B). In order to allow a comparison of the experiments, JNK activation induced by T-cell costimulation was arbitrarily set as 1-fold.

Therefore the mutant Y174F, which contains a single point mutation at the only tyrosine phosphorylation site mapped so far (38), and L278Q, which corresponds to the recently described mutation of leucine 213 in oncogenic Vav1 (39), showed only marginal differences in their ability to activate JNK when compared with wild type Vav1. Deletion mutants lacking the first 67 or 250 amino acids and Vav△PH not only displayed augmented basal JNK activity but still showed further enhanced 32P incorporation into GST-c-Jun upon T-cell costimulation (Fig. 4B). Mutation of leucine 338 to glutamine dramatically increased the ability of Vav to mediate basal and costimulation-induced JNK activation (Fig. 4B). Also Vav C528S, which was mutated in the first cysteine of the cysteinerich region adjacent to the PH domain, behaved as a constitutively active Vav1 mutant. Since the mutation of the corresponding cysteine was found to abolish the transforming ability of oncogenic Vav (11, 39, 40), our results suggest that the potential to transform cells and to activate JNK are uncoupled. DH domains are essential for the GEF activity and contain three highly conserved regions (CRI–3), which form three long helices representing the core of the domain. Vav1 mutants lacking either the entire (Vav△DH) or a part of the DH domain (Vav△319–356) failed to induce JNK activation. VavΔ1–356, a mutant in which the N terminus including a large portion of the DH domain was deleted, did not induce JNK activity. Also a mutant Vav1 protein where amino acids 338-LLL-340 contained in CR3 of Vav1 were changed to 338-QIF-340 did not trigger JNK activity, thus proving the functional importance of these residues. Expression of VavΔ1–356, VavΔDH, and VavLLL/QIF prevented TCR/CD3/CD28-induced JNK activation, showing the relevance of Vav1 for JNK activation induced by T-cell costimulation. However, the TCR/CD3/CD28-triggered JNK response was more efficiently blocked by a dominant negative form of Rac when compared with Vav△319–356 (see Supplemental Material on the JBC website). It remains to be clarified whether the residual JNK activity seen in the presence of dominant negative Vav1 reflects a possible bypass mechanism.

Vav1 Mediates Synergistic Activation of p38 in Response to T-cell Costimulation—Besides JNK, p38 is another member of the MAPK superfamily that has been implicated in cellular responses to environmental stress and proinflammatory cytokines (41). To examine whether costimulation of T-cells results in a synergistic activation of p38 similar to that reported for JNK, Jurkat cells were treated with various combinations of PMA, αTCR/CD3, and αCD28 antibodies. Determination of p38 activation by immunoblotting using a phospho-specific antibody revealed synergistic p38 activation upon coligation of both receptors (Fig. 5A). Maximal p38 phosphorylation occurred upon stimulation of both receptors together with PMA. Since it is known that p38 regulates the transactivation potential of transcription factors such as NF-κB (42), we examined its impact on transcription from the IL-2 promoter. Jurkat T-cells were transfected with a IL-2 promoter-controlled luciferase gene and stimulated with different combinations of αTCR/CD3 and αCD28 antibodies and PMA in the absence or presence of the p38 inhibitor SB203580. Induced expression of IL-2 was significantly impaired in the presence of this inhibitor (Fig. 5B), showing that p38 activity is required for the efficient production of this cytokine. The position of Vav1 as an early signal transducer upstream from Rac prompted us to investigate the influence of Vav1 on p38 signaling. Jurkat cells were transfected with a vector encoding FLAG-tagged p38 together with increasing amounts of Vav1 and costimulated as indicated (Fig. 5C). The tagged p38 protein was immunoprecipitated and analyzed for its Thr-180/Tyr-182 phosphorylation. Vav1 coexpression dose-dependently increased p38 phosphorylation in
response to TCR(CD3)/CD28. The relative importance of Vav1 for the activation of p38 was tested by measuring the impact of coexpressing a dominant negative Vav1 variant on TCR(CD3)/CD28-induced p38 activation. Increasing amounts of Vav D319–356 prevented p38 phosphorylation in a dose-dependent manner (Fig. 5D). The importance of Rac and MKK6 for the Vav1-mediated activation of p38 was investigated by studying the effects of coexpressed DN variants of either protein (Asn-17 Rac and MKK6 Lys/Ala). Both dominant negative forms inhibited the induced p38 activation, showing that they are involved in the p38 activation cascade (Fig. 5E).

Vav1 Independently Promotes TCR(CD3)- and CD28-derived Signals Directed to the CD28RE/AP Element Contained within the IL-2 Promoter—Besides JNK activation, the transcriptional up-regulation of IL-2 is another event displaying the features of costimulation-induced synergism. Mutational studies demonstrated that the specific contribution of CD28-derived signals for IL-2 synthesis is mediated by the so-called CD28-responsive element (CD28RE/AP) (24). We therefore asked whether Vav1 (in addition to its reported ability to potentiate TCR(CD3)-mediated NF-AT activation (43)) is also able to induce the transcriptional activity from the CD28RE/AP element of the IL-2 promoter. A construct controlled by four repeats of the CD28RE/AP element fused to the luciferase reporter gene (4xRE/AP-Luc) was cotransfected with increasing amounts of wild type Vav1, and cells were stimulated as depicted in Fig. 6A. Under these conditions, the maximum response elicited by a triple combination of αCD3, αCD28, and PMA was increased ∼2-fold upon coexpression of Vav1. CD3/CD28 costimulation was augmented 9-fold by Vav1 expression, and CD28/PMA stimulation was increased 7.4-fold in the presence of coexpressed Vav1. In order to test whether Vav1 can independently influence the gene-inductive effects of both individual receptors, Jurkat T-cells were transfected with the CD28RE/AP-dependent reporter gene and increasing amounts of Vav1. Stimulation of either the TCR(CD3) or CD28 together with various concentrations of PMA resulted only in moderate transcriptional effects. The coexpression of Vav1 strongly enhanced CD28RE/AP-dependent transcription from either signaling pathway (Fig. 6B). The enhancing effect of CD3 and CD28 on Vav1-mediated CD28RE/AP transcription was also seen when the cells were stimulated in the absence of PMA (data not shown). We then asked whether the increased expression from the CD28RE/AP element would be due to the enhanced activation of transcription factors binding to this composite element or to other mechanisms. To address this question, we transfected Jurkat cells with an expression vector for Vav1 or the empty control vector. One day later, cells were stimulated with a TCR(CD3)/αCD28 antibodies as indicated, and DNA binding to the CD28RE/AP element was determined by EMSAs (Fig. 6C). These experiments revealed three DNA-protein complexes of distinct electrophoretic mobilities. In unstimulated cells,
coexpression of Vav1 induced binding of proteins contained in complex II. The relatively moderate effects of Vav1 on induced DNA binding of proteins can be attributed to the limited transfection efficiency of Jurkat cells. Costimulation led to enhanced binding of proteins contained in complexes I and II in Vav1-transfected cells, without influencing protein binding to complex III. Supershift experiments using antibodies with specificity for various NF-κB DNA-binding subunits revealed the occurrence of the NF-κB p65 protein in complexes I and II and the predominant localization of c-Rel within complex I (see Supplemental Material). Next, we estimated the relative contribution of Vav1 for the induced transcription from the CD28RE/AP element. The transcriptional activity of CD28RE/AP-mediated gene expression was significantly impaired in the presence of coexpressed VavΔ319–356 (Fig. 6D). Similarly, coexpression of VavΔ319–356 also impaired the induced NF-AT- and IL-2 promoter-dependent transcription to a comparable extent, thus revealing an important, but not exclusive, contribution of Vav1 for these transcriptional events. Taken together, these results show that Vav1 can transmit TCR(CD3)/CD28-derived signals on the level of gene expression by inducing binding of transcription factors to the composite CD28RE/AP element contained within the IL-2 promoter.

Vav1 Potentiates Ca²⁺-dependent and -independent Transcriptional Activation—A prominent feature of CD28-mediated up-regulation of IL-2 secretion is its insensitivity toward the inhibitory effects of cyclosporin A (CsA). CsA blocks the Ca²⁺-dependent activation of the phosphatase calcineurin (44), which is implicated in the costimulation-induced JNK activation by combinations of TCR(CD3)/PMA, TCR(CD3)/CD28, or PMA/Ca²⁺ ionophores (45). One major effect of calcineurin activation seems to be the dephosphorylation and subsequent nuclear translocation of members of the NF-AT family of transcription factors. The recent analysis of Vav1−/− mice by sev-
eral groups revealed a disturbance of Ca\(^{2+}\) homeostasis in T- and B-cells (14, 15, 46). To examine whether Vav1 acts prior to the separation of the diverse Ca\(^{2+}\)-dependent and -independent signaling pathways, we investigated the impact of CsA on Vav1-mediated transcription on IL-2-dependent gene expression and transcription factors binding to the NF-AT-, AP-1-, and CD28RE/AP elements contained within the IL-2 promoter. Jurkat T-cells were transfected with the respective reporter constructs along with empty control vectors or together with Vav1 and were treated as indicated (Fig. 7). Expression of Vav1 augmented the activity of all employed reporter genes. Vav1-induced transcription of the IL-2 promoter and of the AP-1-dependent reporter gene was only partly reduced in the presence of CsA (Fig. 7, A and B). Jurkat cells were transfected either with an expression vector encoding Vav1 or with empty expression vector. The next day, cells were stimulated for 4 h as indicated, and nuclear extracts were prepared. Equal amounts of protein contained in an aliquot of the extract was assayed for binding to a labeled CD28RE/AP element by EMSAs. The positions of the constitutive complex III and the inducible complexes I and II are indicated. Another aliquot of the nuclear extract was tested for expression of Myc-tagged Vav1 by immunoblotting (lower). One of three experiments is displayed. D, Jurkat cells were cotransfected with 5 µg of 4xRE/AP-Luc together with the empty vector or Vav-D319–356. Stimulations were done as indicated and luciferase activity was determined. Bars represent mean values from two experiments performed in duplicate (± S.E.). Unstim., unstimulated; WB, Western blot.

DISCUSSION

In an attempt to identify early points of integration upstream from JNK activation and IL-2 synthesis, we found that TCR- and CD28-generated signals already merge upstream from Rac at the level of Vav1. Costimulation of both receptors caused the following: 1) enhanced and sustained tyrosine phosphorylation of Vav1, 2) significantly prolonged its membrane localization, 3) enhanced the formation of a Vav1-containing TASC, 4) further stimulated the Vav1-submitted signals leading to the activation of JNK and p38, and 5) triggered its activating function for transcription factors transactivating from the CD28RE/AP element. These data are compatible with a kinetic model in which costimulation via CD28
alters the quality and/or quantity of the antigen receptor-derived signal rather than employing a substantially different pathway (47). This delayed kinetics could lower the threshold for the engagement of downstream signals. The various signaling pathways influenced by Vav1 are discussed below and are schematically summarized in Fig. 8.

Since it is known that the enzymatic activity of Vav1 strictly depends on its tyrosine phosphorylation (11), it is tempting to speculate whether Vav1 is differentially phosphorylated following costimulation. Lck has been used in several studies for phosphorylation of Vav1 and its subsequent activation in vitro (11, 12) and in yeast (48), thereby proving that Vav1 phosphorylation by Lck alone is sufficient for its activation. However, the enhancement of Vav1-mediated JNK activation is also compatible with a model where Lck acts in parallel to Vav1. Vav1 is also a substrate for further kinases, including Syk (which phosphorylates a Vav1 fragment containing tyrosine 174 in vitro (38)) and Fyn (which phosphorylates Vav1 after CD28 ligation (49)). Therefore the identification of the kinase(s) which is mainly responsible for this phosphorylation will be a key step toward the understanding of early integrative events in CD28-mediated costimulation.

The impact of Vav1 on the activation of JNK is still a matter of debate. Vav1-deficient T-cells and lymphocytes displayed no defect in the TCR/CD28-induced activation of JNK and ERK (14, 15). However, some of the conclusions described in these two papers were challenged by a recent study that described a lack of TCR/CD28-triggered ERK activation in Vav1−/− mice (50). As discussed by Costello et al. (50), this discrepancy might be due to the different mutation made by Fischer et al. (14) and Holsinger et al. (15) which may not have removed the entire function of Vav. Gain-of-function approaches revealed an important role of Vav for the activation of JNK in T-cells (51–53). The JNK activating capacity of Vav is controlled by interaction with regulatory proteins. Whereas binding to hSiah2 inhibits Vav-mediated signaling pathways and JNK activation (54), interaction of Vav1 with the HIV protein Nef further triggers Vav1-induced activation of JNK (55). These rather conflicting results may be explained by the compensatory function of the recently characterized Vav family members Vav2 and Vav3 in Vav1−/− cells. The Vav2 and Vav3 proteins are also prominently expressed in lymphoid tissues and function as GEFs (56, 57).
Fig. 8. Proposed model for signal transduction by Vav1. Further details are given in the text.

57). Also differences between species, cell types, and the nature of the JNK-inducing stimulus may be taken into account. CD19/mIgM cross-linking-induced JNK activation was only seen in B-cells from Vav1−/− mice but not from Vav1+/− mice (46), suggesting that the functional role of Vav1 may depend on the cell type.

Proteins containing DH domains require their GEF function for the activation of Rho family GTPases. Our detailed mutational analysis revealed the importance of the CR3 region within the DH domain for the ability of Vav1 to transmit downstream signals that lead to the activation of MAPKs and transcription factors binding to the CD28RE/AP element. GEFs of the DBL family including Vav1 are characterized by a PH domain immediately adjacent to their DH domain. The mutual functional interaction of both domains in the Vav1 molecule is supported by recent findings that revealed that binding of phosphatidylinositol 3-kinase products to the PH domain of Vav1 enable its prolonged phosphorylation by Lck (12). This might be due to conformational changes that increase the accessibility of putative regulatory phosphorylation sites. Both the lipid-induced structural changes and the induced phosphorylation may affect the interaction between the DH domain and its appropriate substrate GTPase. In line with this model, a recent report (58) and the results obtained with VavΔPH shown here indicate an inhibitory effect of this domain on the JNK activating ability of Vav1. But also other domains of Vav1 are important for its function, since a recent study demonstrated that mutation of the SH2 domain abrogated activation of PAK kinase, TCR-dependent cytoskeletal rearrangements, and recruitment to glycosphingolipid-enriched microdomains (51, 59). The inhibitory effects of dominant negative Vav1 forms on costimulation-induced signaling processes may be explained by heterodimerization with Vav2 and Vav3 or alternatively by competitive binding of the inactive Vav1 variant to signal transmitting proteins.

This study revealed that the T-cell costimulation-induced Vav1-mediated JNK activation pathway employs a signaling cascade including Rac, HPK1, MLK3, and MKK7. Since this signaling cascade is also used in cells of non-hematopoietic origin, it may well be possible that a scaffold protein such as one of the recently characterized JNK-interacting proteins (60) is responsible for the coordinate sequential interaction of these kinases. It is reasonable to assume that further proteins participate in JNK activation, as also seen by the partial inhibition of JNK activation by dominant negative forms of MEKK1 and MKK4/SEK1. We speculate that the stimulatory effect of Vav1 on CD28RE/AP-dependent transcription may rely on two mechanisms. (i) Vav1 induces DNA binding of transcription factors including members of the NF-κB family to their cognate CD28RE/AP element. This finding is in good agreement with a recent paper that showed a lack of NF-κB activation in T-lymphocytes from Vav1−/− mice (50). (ii) The second mechanism may rely on the contribution of Vav1 to p38 activation, which is required for CD28/RE- and NF-κB-dependent transactivation without influencing induced DNA binding of NF-κB (42).

Our experiments indicate that Vav1 can independently promote Ca2+-dependent and -independent signaling pathways on various elements contained within the IL-2 promoter. The importance of Vav1 for the regulation of intracellular Ca2+ homeostasis in lymphocytes is evident from Vav1-deficient T-cells and B-cells, which fail to sustain elevated levels of intracellular Ca2+ in response to antigen receptor stimulation (14, 15, 46). The analysis of Vav1-deficient B-cells revealed that the defects in Ca2+ signaling can be attributed to an impaired inositol lipid biosynthesis (46, 61). The same holds true for T-cells, since Vav1−/− T-cells released much less inositol 1,4,5-trisphosphate in response to costimulation, thus resulting in an impaired Ca2+ response (50). This study reveals that the presumable double function of Vav1 is well demonstrated by its ability to promote independently CaA-sensitive and -insensitive signals on various elements contained within the IL-2 promoter. This finding is corroborated by a recent study describing that the restoration of intracellular Ca2+ fluxes by ionomycin in Vav1−/− T-cells rescued only the activation of NF-AT but not of NF-κB (50). The pathway bifurcation downstream of Vav1 may be envisioned by the following model. The T-cell costimulation-induced transient phosphorylation links Vav1 to the Ca2+-dependent activation of NF-AT and the actin/myosin-dependent formation of a cap structure (62). On the other hand, the CD28-delivered signals mediate an enhanced tyrosine phosphorylation and lipid agonist binding to Vav, which leads to the activation of Rac and downstream signaling pathways. In T-cells, dominant negative forms of Vav1 were less efficient in the activation of Rac and downstream signaling pathways. In T-cells, dominant negative forms of Vav1 were less efficient in the inhibition of JNK activation and IL-2 production when compared with dominant negative forms of Rac. These findings suggest that Vav1 is not the only point of signal integration and presumably reflects the existence of compensatory pathways or additional signal integrators such as Vav2 or Vav3 (56, 57). Further candidates still await their identification in future studies.

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Vav1 as a Point of Integration for T-cell Costimulation

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Tyrosine-phosphorylated Vav1 as a Point of Integration for T-cell Receptor- and CD28-mediated Activation of JNK, p38, and Interleukin-2 Transcription
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