Synergistic Activation of NF-κB by Functional Cooperation between Vav and PKCθ in T Lymphocytes*

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Here we identified PKCθ as an activator of transcription factor NF-κB in T cells. PKCθ-induced NF-κB activation was synergistically augmented by Vav. Several experimental approaches revealed that PKCθ is located downstream from Vav in the control of the pathway leading to synergistic NF-κB activation. In addition to the synergistic activation cascade, Vav also triggered NF-κB activity on a separate route. CD3/CD28-induced activation of NF-κB was inhibited by dominant negative forms of Vav or PKCθ, revealing their essential role in this activation pathway. The Vav/PKCθ-mediated signals preferentially activated IκB kinase β. Vav and PKCθ were found to be constitutively associated in unstimulated T cells. Only the ligation of the costimulatory CD28 receptor, but not of the T cell receptor, resulted in the transient dissociation of the Vav/PKCθ complex. In contrast, T cell receptor/CD28 costimulation resulted in faster dissociation and slower reassociation kinetics.

Full activation of T cells relies on the simultaneous delivery of signals provided by the TCR-CD3-ζ complex and auxiliary receptors such as CD28 (1). Receptor ligation triggers the activation of protein tyrosine kinases (PTKs) including Lck, that induce the tyrosine phosphorylation of multiple target proteins, thus enabling the formation of multi-protein complexes. These aggregates contain adapter proteins such as LAT or SLP76 and signaling molecules including Vav and PLCγ (2). Activated PLCγ produces inositol triphosphate, which leads to the release of Ca2+ and the production of diacylglycerol, which mediates activation of PKC family members (3). The Ca2+-independent novel PKC isof orm θ (4) is almost exclusively expressed in lymphoid cells and inductively translocates to the zone of TCR clustering in the central core of the supramolecular activation complex (SMAC) present in the contact region between antigen-presenting cells and T cells (5). PKCθ is a potent activator of JNK (6) and cooperates with calcineurin for the activation of this kinase (7).

Tyrosine-phosphorylation of Vav mediates both activation and down-modulation of Vav effector functions (8). Activated Vav displays a GDP/GTP exchange factor (GEF) activity for Rac (9, 10), thereby coupling Vav to downstream Rac effector pathways. But there is also recent evidence for GEF-independent activation pathways of Vav, such as the Vav-mediated activation of nuclear factor AT (11). Gene disruption experiments revealed the importance of Vav for thymic selection, cytoskeletal reorganization, receptor-mediated proliferation, and the activation of NF-κB (12, 13).

This transcription factor is trapped in the cytoplasm of unstimulated cells by association with an inhibitory IκB protein (14). T cell costimulation leads to the activation of two homologous kinases, termed IKKα and IKKβ (15). These kinases are contained in the IκB kinase complex (IKC) and phosphorylate IκB proteins, thus allowing ubiquitinylation and degradation of IκB proteins, resulting in the subsequent activation of NF-κB (16). IKKs are activated by IKK kinases (IKKks) including MEKK1, NIK, and MLK3 and further signaling molecules, which remain to be identified (14). TCR/CD28-induced NF-κB activation involves the Cot kinase, which activates the IKKs via NIK (17), as well as MLK3, a direct binding partner and activator of IKKα and IKKβ (18).

There is recent evidence that Vav and PKCθ functionally interact in the process of TCR-induced T cell activation (19) and IL-4 transcription (20). We have previously shown that Vav and PKCθ target the P1 and PRE-I elements contained in the IL-4 promoter (20). Because both elements are bound by members of the NF-κB family of transcription factors (21), we have tested the effects of Vav and PKCθ on NF-κB activation. This study reveals that Vav and PKCθ cooperatively activate NF-κB in T cells by targeting IKKβ.

EXPERIMENTAL PROCEDURES

Antisera, Plasmids, and Reagents—The following antibodies were purchased from the indicated suppliers: aFLAG-M2, Sigma; αMyc (9E10), Santa Cruz Biotechnology Inc.; aαav, Upstate Biotechnology; αPKCθ, Transduction Laboratories; αHA antibody (12CA8), Roche Molecular Biochemicals. The luciferas e constructs (αB-3-Luc (18) and expression vectors for FLAG-tagged Vav (22), PKCθ A/E, PKCθ K/R, IKKα K/M, IKKβ K/A, MKK7 K/L, MLK3 K/R, SLP76 ASH2, LAT YY/FF (20), MEKK1 Δ K/M, NIK KVA (18), IKKγ (23), Cot K/A (17), RacN17 (24), MEKK4 K/R (25), and Lck K/R (26) were as described.

Electrophoretic Mobility Shift Assays (EMSAs) and Luciferase Determination—EMSAs were performed essentially as described (20). Equal amounts of protein were used for DNA binding to the following oligonucleotide, which contains a single NF-κB-binding site. The sequence is overlined: 5′-AAGTTGAGGGGACATCCAGGCC-3′. Luciferase activity was measured using a luminometer (Duo Lumat LB 9507, Berthold) that was programmed to inject 50 μl of assay buffer and to measure light emission for 10 s after injection according to the instructions of the manufacturer (Promege Inc.). A β-galactosidase reporter plasmid controlled by a constitutive Rous sarcoma virus promoter was cotransfected to ensure comparable transfection efficiencies.

Coprecipitation Experiments and Immunoblotting—Cells were washed with phosphate-buffered saline, and the pellets were resuspended on ice for 15 min and for an additional 15 min at 37 °C in 250 μl
of Triton X-100 lysis buffer (20 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), aprozinin (10 µg/ml), 1% (v/v) Triton X-100, and 10% (v/v) glycerol). Cell debris was pelleted upon centrifugation, and extracts from antibody-stimulated cells were precleared with protein A/G-Sepharose. Equal amounts of protein contained in the supernatants were mixed with 1 to 2 µg of antibody and 25 µl of protein A/G-Sepharose and rotated for 4 h on a spinning wheel at 4 °C. The immunoprecipitates were washed 5 times in Triton X-100 lysis buffer and subsequently boiled in 1× SDS sample buffer prior to SDS polyacrylamide gel electrophoresis and further analysis by Western blotting as described (20).

**Fig. 1.** Synergistic activation of NF-κB by Vav and PKC. A, Jurkat cells were transiently transfected with 5 µg of a NF-κB reporter construct together with increasing amounts of Vav and/or PKCθ A/E at the indicated combinations. Luciferase activity was determined 18 h post-transfection. Gene expression is displayed as average -fold activation relative to vector-transfected cells. Results shown are averages of three independent experiments. B, Jurkat cells were transfected either with empty expression vector or with plasmids encoding Vav and/or PKCθ A/E at the indicated combinations. The next day, cells were stimulated for 4 h with αCD3/αCD28 antibodies as indicated, total cell extracts were prepared, and the DNA binding activity of NF-κB was assayed by EMSAs (upper panel). An autoradiogram is displayed; the filled arrowhead indicates the location of the DNA-NF-κB complex, the circle indicates the position of a constitutively active DNA-binding protein, and the triangle indicates the position of the unbound oligonucleotide. A sample of each lysate was analyzed by Western blotting (WB) for protein expression of Vav and PKCθ (lower panels).

**Fig. 2.** Vav/PKCθ synergistically activate IKKβ. A, the indicated combinations of expression vectors for Vav and/or PKCθ A/E (5 µg, respectively) were transfected either alone or with increasing amounts of vectors encoding the kinase-dead forms of IKKα or IKKβ and 5 µg of the NF-κB-dependent luciferase reporter gene into Jurkat cells. 1 day post-transfection, cells were harvested and tested for luciferase activity and the expression of IKKα and IKKβ. Results from luciferase assays are expressed as average -fold induction relative to unstimulated, vector-transfected cells. Bars indicate standard deviations; mean values of three independent experiments are shown. A fraction of the extract was analyzed by Western blotting for the occurrence of tagged IKKα and IKKβ (lower panel). B, HA-tagged IKKγ (1 µg) was expressed either alone or in combination with PKCθ A/E and/or Vav in Jurkat cells. 18 h post-transfection, cell lysates were prepared, IKKγ was immunoprecipitated, and kinase activity was determined by immunocomplex kinase assays (KA) using purified GST-IκB-α (1–54) as substrate. An autoradiogram from a reducing SDS gel shows phosphorylation of the recombinant substrate protein and a quantitative evaluation obtained by phosphorimaging (upper panel). C, the experiment was performed as in B, with the exception that an expression vector for IKKβ was transfected.
Jurkat T leukemia cells expressing the large T antigen were grown at 37 °C in RPMI 1640 medium containing 10% fetal calf serum, 10 mM HEPES, 1% penicillin/streptomycin (all from Life Technologies), 2 mg/ml G418, and 2 mM glutamine. Cells were electroporated using a gene pulser (Bio-Rad) at 950 microfarad/250 V. Stimulations were performed by adding agonistic sCD3 (final concentration 10 μg/ml, clone OKT3) and/or oCD28 (final concentration 10 μg/ml, clone 9.3) antibodies.

In Vitro Kinase Assays—Immune complex kinases assays using the purified GST-IκB-α (1–54) substrate protein were performed as described (18).

RESULTS

A possible cooperation between Vav and PKCθ for the activation of NF-κB was tested by transection of T cell leukemia Jurkat cells with an NF-κB-dependent luciferase reporter gene together with increasing amounts of expression vectors for Vav and/or constitutively active PKCθ (PKCθ A/E) (Fig. 1A). Vav expression dose-dependently augmented NF-κB activity, but PKCθ A/E activated NF-κB even more potently. No activation of NF-κB was seen upon expression of PKCα, -ε, or -δ, revealing that the PKC isozyme θ for NF-κB activation in T cells (data not shown). However, coexpression of Vav and PKCθ A/E potently activated NF-κB-dependent luciferase activity in a synergistic manner (Fig. 1A). To test whether this synergism is also apparent at the level of induced DNA binding, we transfected Jurkat cells with different combinations of expression vectors for Vav, PKCθ A/E, or the empty expression vector as a control. Cells were either left untreated or stimulated with oCD3/oCD28 antibodies, and NF-κB DNA binding was determined by EMSAs. Expression of either Vav or PKCθ A/E alone induced DNA-binding of NF-κB, but coexpression of both activators enhanced NF-κB DNA binding (Fig. 1B).

To test whether this synergistic activation of NF-κB is mediated by the activation of the IKKs or via an IKK-independent mechanism (28, 29), we analyzed the impact of increasing amounts of coexpressed dominant negative (DN) forms of IKKα and IKKβ on Vav/PKCθ A/E-induced NF-κB activation in Jurkat cells. IKKβ K/A inhibited the Vav/PKCθ-submitted NF-κB activation more completely than kinase-inactive IKKα K/M (Fig. 2A). These results revealed that the Vav- and PKCθ-derived signals depend on IKKβ.

To investigate whether Vav/PKCθ can lead to the activation of IKK kinase activity, Jurkat cells were transfected with various combinations of PKCθ A/E and Vav expression vectors together with a low amount of an HA-tagged IKKγ expression vector that allows its incorporation into functional high molecular weight IKCs. The tagged IKKγ protein was immunoprecipitated, and the activity of coprecipitating IKKs was examined by measuring the phosphorylation of the exogenously added substrate protein (GST-IκB-α (1–54)) by immune complex kinase assays (Fig. 2B). These experiments revealed that the IKC was activated by Vav and even more by PKCθ A/E alone. However, the combined expression of both proteins strongly augmented the enzymatic activity of the IKC. The activation of IKKβ was investigated by employing a similar experimental approach with the exception that a tagged IKKβ protein was transfected instead of IKKγ (Fig. 2C). IKKβ activity induced by the individual activators was strongly enhanced upon coexpression of both activators, revealing that the observed transcriptional synergism is also apparent at the kinase level. In contrast, neither PKCθ nor Vav were able to significantly induce IKKα activity (data not shown).

The activation signals for the IKC are not completely understood, and the relative contributions of the three IKKKs described so far are not clear. We therefore investigated the role of all three IKKKs for the activation signals derived from Vav, PKCθ, or both. Jurkat cells were transfected with an NF-κB-dependent reporter gene and various combinations of Vav and PKCθ A/E in the absence or presence of DN forms of NIK, MEKK1, and MLK3 (Fig. 3A). The Vav-derived signals were only partially inhibited by DN forms of each of the three IKKKs, raising the possibility that Vav activates the IKC by another, so far unknown pathway. In contrast, the PKCθ- and PKCθ/Vav-generated signals were absolutely dependent on NIK but only incompletely inhibited by DN forms of MEKK1 and MLK3. The upstream signaling events were further characterized by a similar experimental approach employing coexpression of DN forms of various signal transducing and adaptor proteins (Fig. 3A). DN forms of Rac and M KK4 interfered with Vav-mediated NF-κB activation without affect-

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2 O. Dienz, unpublished observation.
ing PKC\(\theta\)- and Vav/PKC\(\theta\)-induced activation pathways. DN forms of MKK7, Cot, Lck, and LAT preferentially inhibited PKC\(\theta\)- and Vav/PKC\(\theta\)-mediated signals. Surprisingly, coexpression of a SLP76 variant lacking the SH2 domain did not significantly affect Vav-mediated NF-\(\kappa\)B activation, but further boosted Vav/PKC\(\theta\)-induced NF-\(\kappa\)B transcription. This behavior might be explained by the loss of an inhibitory interaction. We also tested the impact of pathway-specific inhibitory compounds on Vav- and/or PKC\(\theta\)-induced NF-\(\kappa\)B activation. Cyclosporin A (which blocks Ca\(^{2+}\)/calcineurin-dependent signaling events) and herbimycin A (a PTK inhibitor), interfered preferentially with Vav-derived signaling steps but did not inhibit PKC\(\theta\)- and Vav/PKC\(\theta\)-mediated NF-\(\kappa\)B activation (data not shown). In summary, these results suggest that any efficient inhibition of PKC\(\theta\) is concomitant with the loss of Vav/PKC\(\theta\)-mediated NF-\(\kappa\)B activation, suggesting that PKC\(\theta\) acts downstream from Vav. To address this question directly, Jurkat cells were transfected with an NF-\(\kappa\)B-dependent luciferase reporter gene along with various combinations of active and inactive variants of Vav and PKC\(\theta\) prior to stimulation with aCD3/aCD28 antibodies as indicated (Fig. 3B). NF-\(\kappa\)B-driven gene expression was augmented by Vav expression and further triggered upon T cell costimulation. NF-\(\kappa\)B-dependent transcription induced by Vav and/or CD3/CD28 was inhibited upon coexpression of kinase-dead PKC\(\theta\) K/R (Fig. 3B) or the PKC inhibitor GF109203X (data not shown). In contrast, expression of the Vav variant Vav\(\Delta 1\)–249 inhibited CD3/CD28-triggered NF-\(\kappa\)B activation but failed to impair PKC\(\theta\) \(A/\beta\)-induced transcription (Fig. 3B).

Vav and PKC\(\theta\) were found to be constitutively associated in T cells (20, 30). To map the Vav domain mediating this interaction, we expressed the FLAG-tagged Vav protein and various mutants thereof in Jurkat cells. After immunoprecipitation of the endogenous PKC\(\theta\) protein, the associated Vav proteins were detected by immunoblotting (Fig. 4A). These experiments revealed that N-terminally deleted Vav variants were still able to coprecipitate with Vav, whereas Vav\(\Delta 319\)–356 (lacking the Dbl homology domain) displayed an impaired Vav-PKC\(\theta\) interaction. Vav\(\Delta 501\)–845 showed only a faint residual coprecipitation with PKC\(\theta\), revealing the importance of the C-terminal portion for this protein-protein interaction. To address the question whether the mutual binding of both proteins is affected by T cell stimulation events, Jurkat cells were stimulated by aCD3 and/or aCD28 antibodies for 8 min and further analyzed as for B.

**DISCUSSION**

Here we identify the PKC isoform \(\theta\) as a potent inducer of NF-\(\kappa\)B. PKC\(\theta\) synergized with Vav for the activation of NF-\(\kappa\)B, a transcription factor required for the expression of numerous immunologically relevant target genes (16). Vav and PKC\(\theta\) also cooperate for the induction of JNK, as well as transcription of the IL-2 (19) and IL-4 genes (20), thereby mediating a strong enhancement of upstream signals. Such signal amplification events may be important in the early phase of infection, when only low concentrations of pathogens are present. The described interaction between Vav and PKC\(\theta\) (20, 30) was not confirmed by Villalba et al. (19), which may be because of differences in the lysis conditions or binding buffers. Here we show mutual binding and induced dissociation of both proteins...
Synergistic Activation of NF-κB by Vav and PKCθ

in primary human T cells, thereby revealing the physiological importance of this regulatory protein-protein interaction. T cell costimulation leads to the dissociation of the Vav-PKCθ complex and the PTK-dependent phosphorylation and plasma membrane recruitment of Vav. Vav itself induces actin polymerization and TCR capping by a PKCθ-independent pathway, but these events are essential for the PKCθ translocation into the SMACs (19). Along this line, there is recent evidence that Vav expression promotes PKCθ translocation from the cytosol to the membrane and cytoskeleton (19). These results fit our finding that all inhibitors of PKCθ also abrogated the Vav/PKCθ-induced transcriptional synergism, suggesting that Vav is located upstream from PKCθ in the control of the pathway leading to synergistic NF-κB activation. The downstream targets of PKCθ are largely unknown, and it remains to be investigated whether PKCθ acts directly on IKKβ, as described for several atypical PKCs and PKCa (31). However, NF-κB activation by Vav and PKCθ might not only be mediated by the overlapping synergism pathway but also on separate routes. Cyclosporin A inhibited exclusively the Vav-mediated NF-κB activation, suggesting that only Vav, but not the transcriptional synergism, depends on Ca²⁺/calcineurin-dependent signaling steps. Similarly, DN forms of Rac and M KK4 impaired only Vav-dependent processes without affecting NF-κB activation induced by the synergistic Vav/PKCθ module. The CD28-dependent dissociation of Vav and PKCθ indicates that mutual binding of both proteins is not required for synergistic NF-κB activation. Along this line, VavΔ501–845 was unable to efficiently bind to PKCθ, but still synergized with PKCθ for NF-κB activation. The signal(s) triggering this dissociation is presently not identified. One possibility is a PKCθ-mediated phosphorylation of Vav, but we failed to detect this by in vitro and in vivo phosphorylation experiments (data not shown). Alternatively, the dissociation may be regulated by the 14–3–3-τ protein, which prevents the costimulation-induced translocation of PKCθ (32). Another candidate is Lck, a regulator of PKCθ function (33). Vav/PKCθ acted preferentially on IKKβ, which in line with results from gene disruption experiments showing a predominant role of IKKβ for stimulus-induced phosphorylation and degradation of IκB-α (34). It remains to be seen in future studies whether Vav/PKCθ targets the "classical" IKC consisting of IKKa, IKKβ, and IKKy proteins or differentially composed IKK complexes the recently discovered IKKe (35).

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