Activation of p21-CDC42/Rac-Activated Kinases by CD28 Signaling: p21-Activated Kinase (PAK) and MEK Kinase 1 (MEKK1) May Mediate the Interplay Between CD3 and CD28 Signals

Shuji Kaga, Scott Ragg, Kem A. Rogers and Atsuo Ochi

*J Immunol* 1998; 160:4182-4189; ;
http://www.jimmunol.org/content/160/9/4182
Activation of p21-CDC42/Rac-Activated Kinases by CD28 Signaling: p21-Activated Kinase (PAK) and MEK Kinase 1 (MEKK1) May Mediate the Interplay Between CD3 and CD28 Signals1

Shuji Kaga,*† Scott Ragg,*† Ken A. Rogers,‡ and Atsuo Ochi2*†

CD28, a T cell costimulatory receptor, provides a signal that induces both optimal proliferation and the production of IL-2 by TCR-activated T cells. We show that the stimulation of CD28 leads to the activation of p21-activated kinase and MEK kinase 1. The same pathway was also stimulated in T cells treated with the cell-permeable ceramide analogue, C2-ceramide. The combined stimulation of either CD3 and CD28 or CD3 concurrently with C2-ceramide largely enhanced the activity of p21-activated kinase and MEK kinase 1. Therefore the Rac1/CDC42-coupled pathway(s) is a candidate that transduces and facilitates cross-talk between the CD28 costimulatory signal and the TCR signal.

Received for publication September 22, 1997. Accepted for publication December 29, 1997.


*John P. Robarts Research Institute, †The Department of Anatomy and Cell Biology, The University of Western Ontario, London, Ontario, Canada.
‡Division of Immunology and Hematology, Department of Medicine, University of Toronto, Toronto, Ontario, Canada.
1Copyright © 1998 by The American Association of Immunologists 0022-1767/98/$02.00
2Address correspondence and reprint requests to Dr. Atsuo Ochi, John P. Robarts Research Institute, 1400 Western Road, London, Ontario N6G 2V4, Canada. E-mail address: ochi@rri.on.ca
3Abbreviations used in this paper: PI3K, phosphatidylinositol-3-phosphate kinase; JNK, c-Jun NH2-terminal kinase; PAK, p21-activated kinase; MEK, MEK kinase; SEK, stress-activated protein kinase/extracellular signal-related protein kinase; ERK, extracellular signal-related kinase; GST, glutathione S-transferase; MBP, myelin basic protein.
The treatment of T cells with C2-ceramide mimics CD28 stimulation by PAK and MEKK1 activation. CD3 stimulation also activates this kinase cascade; however, coligation of CD3 and CD28 greatly increases the activation of the PAK cascade, while the same stimulation promotes c-Jun phosphorylation in the nucleus. C2-ceramide also augments the activation of the PAK and MEKK1 cascades after CD3 stimulation. These data demonstrate that CD28 costimulation involves coupling of the sphingomyelin/ceramide turnover-mediated signaling and the PAK/MEKK1/SEK/JNK pathways. The data also suggest that the TCR signal and the CD28 signal merge primarily at the level of a cytoskeletal signaling process which is mediated by Rho family G proteins.

Materials and Methods

Abs, proteins, and chemicals

The Abs used in this work were as follows: anti-human CD3 (UCHT1) from PharMingen (San Diego, CA); anti-human CD28 (YT9H13.2) from Serotec (Oxford, U.K.); and anti-PAK (C-19 and N-20), anti-MEKK1 (43Y and C-20), anti-c-Jun (KM-1), FITC-goat F(ab')2, anti-rabbit IgG, and horseradish peroxidase-conjugated anti-mouse IgG from Santa Cruz Biotechnology (Santa Cruz, CA). The Abs to mouse CD3 (2C11) and mouse CD28 (37.51) were purified from culture supernatants. Glutathione S-transferase (GST)-Ie-Bs and kinase-deficient MEKK1, MEKK1(1–301), were both purchased from Santa Cruz Biotechnology. Myelin basic protein (MBP) was obtained from Sigma (St. Louis, MO). Wortmannin, C2-ceramide, and dehydro-c2-ceramide were obtained from Biomol (Plymouth Meeting, PA). Immunizing peptides (blocking peptides) for PAK-specific Abs were purchased from Santa Cruz Biotechnology.

Cell culture, cell stimulation, and immunoprecipitation

Jurkat and EL4 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 with 5% FCS and 40 μg/ml gentamicin. Jurkat cells or EL4 cells were either stimulated with anti-CD3 (at a final concentration of 10 μg/ml) and/or anti-CD28 (at a final concentration of 15 μg/ml) without cross-linking by secondary Abs or treated with C2-ceramide or dehydro-C2-ceramide in FCS-free RPMI 1640 as indicated. Following stimulation, cells were lysed in RIPA buffer (20 mM Tris (pH 7.4), 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM sodium molybdate, 1 mM sodium orthovanadate, and 20 μg/ml aprotinin). After preclearing with protein A/G agarose (Santa Cruz Biotechnology), immunoprecipitates were prepared using both Abs to PAK or MEKK1 and protein A/G agarose.

To demonstrate the specificity of immunoprecipitation, Ab was neutralized by incubating with an excess amount of immunizing peptide for 2 h at room temperature before use.

Protein kinase assays

Immune complexes were washed four times in RIPA buffer and twice in kinase buffer (50 mM HEPES (pH 7.5), 10 mM MnCl$_2$, 10 mM MgCl$_2$, and 1 mM DTT). Autokinase reactions for PAK and MEKK1 were performed in kinase buffer containing 2 μM ATP (15 μCi/μM). For PAK activity toward MBP, kinase-deficient MEKK1 (MEKK1(1–301)), or GST-Ie-B, immunoprecipitates were incubated in kinase buffer containing 10 μg of MBP and 5 μM ATP (4 μCi/μM), 1 μg of MEKK1(1–301) and 5 μM ATP (4 μCi/μM), or 1 μg of GST-Ie-B and 5 μM ATP (4 μCi/μM), respectively. After a 15-min incubation at 30°C, the reactions were stopped by the addition of denaturing SDS-PAGE buffer. 32P-labeled proteins were resolved by SDS-PAGE and analyzed using a Molecular Imager (Bio-Rad, Hercules, CA).

Fluorescence microscopy study of phosphorylated c-Jun

To measure the phosphorylation of c-Jun in vivo, EL4 cells were cultured overnight on 22-mm square microscope cover slips. Cells on the cover slips were stimulated for 30 min with Abs (2 μg/ml for each Ab), washed, permeabilized, and fixed using standard methods (23). Cells were stained with mouse anti-phosphorylated c-Jun mAb (IgG1) at a 1:20 dilution followed by FITC-goat F(ab')2, anti-mouse IgG (1:20) as described previously (24).

Samples were examined using a Zeiss Axioscope microscope (Stuttgart, Germany) equipped with epifluorescence filters.

Results

CD28 stimulation activates PAK and MEKK1

Our recent experiments have demonstrated that CD28 stimulates focal adhesion, such as molecular changes in T cells, which is apparently controlled by the Rho family G proteins CDC42 and Rac1 (24). The data indicated the possibility that the CD28 signal activates a Ser/Thr kinase, PAK, which is known to associate with and be activated by GTP-bound Rac1 or CDC42. Therefore, we extended our investigation to measure the activity of PAK in CD28-stimulated T cells. Both an autokinase assay (Fig. 1A) and an in vitro kinase assay against MBP (Fig. 1B) clearly demonstrated a transient activation of PAK in anti-CD28-treated human T cell line Jurkat cells. The immunizing peptide successfully inhibited that immunoprecipitation of PAK.

In budding yeast, a PAK homologue, Ste20, mediates the mating signal as it activates Ste11 (a MEKK homologue) by phosphorylation (25). In a manner similar to yeast, PAK may activate MEKK1 in CD28-stimulated Jurkat cells. We used this N-terminal domain (1–301), which does not contain the kinase domain, as the substrate for PAK. In vitro kinase assays showed that the activation of PAK following anti-CD28 stimulation leads to the phosphorylation of the N-terminal domain of MEKK1 but not IeBo used as the control substrate (Fig. 1, C and D). Thus MEKK1 appeared to be a substrate of PAK in CD28-stimulated T cells. Similar results were obtained using the EL4 mouse T cell line (data not shown).

Recently, MEKK1 has been cloned in its full length as a 195-kDa protein (26). In determining if CD28 stimulation activates MEKK1, the Jurkat cell was not suitable, since it expresses MEKK2 and not MEKK1; only anti-MEKK1 Ab is currently available for immunoprecipitation assays. Therefore, we used EL4 cells for all MEKK1 assays. When we assayed the expression of MEKK1, both anti-MEKK1(43Y) Ab to 1–301 amino acids of originally cloned MEKK1 and anti-MEKK1(C-22) Ab to C-terminal 22 amino acids immunoprecipitated a ~190-kDa protein (Fig. 1E). This protein band demonstrated autophosphorylation activity, and the stimulation of these cells with anti-CD28 clearly increased this autophosphorylation activity (Fig. 1F).

The cell-permeable ceramide analogue can activate PAK

Next, we hypothesized that the signaling pathway stimulated by ceramide overlaps with that activated by PAK. In vitro kinase assays were used to test the effect of a cell-permeable ceramide analogue on PAK activation. C2-ceramide activated PAK, while dehydro-C2-ceramide, a biologically inactive form of C2-ceramide (27), did not (Fig. 2, A and B). The activation of PAK occurred in a dose-dependent fashion (Fig. 2B). The activity of PAK induced by C2-ceramide was confirmed using MEKK1(1–301) as the substrate (Fig. 2C).

The CD3 signal synergizes with the CD28 signal or C2-ceramide to augment the activation of the PAk and MEKK1

Given that JNK activity is under the control of PAK/MEKK1, CD28 and CD3 signals may merge at the level of PAK/MEKK1 activation. To investigate this possibility, PAK and MEKK1 activity were studied in Jurkat or EL4 cells stimulated with anti-CD3 plus anti-CD28. Interestingly, CD3 activation significantly stimulated PAK; however, when cells were stimulated with both CD28 and CD3, the activity of PAK was dramatically increased (Fig. 3, A and B). We then examined whether MEKK1 activity parallels that of PAK (Fig. 3, C and D). The increase in phosphorylation resulting from autokinase activity was lower than that observed in the PAK assay, but CD3 plus CD28-stimulated cells evidently had...
FIGURE 1. Stimulation of CD28 activates PAK and MEKK1. **A** shows the autokinase assay of PAK. Jurkat cells were stimulated with anti-CD28 Ab (YTH913.2, rat IgG) or normal rat IgG (NRIg) for 2, 5, or 10 min as indicated above each lane. PAK activity was measured using an autokinase reaction. In lanes 5 and 6, PAK was immunoprecipitated in the presence of the immunizing peptide. The control indicates nonstimulated cells. The amounts of immunoprecipitated PAK were determined by Western blotting (shown in the bottom). **B** shows the kinase activity of PAK toward MBP. Jurkat cells were stimulated with anti-CD28 Ab (YTH913.2) for 2, 5, or 10 min as indicated above each lane. PAK activity was measured using MBP as the substrate. In lanes 5 and 6, PAK was immunoprecipitated in the presence of the immunizing peptide. The control indicates nonstimulated cells. The 18-kDa bands indicate phosphorylated MBP. **C** indicates the kinase activity of PAK against MEKK1(1–301). Jurkat cells were stimulated with anti-CD28 Ab (YTH913.2) for 2, 5, or 10 min as indicated above each lane. PAK activity was measured using kinase-deficient MEKK1 (MEKK1(1–301)) as the substrate. In lanes 5 and 6, PAK was immunoprecipitated in the presence of the immunizing peptide. The 50-kDa bands indicate phosphorylated MEKK1(1–301). The control indicates nonstimulated cells. **D**, PAK phosphorylates MEKK1(1–301) but not I-κBα. Jurkat cells were stimulated with anti-CD28 Ab (YTH913.2) or normal rat IgG (NRIg) for the periods indicated above. In lanes 1, 2, 5, and 6, MEKK1(1–301) was used as the substrate. The GST fusion protein of full length I-κBα (70 kDa) was used as the substrate in lanes 3 and 4. **E**, Anti-MEKK1 detects an 190-kDa protein in the EL4 cell extract. Immunoprecipitates were made using normal rabbit IgG (lane 1), anti-MEKK1(43Y) (lane 2), or anti-MEKK1(C-20) (lane 3) from EL4 cell lysates. MEKK1 was visualized by Western blotting using anti-MEKK1(43Y) as the primary Ab. **F**, Stimulation of CD28 activates MEKK1. EL4 cells were stimulated with anti-CD28 Ab (37.51) for 2, 5, or 10 min as indicated above each lane. Immune complex autokinase assays were performed using anti-MEKK1(43Y) Ab. In lanes 5 and 6, immunoprecipitates were prepared using normal rabbit IgG. The control indicates nonstimulated cells. Amounts of immunoprecipitated MEKK1 were determined by Western blotting (shown in the bottom).
transcription factor c-Jun at Ser63 and Ser72 (reviewed in Ref. 28).

cytoplasm, JNK translocates to the nucleus to phosphorylate the

18 and our unpublished observations). Following activation in the

both signals were necessary to cause optimal T cell activation (Ref.

level as assayed by measuring the kinase activity in vitro. Thus,

that CD28 and CD3 in synergism stimulate JNK to a significant

The results described above strongly suggested that the CD3 signal

CD28 stimulation in vivo

The phosphorylation of nuclear c-Jun increases upon CD3 and

CD28 stimulation in vivo

The results described above strongly suggested that the CD3 signal

and the CD28 signal merge at PKA/MEKK1, possibly resulting in

the augmentation of JNK, we examined the level of c-Jun phosphorylation in

and CD28 does indeed increase the active form of the c-Jun tran-

scription factor in cells, as was expected from the increased activity

of JNK, we examined the level of c-Jun phosphorylation in

cells after CD3 stimulation with or without CD28 costimulation.

EL4 cells were stimulated with anti-CD3 and/or anti-CD28, and

the phosphorylated c-Jun was detected using a mAb against the

Ser63-phosphorylated form of c-Jun. In comparison with the con-

control or with stimulation with anti-CD28 or anti-CD3 alone, the

phosphorylation of c-Jun in the nucleus was significantly increased

when CD28 and CD3 were costimulated (Fig. 4). Thus, the aug-

mentation of PAK and MEKK1 activity resulting from concomi-

stunt stimulation of CD3 and CD28 appeared to facilitate the phos-

phosphorylation of c-Jun in the nucleus was significantly increased

in the control or with stimulation with anti-CD28 or anti-CD3 alone, the

phosphorylation of c-Jun in the nucleus was significantly increased

when CD28 and CD3 were costimulated (Fig. 4). Thus, the aug-

mentation of PAK and MEKK1 activity resulting from concomi-

stunt stimulation of CD3 and CD28 appeared to facilitate the phos-

phosphorylation of c-Jun most likely by the nuclear translocation of

active JNK.

Discussion

The G protein cascade integrates different types of extracellular

signals

Previous studies have shown that the interaction among low m.w.
G proteins, arranged in a cascade, is involved in cell surface recep-
tor-mediated cell activation (29). In budding yeast, the BUD1

G protein cycle controls bud site selection; BUD1 controls

CDC42, which in turn probably controls Rho. In mammalian cells,
a similar G protein cascade consisting of Ras, CDC42, Rac, and

Rho appears to exist. According to the results of experiments in

which activated forms of each G protein have been microinjected

into fibroblasts, CDC42, Rac, and Rho behave as a linear cascade,

with CDC42 activating Rac, and Rac, in turn, activating Rho (30).

Ras may be upstream of these Rho family G proteins, since the

negative, dominant form of Rac blocked both the induction of

membrane ruffling caused by activated Ras (31) and the transfor-
mation activity of oncogenic Ras (32). Different extracellular signals

are known to activate the G protein cascade at different points. For

instance, in fibroblasts, platelet-derived growth factor activates

Ras, followed by Rac and Rho (23, 33, 34), while other signals,
such as lysophosphatidic acid, activate Rho only (31). It is prob-

able that the same G protein cascade is conserved in T cells and

integrates various activation signals. TCR stimulation also activa-

ates Ras (35); therefore, it is likely that TCR stimulates those G

proteins that are regulated by Ras, namely CDC42, Rac, and Rho.

Strikingly, we have also observed that CD28 stimulation initiates

GDP/GTP Rac1 turnover and that combined activation of CD28

and CD3 increased this turnover by 50% (H. Hanawa and A. Ochi,

manuscript in preparation). Our results indicate that CD3 or CD28

stimulation alone activates CDC42 and/or Rac; however, coliga-
tion of CD3 with CD28 greatly potentiates the activation of these

G proteins. Furthermore, we found that one of the kinases down-

stream of Rho is activated by CD3 (data not shown). This finding

seems to support the role of the Ras/CDC42/Rac/Rho pathway in

T cell activation.

Is MEKK1 the kinase that is downstream of PAK?

Our data indicated that immunoprecipitated PAK derived from

CD28-stimulated T cells phosphorylates the N-terminal domain

(1–301) of MEKK1. These data seem to contradict a recent pub-
llication which denies the direct kinase activity of PAK against

MEKK1 (36). In that study, cloned PAK3 gene is expressed in

insect cells and protein is affinity-purified using GST-CDC42Hs-

conjugated beads. The study also described a negative result on the
kinase reaction against MEKK1 by the active form of PAK. However, it should be noted that the experimental system used by those researchers was largely different from ours; the following are likely the reasons for the discrepancy: First, PAK activity is expected to be induced after contact with GTP-bound CDC42 or Rac1. Unless the interaction of PAK with GTP-bound CDC42 is allowed prior to kinase reaction, insect cell-expressed PAK may not be able to phosphorylate MEKK1. Second, it is our experience that PAK kinase assay against MBP often has high basal activity, while the activity against MEKK1 does not. Thus, PAK activity against MEKK1 may be regulated in more specific manner than that against MBP by external stimulation. These two points may explain the failure to phosphorylate MEKK1 with PAK purified with GST-CDC42Hs. Finally, it is possible that the kinase reaction of PAK may require a protein that promotes binding with MEKK1, and that the PAK-specific Ab could allow us to immunoprecipitate PAK complexed with such a "docking protein". Another possibility may be that our PAK precipitate is contaminated with a kinase that intermediates between PAK and MEKK1. Fanger reported that MEKK1, like PAK, directly interacted with Rac/CDC42 to involve the SEK/JNK pathway of activation in COS cells (37). The data indicated that PAK and MEKK1 locate in parallel as the elements of Rac/CDC42-coupled signaling. We also realized that MEKK1(1–301) used as the substrate for PAK does not include the phosphorylation sites required for MEKK1 activation (36).
Therefore, it is possible that phosphorylation by PAK does not activate MEKK1 but may regulate the caspase association and the cleavage of MEKK1 as reported recently (38). Further studies will be required to elucidate the possibility of involvement of other kinases and docking proteins between the PAK and MEKK1 cascade.

How does ceramide mediate the CD28 costimulatory signal?

We have demonstrated that cell-permeable ceramide augments PAK action in CD3-stimulated T cells. Although this result may imply that ceramide could be a mediator of the CD28 costimulatory signal, ceramide is also known as a mediator of apoptosis (39, 40). Therefore, a molecular mechanism that allows ceramide to cause programmed cell death or proliferation must exist. Accordingly, at this point, three G protein-coupled mitogen-activated protein kinase cascades that result in the activation of extracellular signal-related kinase (ERK), JNK, or p38-K have been identified in mammalian cells (reviewed in Ref. 20). Accordingly, it is possible that the multiplicity of mitogen-activated protein kinase cascades contributes, through the integration of each signal, to the determination of death or proliferation in response to various stimuli. Indeed, the activation of JNK and p38-K and the concurrent inhibition of ERK appear to be critical in the induction of apoptosis in PC12 cells after nerve growth factor withdrawal (41). Alternatively, the duration of activation of each kinase may be critical in determining the outcome; for example, the transient activation of ERK by epidermal growth factor induces cell proliferation, whereas the sustained activation of ERK by nerve growth factor induces cell differentiation in PC12 cells (42). In the case of CD28 signal transduction, the activation of ERK by CD3 and the concomitant and transient activation of JNK by the PAK cascade through the effect of ceramide may be essential to the costimulatory activity.

How does PI3K fit into the G protein cascade model?

We attempted to verify the importance of PI3K in PAK-mediated CD28 costimulatory signaling using the specific inhibitor wortmannin. In our study, wortmannin inhibited the activation of PAK following CD28, CD3, or CD3 plus CD28 stimulation (Fig. 5). Presumably P3K activation by Ras upon CD3 stimulation was blocked by wortmannin (43). This result suggests that wortmannin inhibits P3K activation downstream of both the CD3 and CD28 pathways. Therefore, since P3K is involved in CD28 and CD3 signal transduction, wortmannin is not suitable for dissecting the role of P3K in the CD28 costimulatory signaling pathway.

CD28 and TCR signaling pathways are regulated by Rho family small G proteins and associating kinases

The CD28 costimulatory signaling pathway that interplays with TCR signaling may be summarized as shown in Figure 6. TCR stimulation causes the activation of Ras, and then Ras induces the activation of the Raf/MEK/ERK module. Ras activation may induce CDC42 and/or Rac activation through the PI3K pathway. Phosphatidylinositol 3,4,5-triphosphate delivered by PI3K causes dissociation of the Rho GDP-dissociation inhibitor from the GDP-binding form of CDC42 and Rac (44), following which CDC42 and Rac become accessible to the GDP/GTP dissociation stimulator. CD28 stimulation activates PI3K and sphingomyelinase, releasing the metabolites phosphatidylinositol 3,4,5-triphosphate and ceramide which activate CDC42 and Rac. In addition, ceramide may activate PAK directly. CD28 and CD3 signal transduction, wortmannin is not suitable for dissecting the role of P3K in the CD28 costimulatory signaling pathway.

FIGURE 5. Inhibition of CD3 and CD28-induced PAK activation by wortmannin. Jurkat cells were treated with wortmannin (100 nM) or vehicle (DMSO) for 15 min before cell stimulation. Treatment with wortmannin or DMSO was indicated by a + or − at the top, respectively. Cells were stimulated as indicated above, and PAK activity was measured using MEKK1(1–301) as the substrate.

FIGURE 4. Concomitant stimulation of CD3 and CD28 promotes the phosphorylation of c-Jun in the nucleus. EL4 cells were grown on cover slips and stimulated with anti-CD28 (B), anti-CD3 (C), or anti-CD28 plus anti-CD3 (D) for 30 min. The phosphorylated form of c-Jun was monitored by fluorescence microscopy. Nonstimulated cells are shown in A. The size bar in A indicates 100 μm.
Our model suggests that many different signals are integrated through low m.w. G proteins. Our findings document the importance of PAK/MEKK1 in a costimulatory signal transduction pathway and should lead to additional studies that will clarify the function of low m.w. G proteins in cell proliferation, anergy induction, and apoptosis in T cells.

Acknowledgments

We thank J. Allison and J. Bluestone for the gift of the B cell hybridoma lines for anti-CD28 and anti-CD3 and A. Abo and A. Hall for valuable discussions. M. Cameron, H. Chou, and B. Sogbein are also acknowledged for technical assistance.

References


