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Discs large (Dlg1) complexes in lymphocyte activation

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Among candidate molecules with similar functions outside the immune system, the members of a class of proteins called PDZ proteins are of particular interest (Montgomery et al., 2004). The PDZ domains of these proteins form at least two kinds of protein–protein contact (Songyang et al., 1997). The best studied is an interaction with the carboxy-terminal residues of proteins that terminate in a variably conserved sequence of form S/T-X-V or Y/F-X-V although considerable latitude is observed. PDZ domains also appear to be able to interact with internal residues on some proteins, including PDZ domains themselves. Thus, the PDZ domain itself has the attributes expected of a scaffolding structure supporting heterotypic and homotypic interactions.

Recent studies have described a role for membrane-associated guanylate kinases (MAGUKs) in T cell activation. Compromise of expression of the MAGUK protein, CARMA-1, by germline targeting, somatic mutation, or RNA interference inhibits antigen receptor mediated NF-κB activation (Jun and Goodnow, 2003; Thome, 2004). The human homologue of Drosophila Discs large (Dlg1) is a MAGUK found in postsynaptic densities in the central nervous system (Muller et al., 1995). Dlg1 has been implicated in the formation of tight junctions, in epithelial cell polarity, and in the control of proliferation of Drosophila imaginal discs. It consists of an amino-terminal proline-rich...

Abbreviations used in this paper: APC, antigen-presenting cell; Dlg1, Discs large; G/K, guanylate kinase; MAGUK, membrane-associated guanylate kinase; SEE, staphylococcal enterotoxin E; shRNA, short hairpin RNA; TCR, T cell antigen receptor.
domain, multiple PDZ domains, an SH3 domain, HOOK domain, i3 domain, and a guanylate kinase (GK)–like domain.

We present evidence that Dlg1 localizes with the actin cytoskeleton in T cells, associates with early participants in the signaling process and functions as a negative regulator of T cell activation.

**Results and discussion**

To explore the possible roles of PDZ proteins in T cell activation, we conducted a survey of expression in the Jurkat T cell line, focusing on proteins that might associate with lipid rafts, plasma membrane, or signaling intermediates. Dlg1 is expressed throughout all T cell developmental stages and in previous studies has been shown to form a stable complex with the Src family kinase Lck. (Hanada et al., 1997). The i3 domain of Dlg1 is thought to interact with ezrin–radixin-moesin family proteins, which couple membrane proteins to the actin skeleton (Lue et al., 1996; Wu et al., 1998); in T cells ezrin associates with the immunological synapse (Roumier et al., 2001). We analyzed the distribution of actin and Dlg1 in Jurkat cells allowed to settle on coverslips coated with polylysine, anti-CD3 antibody, or anti-CD3 plus anti-CD28 antibodies. Cells were fixed and permeabilized and stained with phalloidin conjugated with Alexa Fluor 594 (for visualization of actin) and polyclonal rabbit anti-Dlg1. (B) Images acquired 5 min after exposure of cells to beads bearing anti-CD3 and anti-CD28 antibody. Dlg1 and actin were visualized with anti-Dlg1 antibody and fluorescent phalloidin, respectively. Dlg1 does not localize at the contact region between Jurkat cells and beads conjugated with anti-CD19 antibody. (C) Images acquired 15 min after exposure of cells to beads bearing anti-CD3 and anti-CD28 antibody. Four representative images demonstrate the redistribution of endogenous Dlg1 away from the contact surface.

Activation of T cells by anti-CD3 and anti-CD28 beads is a related, well-validated system for studying the large-scale reorganization of cellular constituents after exposure to agonistic stimuli (Roumier et al., 2001). As shown in Fig. 1 B, upon contact with CD3/CD28-antibody–coated beads, endogenous Dlg1 is redistributed to membrane caps and extensively colocalizes with actin. Recruitment of Dlg1 to the bead–cell interface is transient: 15 min after stimulation, Dlg1 is no longer enriched at the contact zone (Fig. 1 C). In contrast, CD19-coated beads do not have a significant effect on Dlg1 localization to the bead–cell interface (Fig. 1 B). To study recruitment of Dlg1 to the T cell antigen-presenting cell (APC) interface we performed immunolocalization studies (Roumier et al., 2001; Lee et al., 2002) after stimulation with superantigen or antigenic peptide. Jurkat cells were incubated with Raji cells pulsed with staphylococcal enterotoxin E (SEE) and the distribution of actin and Dlg1 was analyzed by immunofluorescence microscopy. In the absence of SEE, Dlg1 and actin were evenly distributed in Jurkat cells, whereas in the presence of SEE, Dlg1 and actin were concentrated at the cell contact interface at 5 and 10 min (Fig. 2 A; not depicted). Within 5 min of exposure to SEE, TCR× can be seen to translocate to the contact zone and colocalize with Dlg1 (Fig. 2 B). Dlg1 colocalization at the Raji–Jurkat interface was seen to diminish after prolonged association of the presenting and responding cells.

We next examined if Dlg1 was recruited to the immune synapse when antigen-loaded B cells (prepared as LPS-elic-
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 gated blast cells) were incubated with MHC-matched splenic T cells obtained from DO11.10 TCR transgenic mice bearing T cell receptors specific for the peptide antigen. In this system Dlg1 was recruited transiently to the immune synapse at 5 min (Fig. 2 C). Dlg1 was also detected in the LPS blasts and B cell lines and has been described in B lymphocytes (Lue et al., 1994). Using quantitative PCR we have shown that Dlg1 expression is regulated in B cell subsets (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200309044/DC1).

To better understand the association patterns of Dlg1 with other molecules, we immunoprecipitated Dlg1 from the cytosolic and membrane fractions of Jurkat T cells that had been exposed to agonistic antibody (anti-CD3 and anti-CD28) stimulation. We first confirmed that Dlg1 specifi-
Figure 4. Effects ofDlg1 expression on transcriptional activity in Jurkat cells. (A) Overexpression ofDlg1 attenuatesVav1-induced NFAT transcriptional activity. NFAT activity was measured after cotransfection ofJurkat cells with the indicated plasmids and an NFAT-luciferase reporter construct. After stimulation, cells were lysed and luciferase activity was measured. Relative activity is corrected for transfection efficiency. (Bottom) Immunoblot analysis confirms expression of constructs used in the reporter assay. (B) Effects ofDlg1 onVav1 basal and anti-CD3 stimulated NFAT activation in Jurkat cells. Overexpression ofDlg1 inhibitsVav1 induction ofNFAT activity, whereas overexpression ofDlg1 carboxy-terminal fragment potentiates theVav1 effect. (Bottom) Immunoblot illustrates relative expression of various constructs used in reporter assay. (C) Overexpression ofDlg1 suppressesNFAT activation by coexpressedVav1 in Jurkat cells stimulated by superantigen pulsedRaji cells. Jurkat T cells were transfected with NFAT-luciferase reporter, Vav1 andDlg1 constructs. 20 h after transfection, cells were stimulated withRaji cells pulsed withSEE for 8 h and lysed for luciferase assay. (Bottom) Immunoblot analysis confirms expression of constructs used in the reporter assay. White lines indicate that intervening lanes have been spliced out. Error bars represent SEM.

Cally interacts withLck in Jurkat cells (Hanada et al., 1997). We next showed that endogenousDlg1 specifically affiliates with the signaling moleculesTCRζ andCbl. TheTCRζ species complexed withDlg1 contains both phosphorylated and nonphosphorylated components, and the phosphorylation status depends on the activities ofLck andZap-70 (Fig. 3, A and B; not depicted).

Overexpression ofVav1 has been shown to enhanceCD3-dependent NFAT reporter activation (Wu et al., 1995). As demonstrated in Fig. 4 (A and B), coexpression ofDlg1 andVav1 inhibitsVav1-induced basal andCD3-potentiated NFAT activity. A carboxy-terminalDlg1 fragment that contains thePDZ3-SH3-I3-GKdomains enhanced NFAT activity in this system. Coexpression ofDlg1 withVav1 attenuatedNFAT reporter activity in Jurkat cells stimulated bySEE-loadedRaji cells (Fig. 4 C).

Suppression of endogenous gene expression by short hairpin RNA (shRNA) has been used to create hypomorphic phenotypes in mammalian cells (Brummelkamp et al., 2002). We createdshRNAs targeted toDlg1 sequences in theSH3 (shRNA #1) andGK domains (shRNA #2). Lysates from pooled stable transfectants expressingshRNA were blotted with an anti-Dlg1 antibody. As shown in Fig. 5 A, shRNA #2 gave significant suppression of expression. Fig. 5 B shows thatDlg1 suppression caused an increase in basalNFAT activity and a potentiation of the response toanti-CD3 stimulation. We found similar effects onNFAT reporterafter stable and transient transfection ofshRNA inJurkat cells (not depicted). To determine ifDlg1 knockdown had a similar effect on superantigen-mediatedNFAT activation, Jurkat cells expressingshRNAs against theSH3 andGK domains were activated withSEE pulsedRaji B cells. As shown in Fig. 5 C, RNA interference ofDlg1 enhanced superantigen-mediatedNFAT activation.

In subcellularfractionation studies we have demonstrated thatDlg1 complex inT cells containsLck,TCRζ, andCbl and colocalization studies lend support for these biochemical observations (unpublished data). In neuronal synaptic signaling andDrosophilaembryogenesisDlg1 associates with a subset ofAMPA receptors, Kv channels, and membrane proteins on endomembranes and appears to regulate the abundance of these proteins at the plasma membranes (Tiffany et al., 2000; Lee et al., 2003). Dlg1 has been shown to associate withAKAP-79 inhippocampal neurons (Colledge et al., 2000), and AKAP-79 interactions with calcineurin and protein kinase A are known to inhibitNFAT activation (Kashishian et al., 1998; Crabtree and Olson, 2002). Additional studies will be required to determine ifDlg1 plays a similar role in delivery of components of theTCR signal complex to the plasma membrane during lymphocyte differentiation and activation. Dlg1 may also play a role in signaling via theBCR as well.

The interpretation thatDlg1 acts as an activation antagonist is consistent with findings from other systems. The phenotype that originally led to the discovery ofDlg1, grossly enlarged imaginal disks inDrosophila, suggested a role for the protein in restraint of imaginal disk cell proliferation, andDlg1 has been considered to be a kind of tumor suppressor inDrosophila (Dimitratos et al., 1999).
important role of Dlg1 may be to promote receptor internalization and/or recycling after the initial phase of contact and engagement.

Materials and methods

Reagents

The 10.10.4 Jurkat subclone used in this work has been engineered to express SV40 large T antigen and inducible cell surface proteins, and selected for high (>90%) expression of CD3. Antibodies obtained include: C6M, PKC-8, CD45 (Transduction Laboratories); actin (ICN Biomedicals); chapsyn (Calbiochem); CD3γ (BD Biosciences); Dlg1, TCRα, GSK, and Lck (BD Biosciences); Dlg1, TCRα, GSK, and Lck.

Expression constructs

Construction of rat Dlg1-GFP and deletion constructs have been described elsewhere (Wu et al., 1998). These constructs were prepared according to the manufacturer's instructions (Promega). The electroporation efficiency was normalized by assessing GFP expression by flow cytometry. Expression of various constructs was confirmed by immunoblots.

Immunoprecipitations

105 Jurkat cells were stimulated with 2 μg/ml of anti-CD3 antibody (OKT3) and 2 μg/ml of anti-CD28 (clone 9.3), washed twice in 1 ml of ice cold PBS and resuspended in 750 μl of hypotonic lysis buffer (20 mM Tris HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 5 mM EGTA, 10 mM β-ME, one protease inhibitor cocktail tablet (Roche) per 10 ml, 1 mM Na3VO4, 20 mM NaF), transferred to a 1-ml syringe, and sheared by passing 30 times through a 25-gauge needle. The lysates were centrifuged at 1,000 rpm for 7 min to precipitate nuclei and the supernatant was collected. 50 μl of the total cell extract was saved, and the remainder was centrifuged at 16,000 rpm for 20 min. The supernatant was collected and the pellet resuspended in lysis buffer with 1% NP-40 for 30 min with gentle vortexing every 5 min on ice. The supernatant and membrane fractions were immunoprecipitated for 4 h at 4°C with Dlg1 (Santa Cruz Biotechnology, Inc.) specific antisera. Isotype controls were performed for each immunoprecipitation. Immune complexes were collected with goat anti-mouse beads (Amersham Biosciences) and washed three times with lysis buffer. Bound proteins were eluted with 50 μl SDS sample buffer and resolved by SDS-PAGE. Proteins were transferred to PVDF membranes, which were blocked overnight and probed with indicated antibody. After washing, filters were incubated with a 1:5,000 dilution of anti-mouse or anti-rabbit HRP conjugate (DakoCytomation) in TBST for 1 h. Protein bands were detected by ECL (Amersham Biosciences).

Luciferase assays

Determination of NFAT activity was performed as described previously (Wu et al., 1995; Roumier et al., 2001). In brief, 5 × 104 Jurkat cells were sedimented, resuspended in 0.25 ml IMDM, and electroporated (250 V/960 μF) with 10 μg of NFATx3–luciferase reporter construct, 10 μg of full-length or fragments of Dlg1-GFP or SH3-I3-GK domain (shRNA1) or GK domain (shRNA2). Cells were transfected with NFAT-luciferase reporter. 24 h after transfection, cells were stimulated with anti-CD3 (5 μg/ml) for 8 h and lysed for assay. (C) Activation of NFAT luciferase reporter in Jurkat cells stably transfected to express shRNA targeted against the Dlg1 SH3 domain (shRNA1) or GK domain (shRNA2). Pooled stable cells were transfected with NFAT-luciferase reporter construct. 24 h after transfection, cells were stimulated with Raji cells pulsed with SEE for 8 h and lysed for luciferase assay. (Bottom) Immunoblot analysis confirms shRNA suppression of Dlg1 expression. White lines indicate that intervening lanes have been spliced out. Error bars represent SEM.

Figure 5. Stable RNA interference of Dlg1 in Jurkat cells enhances NFAT activity. (A) Cells expressing shRNA were lysed in 1% Triton X-100 buffer and an equal amount protein from each cell line was resolved on a 7.5% SDS gel and blotted with antibodies recognizing Dlg1, actin, GSK, and Lck. (B) Activation of NFAT luciferase reporter in Jurkat cells stably transfected to express shRNA targeted against the Dlg1 SH3 domain (shRNA1) or GK domain (shRNA2). Cells were transfected with NFAT-luciferase reporter. 24 h after transfection, cells were stimulated with anti-CD3 (5 μg/ml) for 8 h and lysed for assay. (C) Activation of NFAT luciferase reporter in Jurkat cells stably transfected to express shRNA targeted against the Dlg1 SH3 domain (shRNA1) or GK domain (shRNA2). Pooled stable cells were transfected with NFAT-luciferase reporter construct. 24 h after transfection, cells were stimulated with Raji cells pulsed with SEE for 8 h and lysed for luciferase assay. (Bottom) Immunoblot analysis confirms shRNA suppression of Dlg1 expression. White lines indicate that intervening lanes have been spliced out. Error bars represent SEM.
and fixed for 10 min as above. To distinguish APCs from Jurkat cells, APCs were loaded with cell tracker CMAC blue (CT blue – molecular probes).

T cells were obtained from DO11.10 transgenic mice as described previously (Lee et al., 2002). DO11.10 transgenic T cells were induced with OVA peptide in bulk spleenocyte cultures for 5–6 d, washed extensively and spun over Lympholyte M to remove dead cells and debris. T cells were rested in media supplemented with IL2 for several hours or overnight. To initiate conjugate formation, T cells were centrifuged briefly with LPS-induced B cell blasts (used as APCs) pulsed with 1–10 μM OVA peptide for 2–3 h at 37°C and incubated for 1, 2, 5, or 10 min. Conjugates were examined for endogenous Dlg1 recruitment to the immune synapse by immunofluorescence microscopy.

After blocking with PBS including 1% BSA, the cells were labeled with rabbit anti-Dlg1, mouse anti-CD3 antibody in PBS including 1% BSA, followed by three washes of 5 min each. After labeling with secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes), phalloidin conjugated with Alexa Fluor 594 (Molecular Probes) and DAPI, the slides were mounted with Aqua Poly/Mount (Polysciences). F-actin polymerization was analyzed by immunofluorescence microscopy using an Axio- scope (Carl Zeiss MicroImaging, Inc.) microscope, with focus adjusted to the plane of maximum staining intensity. More than 100 cells were analyzed for each condition (either resting or after TCR activation).

Online supplemental material

Fig. S1 is an analysis of Dlg1 expression in B cell developmental subsets by real-time PCR. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200309044/DC1.

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