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Discs large (Dlg1) complexes in lymphocyte activation

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Discs large (Dlg1) complexes in lymphocyte activation by germline targeting, somatic mutation, or RNA interference enhances both CD3- and superantigen-mediated NFAT activation. Attenuation of antigen receptor signaling appears to be a complex, highly orchestrated event that involves the mutual segregation of important elements of the early signaling complex.

Introduction

Engagement of the T cell antigen receptor (TCR) during antigen presentation initiates a sensitive, highly regulated response that relies on the coordinated action of a large number of signaling proteins. Recent evidence has shown that extensive rearrangements of membrane and cytoskeletal elements attend the activation response, and agents that disrupt the organization or localization of these elements interfere with antigen recognition (Dustin and Cooper, 2000). Under certain conditions receptor engagement leads to the assembly of a characteristic supramolecular activation cluster on the T lymphocyte side of the interface (Monks et al., 1998).

Molecules that promote and regulate the association of membrane signaling with the cytoskeleton are expected to have, at minimum, a mechanism for association with the signaling molecule and/or membrane, as well as a mechanism for association with the cytoskeleton. Because such molecules are expected to coordinate the movement and regulation of large signaling assemblies, they would likely behave as molecular scaffolds and have mechanisms for interacting with one another as well as with signaling proteins.

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 kinas (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

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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). 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).

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, TCRδ 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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edited 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 after activation. Raji cells are labeled with CMAC tracker blue (CT blue). (C) Dlg1 localizes transiently at the immune synapse after antigenic stimulation of T cells. LPS-activated B cells (labeled blue) were pulsed with ova-peptide and mixed with DO11.10 transgenic T cells. F-actin and Dlg1 were recruited to the contact area at 5 min.

Figure 2. APC-T cell activation induces Dlg1 relocalization. (A) Dlg1 accumulates at the T cell–B cell contact site. Jurkat cells were incubated with Raji cells that had been exposed to medium with or without SEE for 5, 10, 15, and 20 min. Raji cells were labeled with CMAC cell tracker blue. F-actin and Dlg1 accumulation were induced by SEE pulsed Raji cells at 5 and 10 min (not depicted). Representative images show no increase in Dlg1 accumulation at cell–cell contact site at 20 min. (B) Codistribution of CD3ζ and Dlg1 after activation of Jurkat cells with Raji cells loaded with SEE. CD3ζ and Dlg1 colocalize at the cell–cell contact site at early time points (5 min) after activation. Raji cells are labeled with CMAC tracker blue (CT blue). (C) Dlg1 localizes transiently at the immune synapse after antigenic stimulation of T cells. LPS-activated B cells (labeled blue) were pulsed with ova-peptide and mixed with DO11.10 transgenic T cells. F-actin and Dlg1 were recruited to the contact area at 5 min.

Figure 3. Proteins coordinated by Dlg1. (A) Multiple signaling molecules associate with Dlg1 in T cells. Membrane and cytosolic fractions from unstimulated and anti-CD3 plus CD28-stimulated Jurkat cells were immunoprecipitated with Dlg1 antibody, resolved by SDS-PAGE and immunoblotted with antibodies recognizing the proteins shown. TL represents 10 μl of non-immunoprecipitated total lysate loaded with each blot to confirm antibody specificity. Iso represents the result of immunoblotting lysates prepared by immunoprecipitation with an isotype-matched control antibody. (B) Phospho-ζ (p23ζ) association with Dlg1. Dlg1 immunoprecipitates from cytosol, membrane fractions, and nucleus fractions were probed with anti-TCRζ (left) or with anti-phosphotyrosine antibody 4G10 (right). The top bands in the immunoblots represent immunoglobulin light chain. White lines indicate that intervening lanes have been spliced out.
We next showed that endogenous Dlg1 specifically affiliates with the signaling molecules TCRγH9256 and Cbl. The TCRγH9256 species complexed with Dlg1 contains both phosphorylated and nonphosphorylated components, and the phosphorylation status depends on the activities of Lck and Zap-70 (Fig. 3, A and B; not depicted).

Overexpression of Vav1 has been shown to enhance CD3-dependent NFAT reporter activation (Wu et al., 1995). As demonstrated in Fig. 4 (A and B), coexpression of Dlg1 and Vav1 inhibits Vav1-induced basal and CD3-potentiated NFAT activity. A carboxy-terminal Dlg1 fragment that contains the PDZ3-SH3-I3-GK domains enhanced NFAT activity in this system. Coexpression of Dlg1 with Vav1 attenuated NFAT reporter activity in Jurkat cells stimulated by SEE-loaded Raji 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 created shRNAs targeted to Dlg1 sequences in the SH3 (shRNA #1) and GK domains (shRNA#2). Lysates from pooled stable transfectants expressing shRNA were blotted with an anti-Dlg1 antibody. As shown in Fig. 5 A, shRNA#2 gave significant suppression of expression. Fig. 5 B shows that Dlg1 suppression caused an increase in basal NFAT activity and a potentiation of the response to anti-CD3 stimulation. We found similar effects on NFAT reporter activity after stable and transient transfection of shRNA in Jurkat cells (not depicted). To determine if Dlg1 knockdown had a similar effect on superantigen-mediated NFAT activation, Jurkat cells expressing shRNAs against the SH3 and GK domains were activated with SEE pulsed Raji B cells. As shown in Fig. 5 C, RNA interference of Dlg1 enhanced superantigen-mediated NFAT activation.

In subcellular fractionation studies we have demonstrated that Dlg1 complex in T cells contains Lck, TCRγH9256, and Cbl and colocalization studies lend support for these biochemical observations (unpublished data). In neuronal synaptic signaling and Drosophila embryogenesis Dlg1 associates with a subset of AMPA 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 with AKAP-79 in hippocampal neurons (Colledge et al., 2000), and AKAP-79 interactions with calcineurin and protein kinase A are known to inhibit NFAT activation (Kashishian et al., 1998; Crabtree and Olson, 2002). Additional studies will be required to determine if Dlg1 plays a similar role in delivery of components of the TCR signal complex to the plasma membrane during lymphocyte differentiation and activation. Dlg1 may also play a role in signaling via the BCR as well.

The interpretation that Dlg1 acts as an activation antagonist is consistent with findings from other systems. The phenotype that originally led to the discovery of Dlg1, grossly enlarged imaginal disks in Drosophila, suggested a role for the protein in restraint of imaginal disk cell proliferation, and Dlg1 has been considered to be a kind of tumor suppressor in Drosophila (Dimitratos et al., 1999). The connection with the cortical actin skeleton suggests that one
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: Cbl, PKC-θ, CD45 (Transduction Laboratories); actin (ICN Biomedicals); c-Jun (Calbiochem); CD3ζ (BD Biosciences); Dlg1, TCRα/β (Santa Cruz Biotechnology, Inc.); GADS, Lck, Zap-70, Vav-1 (Upstate Biotechnology); and 540–906 (SH3-I3-GK). Human Dlg1 was isolated by PCR amplification from a Raji cell library and inserted between HindIII and NotI sites downstream of an EF1α promoter.

Immunoprecipitation

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 NaVO3, 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 activities was performed as described previously (Wu et al., 1998). These constructs were transfected with NFAT-luciferase reporter construct. 20 h after transfection, cells were stimulated with SEE. The supernatants were collected for luciferase assay.

RNA interference

The vector pshRNApress containing the RNA polymerase III-dependent H1 RNA gene promoter was provided by D. Billadeau (Division of Oncology Research, Mayo Clinic, Rochester, MN). Complementary oligonucleotides designed against sequences in the SH3 domain of Dlg1 were cloned into unique BglII–XhoI site of pcDNA3-Vav1 and pcDNA3-Vav1. Upon electroporation, cells were diluted in 2 ml IMDM, placed in wells of a 6-well plate, and incubated for 16 h. 0.5-ml aliquots were then transferred to wells of a 24-well plate and exposed to 5 μg/ml OKT3. Alternatively, Raji cells pulsed with SEE for 30 min were incubated with T cells overexpressing or knockdown of Dlg1 for 6 h at 37°C. Luciferase assays were performed according to the manufacturer’s instructions (Promega). The electrophoration efficiency was normalized by assessing GFP expression by flow cytometry. Expression of various constructs was confirmed by immunoblots.

Fluorescence microscopy

Jurkat T cells were stimulated with anti-CD3 antibody (OKT3) on coverslips as described previously (Bunnell et al., 2001). Coverslips were coated with 10 μg/ml anti-CD3 antibody and 10 μg/ml anti-CD28 for 2 h at 37°C in a moist chamber. The slides were washed with PBS four times. Spreadings were initiated by placing 10 μl of a concentrated Jurkat cell suspension on anti-CD3–coated coverslips. After incubation at 37°C for 2 or 5 min, the cells were fixed for 10 min with 3.5% PFA and 0.1% Tween-20 in PBS at RT. Jurkat T cells were stimulated with antibody-coated beads and APCs (Raji cells) pulsed with SEE as described previously (Roumier et al., 2001). In brief, the cells were mixed with beads coated with CD3/CD28 (Dynal) at a ratio of two beads/cell. After centrifugation for 5 min at 100 g, the cell–bead mixture was incubated for an additional 5–15 min at 37°C. Conjugates were then resuspended, plated onto poly-I-lysine–coated slides,
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 (Polyscience). F-actin polymerization was analyzed by immunofluorescence microscopy using an Axio-Imager 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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