Dlgh1 coordinates actin polymerization, synaptic T cell receptor and lipid raft aggregation, and effector function in T cells

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Lipid raft membrane compartmentalization and membrane-associated guanylate kinase (MAGUK) family molecular scaffolds function in establishing cell polarity and organizing signal transducers within epithelial cell junctions and neuronal synapses. Here, we elucidate a role for the MAGUK protein, Dlgh1, in polarized T cell synapse assembly and T cell function. We find that Dlgh1 translocates to the immune synapse and lipid rafts in response to T cell receptor (TCR)/CD28 engagement and that LckSH3-mediated interactions with Dlgh1 control its membrane targeting. TCR/CD28 engagement induces the formation of endogenous Lck–Dlgh1–Zap70–Wiskott-Aldrich syndrome protein (WASp) complexes in which Dlgh1 acts to facilitate interactions of Lck with Zap70 and WASp. Using small interfering RNA and overexpression approaches, we show that Dlgh1 promotes antigen-induced actin polymerization, synaptic raft and TCR clustering, nuclear factor of activated T cell activity, and cytokine production. We propose that Dlgh1 coordinates TCR/CD28-induced actin-driven T cell synapse assembly, signal transduction, and effector function. These findings highlight common molecular strategies used to regulate cell polarity, synapse assembly, and transducer organization in diverse cellular systems.

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Abbreviations used: GST, glutathione S-transferase; IRF, IFN regulatory factor; MAGUK, membrane-associated guanylate kinase; siRNA, small interfering RNA; WASp, Wiskott-Aldrich syndrome protein.

Proper T cell activation is central to the generation of protective adaptive immunity and in the maintenance of self-tolerance. T cell activation is initiated when the TCR encounters specific antigen–MHC complexes and costimulatory ligands on the surface of an APC. This recognition induces dramatic T cell polarization and the formation of a specialized "immunological synapse" at the T cell–APC junction (1).

Recent studies indicate that the organized immune synapse is a multitasking platform performing several functions essential to the determination of TCR sensitivity and responsiveness. The synapse enhances TCR engagement and signal transduction through the recruitment, concentration, and juxtaposition of receptors and transducers (1, 2). The migration of cholesterol and sphingolipid–rich "lipid rafts" to the synaptic contact likely facilitates these processes (3–6). Paradoxically, the immune synapse also attenuates TCR signal transduction by directing TCR endocytosis, transducer ubiquitination, and proteolysis and by functioning as a target for the delivery of down-

regulatory CTLA-4 (1, 2, 7). Coordinate regulation of these opposing activities allows the synapse to "fine tune" TCR signal transduction and T cell responsiveness. Finally, the synapse orients and orchestrates a microtubule array that directs the TCR–regulated secretion of cytokines and cytotoxic lytic effectors toward the APC so that effectors selectively act on target cells (8, 9).

Continued TCR engagement and signal transduction is required for both synapse maintenance and realization of maximal T cell proliferation and effector function (10–12). Although the molecular basis of T cell polarization, synapse assembly, and sustained signaling remain poorly defined, actin cytoskeletal remodeling is central to each of these processes (13). In lymphocytes, de novo actin polymerization is controlled by Wiskott-Aldrich syndrome protein (WASp; reference 13). Immunoreceptor engagement induces Src, Btk, and Syk family up-regulation of WASp activity (9, 14–16). In T cells, WASp activity is required for TCR and synaptic lipid raft clustering,

TCR endocytosis, sustained signaling, and cytokine gene transcription (13, 17–19). However, intermediates linking receptor engagement to WASp activity remain incompletely characterized.

Recent reports have described associations between the neuronal synaptic scaffolding molecule hDlg/Dlgh1 and T cell transducers involved in TCR signal transduction and cytoskeletal reorganization in T cells (20–23). Dlgh1, the mouse homologue of the human hDlg and rat SAP97, is a member of the membrane-associated guanylate kinase protein family (MAGUK). MAGUKs are characterized by the presence of one to three PDZ domains, an SH3 domain, and a guanylate kinase domain that lacks enzymatic activity. These modular domains have been shown to mediate multiple interactions with several proteins concomitantly (24). MAGUKs anchor voltage and ligand-gated ion channels and other receptors to the neuronal synapse and organize signaling complexes within the synaptic contact. Dlgh1 is also found in epithelial cells where it can localize to the cellular membrane and associate with the cortical actin cytoskeleton. In epithelial cells, Dlgh1 regulates apical-basal cell polarity

and organizes junctional structure (25). It has thus been suggested that Dlgh1 may act as a molecular scaffold to mediate the structural integrity of various types of membrane specializations (26). Although Dlgh1 has been well characterized in both neuronal and epithelial cells, its role in modulating T cell synaptogenesis, activation, and function remain largely uncharacterized.

Here, we demonstrate that Dlgh1 translocates to the immune synapse and detergent-insoluble lipid rafts in response to TCR/costimulator engagement. Furthermore, we demonstrate that Lck–Dlgh1 interactions are mediated by the LckSH3 domain and are required for proper Dlgh1 membrane association. We identify Zap70 and WASp as novel constituents in Dlgh1 molecular complexes and demonstrate that Dlgh1 functions in facilitating WASp and Zap70 interactions with Lck. We hypothesize that Dlgh1 facilitates activation of these transducers to coordinate actin-mediated T cell synapse assembly and effector function. Consistently, small interfering RNA (siRNA)-mediated knockdown of Dlgh1 expression in T cells leads to impaired antigen-induced actin polymerization, TCR and raft synaptic clustering, and
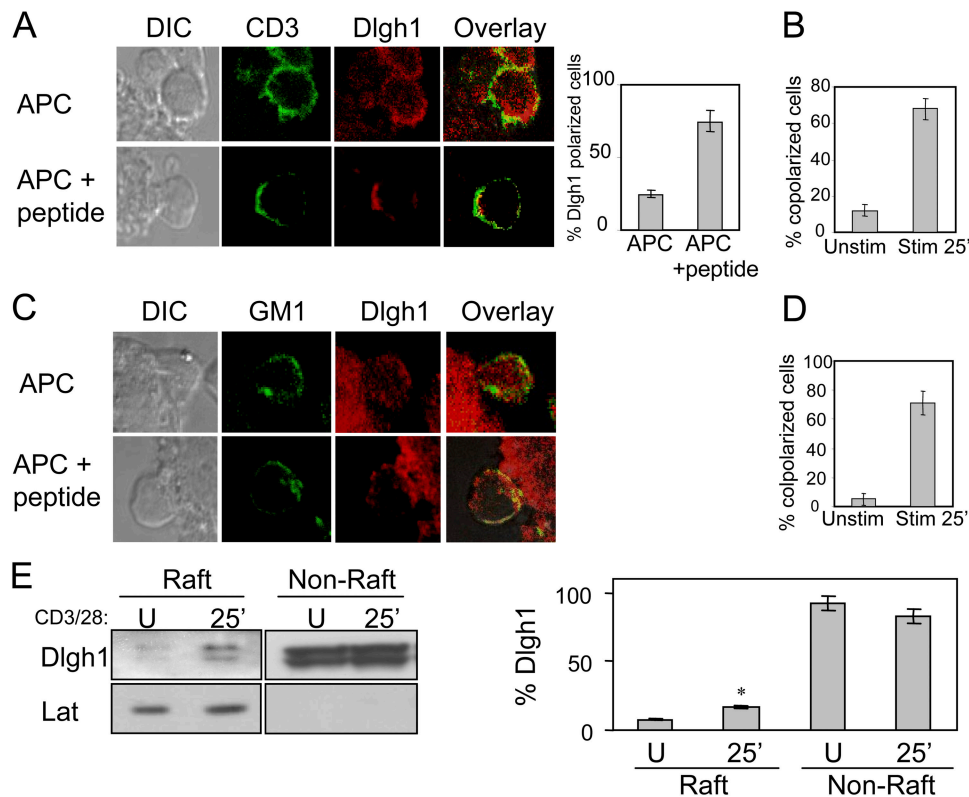

Figure 1. Dlgh1 localizes with lipid rafts and CD3 in the immunological synapse. (A–D) Lymphocytes were isolated from the spleen and lymph nodes of an OT-1 TCR transgenic RAG $^{-/-}$ mouse and stimulated with APCs lacking MHC/peptide or antigen/APCs for 0 (Unstim) and 25 min. Cells were then stained with FITC-conjugated CD3 (A and B) or FITC-conjugated cholera toxin (CTX) β subunit (C and D) and Dlgh1 antibody (A–D). 100 T cell–APC conjugates were counted per experiment and cells were scored positive for CD3 or Dlgh1 polarization or synaptic clustering when >50% of the CD3, Dlgh1, or GM1 was polarized toward the T cell–APC junction. The graph represents the percentage of cells with Dlgh1 polarized at the synapse under each experimental condition from two experiments. The percentages of cells in which CD3 or GM1 copolarized with Dlgh1 at the T cell synapse from four experiments is represented in B and D. (E) Cells were left unstimulated or stimulated with anti-CD3/CD28 for the indicated times. Detergent-soluble and -insoluble fractions were isolated and immunoblotted with antibodies directed against Dlgh1 or Lat. Relative densitometry values from four experiments are represented in the graph. *, statistical significance according to a standard Student's t test.

T cell function. Additionally, Dlgh1 overexpression enhances TCR-induced NFAT activation and lymphokine production. Taken together, our findings elucidate a positive role for Dlgh1 in the orchestration of T cell activation.

RESULTS

Dlgh1 localizes to the immune synapse and can reside in the lipid raft compartment

To characterize Dlgh1 localization in T cells, we visualized Dlgh1, CD3, and the raft marker GM1 in freshly isolated CD8$^+$ OT-1 TCR transgenic T cells during the course of activation and immune synapse formation. Dlgh1 patterns are similar in T cells stimulated with APCs in the absence of MHC/peptide and T cells before stimulation, demonstrating nonpolarized Dlgh1, GM1, and CD3 distribution (Fig. 1, A–C, and not depicted). Alternatively, a bulk of Dlgh1 accumulates at the synapse and a portion of Dlgh1 colocalizes with both CD3 and GM1 (Fig. 1, A–D) within 25 min of antigenic stimulation. Polarization of CD3, GM1, and Dlgh1 at the synapse is not seen at an earlier 5-min time point (not depicted). Dlgh1 synaptic localization requires TCR engagement because APCs that do not express MHC/peptide do not induce Dlgh1 synaptic translocation (Fig. 1 A, APC).

To further characterize Dlgh1 subcellular distribution, we fractionated T cells into detergent-soluble lipid raft and nonraft fractions using sucrose density centrifugation. We found that a subset of Dlgh1 constitutively partitions in the raft fraction, and TCR/CD28 stimulation increases Dlgh1 raft partitioning twofold (Fig. 1 E). Although this increase is modest, it is reproducible, as indicated in the graph representing relative densitometry values from four experiments. Most of Dlgh1 is found in detergent-soluble, membrane-associated and cytosolic proteins. Biochemical partitioning of raft–associated proteins often requires more stringent association than is required for coassociation measured using raft vi-

sualization approaches and often underestimates the amount of protein within a compartment, as is the case for TCR (5, 27). Taken together, our findings indicate that a subset of Dlgh1 translocates to the immune synapse in response to antigenic stimulation and can associate with lipid raft microdomains within the synaptic contact.

Dlgh1 binds specifically to the SH3 domain of Lck

We have previously established a role for the LckSH3 domain in T cell activation; however, the molecular basis of LckSH3 activity remains incompletely characterized (11). A previous description of an association between the Dlgh1 NH$_2$-terminal proline–rich domain and Lck (20) prompted us to investigate whether the LckSH3 domain is required for Dlgh1–Lck coassociation and determine whether this association is important for localization of Dlgh1. As reported for Jurkat T cells, we find that Lck and Dlgh1 coprecipitate in murine BI-141 and primary CD8$^+$ T cells (Figs. 2 C and 3, A–C). To determine what role the LckSH3 domain plays in mediating this interaction, purified glutathione S-transferase (GST) fusion proteins containing wild-type or mutated LckSH3 domains were used to precipitate Dlgh1 from T cell lysates. As shown in Fig. 2 A, Dlgh1 specifically associates with wild-type LckSH3 domain, but not with LckSH3 domains mutated in residues within the canonical PXXP binding pocket, AF and YLDY (11). Dlgh1 was not detected in precipitates of Fyn, Abl, Grb2, and Itk SH3 domain GST fusions, further demonstrating the specificity of the LckSH3–Dlgh1 interaction (Fig. 2 B).

Proper Dlgh1 membrane localization depends on an intact Lck SH3 domain

Dlgh1 contains no transmembrane domain or putative sites for lipid modification that would allow it to associate with the plasma membrane or the lipid raft compartment (28).
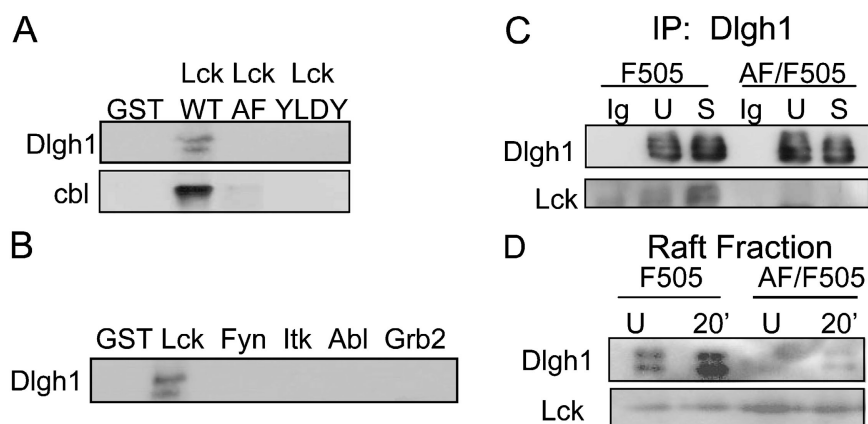

Figure 2. Dlgh1 specifically associates with the Lck SH3 domain, and this interaction is required for Dlgh1 association with detergent–insoluble lipid rafts. (A and B) GST fusion proteins containing the indicated SH3 domains were purified and incubated with TNE lysates collected from BI-141 T cells and immunoblotted with anti-Dlgh1, anti-Lck, or anti-Cbl (as a positive control for Lck SH3 binding). (C and D) Unstimulated (U) or CD3/CD28-stimulated (for 15 min) BI-141 T cells expressing constitutively active Lck (F505) or SH3-impaired Lck (F505/AF) were lysed in TNE and immunoprecipitated with anti-Dlgh1 (C) or fractionated (D) into detergent-soluble and -insoluble lipid rafts. Samples were immunoblotted with anti-Dlgh1 or Lck. All panels are representative of a minimum of three experiments.

Therefore, membrane localization of Dlgh1 likely depends on its associations with other proteins. To determine whether the LckSH3–Dlgh1 interaction functions in subcellular localization of Dlgh1, we analyzed the membrane microdomain partitioning of Dlgh1 in BI-141 cells expressing active (F505) or SH3-impaired (AF/F505) Lck (11). Dlgh1 and Lck interactions are abolished in the AF/F505 cells, corroborating a role for LckSH3 in mediating Dlgh1–Lck interaction in intact T cells (Fig. 2 C). Furthermore, Dlgh1 targeting to lipid rafts is impaired in AF/F505 SH3 mutant–expressing T cells, both before and in response to TCR/CD28 costimulation (Fig. 2 D). Taken together, these findings elucidate a role for the LckSH3 domain in Dlgh1 T cell lipid raft membrane association.

Dlgh1 complexes with WASp, Lck, and the activated form of Zap70

In T cells, Dlgh1 has been reported to associate with several transducers implicated in T cell signal transduction and cytoskeletal anchoring and remodeling (20–23). Furthermore, Dlgh1 is self-oligomerizing, a feature proposed to enhance its activity as a scaffolding protein that coordinates events at the cellular membrane. Therefore, we investigated a potential role for Dlgh1 as a molecular scaffold involved in orchestrating immune synapse assembly and signal transduction.

The signal transducers Lck, Lat, Zap70, and WASp are all required for T cell polarization and activation. In addition, each of these proteins localizes within the immune synapse, is regulated by protein tyrosine kinase activity in response to TCR engagement, and has been implicated in synaptic cytoskeletal reorganization (11, 17, 29–31). Therefore, we probed Dlgh1 immunoprecipitates from resting or TCR/CD28-costimulated T cells for the presence of phosphorylated proteins in general and Lck, Lat, Zap70, and WASp in particular. We found Dlgh1 immunoprecipitates containing WASp, Lck, and Zap70 in both transformed and primary T cell populations (Fig. 3, A–C). Although Dlgh1 association with Lck and WASp appears to be constitutive in BI-141 T hybridoma cells, Zap70 association is increased in response to CD3/CD28 costimulation. Dlgh1 complexes containing Lck, WASp, and Zap70 are also found in Dlgh1 precipitates from primary T cells; however, in this instance, association with Lck also appears to be induced by CD3/CD28 costimulation (Fig. 3, A and C). Reverse immunoprecipitation experiments using antibodies directed against Lck, WASp, and Zap70 indicate that these proteins coexist in a molecular complex (Fig. 3 B). Furthermore, GST fusion proteins containing full-length Dlgh1, but not GST alone, specifically precipitated WASp, further substantiating WASp as a Dlgh1 ligand (Fig. 3 D).
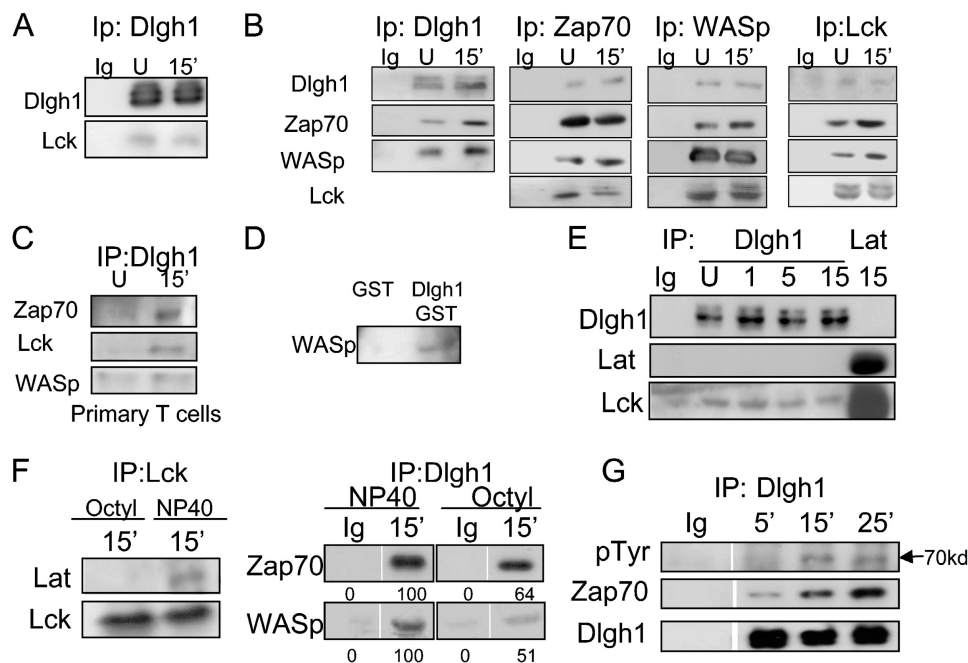

Figure 3.   Dlgh1 complexes with WASp and Lck and the activated form of Zap70, but not Lat. (A and B) Lysates were collected from BI-141 cells stimulated with anti-CD3/CD28 for indicated times and immunoprecipitated and immunoblotted with indicated antibodies. Appropriate isotype controls (Ig) were incubated with lysates from cells stimulated for 15 min. (C) Lymphocytes isolated from the spleen and lymph nodes of an OT-1 TCR transgenic RAG$^{-/-}$ mouse were stimulated with anti-CD3/CD28 for 15 min. TNE lysates were then immunoprecipitated with a Dlgh1 antibody and immunoblotted with indicated antibodies. (D) GST alone or full-length Dlgh1 fused to GST was incubated with BI-141 cell lysates, and complexes were immunoblotted with anti-WASp. (E) BI-141 cells were stimulated with anti-CD3/CD28, and TNE lysates were immunoprecipitated with anti-Dlgh1or anti-Lat and immunoblotted with indicated antibodies. (F) BI-141 T cells were stimulated with anti-CD3/CD28 and lysed in either TNE NP-40 or octylglucoside and immunoprecipitated with either Dlgh1or Lck antibodies and immunoblotted with indicated antibodies. Blots were quantitated and Ig bands were normalized to background and loading controls (numbers below blots). (G) BI-141 cells stimulated with CD3/CD28 were immunoprecipitated with anti-Dlgh1 and immunoblotted with anti–phospho-tyrosine (4G10), anti-Zap70, or Dlgh1 as indicated.

Raft resident Lat does not associate with Dlgh1 under these same conditions, indicating that Dlgh1, Lck, WASp, and Zap70 associations do not simply reflect colocalization in rafts (Fig. 3 E). In keeping with this suggestion, Zap70 and WASp associations with Dlgh1 persist to some extent in the presence of raft-solubilizing octylglucoside (Fig. 3 F). That these associations are partially diminished in the presence of octylglucoside may indicate that coassociation within lipid rafts facilitates formation of Dlgh1 complexes. Lipid raft–dependent association between Lck and Lat is completely disrupted under these same conditions (Fig. 3 F).

Antiphosphotyrosine immunoblot analysis of Dlgh1 immunoprecipitates demonstrates that associated Zap70 is tyrosine phosphorylated (Fig. 3 G), a modification known to be required for efficient Zap70 activation. Maximum Dlgh1–phospho-Zap70 complex formation is observed after 15–25 min of TCR/CD28 costimulation (Fig. 3 G), well after the initial wave of TCR-induced protein tyrosine phosphorylation has peaked.

Dlgh1 functions in positioning Zap70 and WASp within molecular complexes with Lck

In other cell types, Dlgh1 functions in the assembly and organization of signal transducers within polarized membrane specializations by acting as a molecular scaffold (24). Therefore, Dlgh1 might similarly act as a scaffold for coordinating Lck, Zap70, and WASp interactions important for T cell signal transduction, immune synapse assembly, and effector function. To test this possibility, we used an siRNA approach to reduce expression of Dlgh1 in BI-141 cells before stimulating them with CD3/CD28. Significant Dlgh1 knockdown (55%) was confirmed at the RNA (Fig. 4 C) and protein level (Fig. 4 C, inset). We found that Lck–WASp and Lck–Zap70 complex formation is impaired in cells where Dlgh1 expression levels are compromised (Fig. 4

A). Indeed, quantitation of the degree of Lck–Wasp and Lck–Zap70 association in multiple experiments demonstrates a significant and reproducible impairment in Dlgh1-compromised cells (Fig. 4 B). Taken together, these data point to a role for Dlgh1 in functioning as a molecular scaffold to organize signaling complexes at the synapse.

Dlgh1 facilitates actin polymerization and synaptic TCR and lipid raft aggregation

LckSH3 and WASp have been implicated in T cell synaptic raft clustering and IL-2 production (11, 17, 30, 32). Furthermore, previous studies have established roles for Lck and Zap70 family members in regulating WASp activation. Here, we demonstrate that the LckSH3 ligand, Dlgh1, facilitates Lck association with Zap70 and WASp. These findings point to a potential role for Dlgh1 in orchestrating actin-driven synaptic raft clustering and immune synaptogenesis.

Therefore, to assess the role of Dlgh1 in actin-driven synapse assembly, we used siRNA-mediated knockdown in CD8^{+} OT-1 T lymphocytes. Because activated T cells have been reported to be optimal targets for siRNA knockdown (33), we chose to stimulate and expand naive OT-1 T cells in vitro and examine synapse formation 6.5 d later in response to secondary challenge with specific antigen/APC. Because siRNA was added on day 5, at a time when the cultures no longer contain APCs, we avoided potential complications due to "knocking down" Dlgh1 in the stimulating APCs.

Great care was taken in validating the siRNA system for specificity. Two siRNA oligos (Dlgh1 siRNA 1 and 2) targeted at different portions of the Dlgh1 transcript were used to show that Dlgh1 expression is specifically reduced by as much as 71% at both the mRNA and protein levels (Fig. 5, A–C). In addition, three independent control siRNA oligos were used (Figs. 5 B and 6 C and not depicted), including one that only differs from the active Dlgh1 siRNA by two nucle-
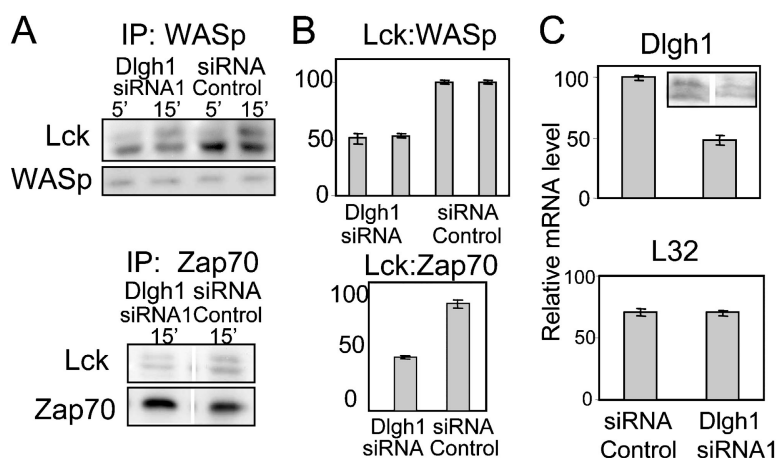
Figure 4. Dlgh1 functions in positioning Zap70 and WASp within molecular complexes with Lck. (A) BI-141 lysates were transfected with Dlgh1 siRNA or control siRNA. 36 h after transfection, cells were stimulated with CD3/CD28 and immunoprecipitated and immunoblotted with indicated antibodies. (B) Quantitation of panels represented in A using Scion Imaging software. Graphs represent Lck levels normalized to either WASp or Zap70 and are representative of at least two experiments. (C) Lysate and RNA was collected from BI-141 cells 36 h after transfection with Dlgh1 siRNA (Dlgh1 siRNA1) or control siRNA. Dlgh1 mRNA levels were determined and normalized to L32 levels. Dlgh1 protein levels are shown as an inset in the top panel with siRNA control on the left and Dlgh1 siRNA on the right.
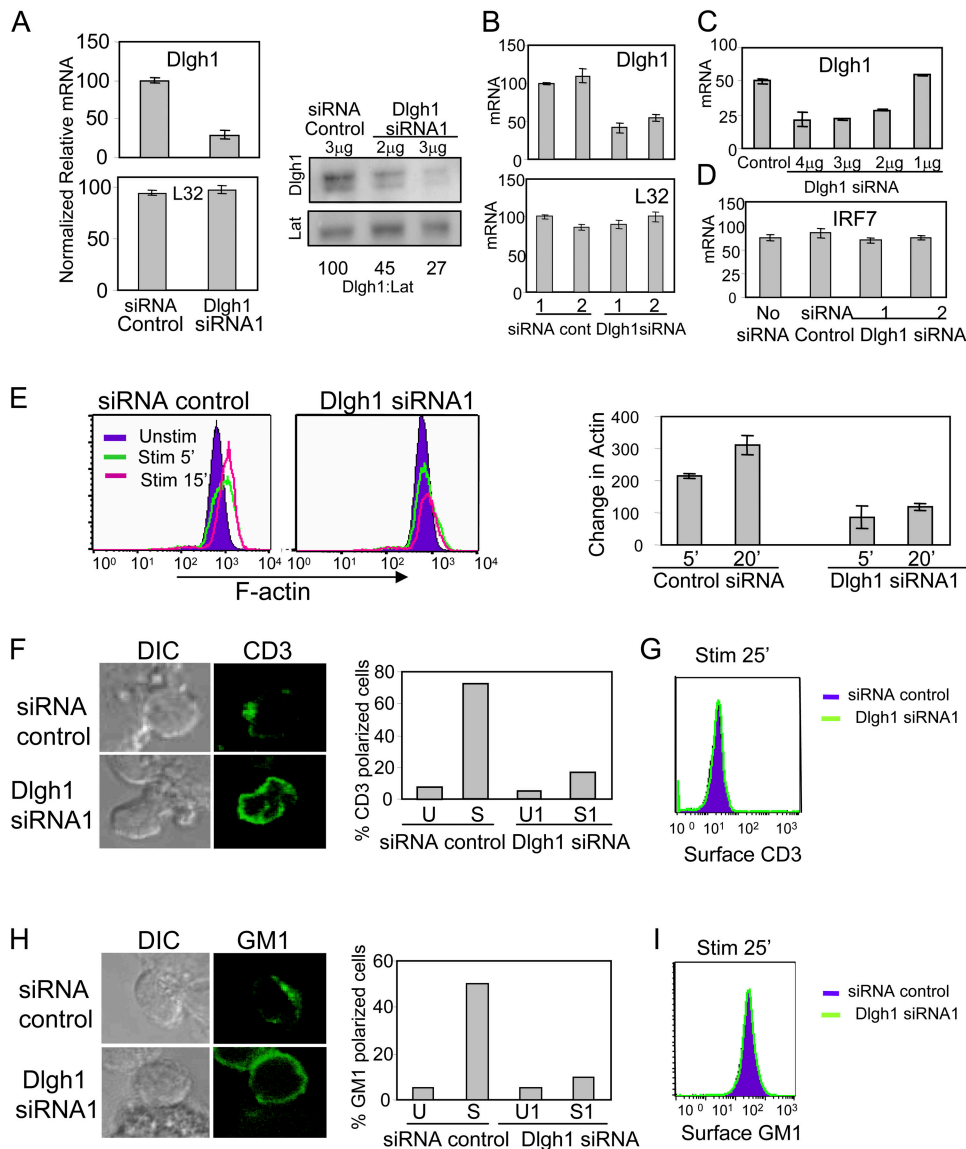
Figure 5. Dlgh1 expression is required for antigen–induced actin polymerization and synaptic TCR and lipid raft clustering. (A and B) OT-1 T cells were stimulated for 5 d before transfection with either of two siRNA oligos directed against Dlgh1 (Dlgh1 1 or Dlgh1 2) or siRNA controls (siRNA control 1 and 2). 36 h after transfection, Dlgh1 and L32 mRNA and protein levels were quantitated or immunoblotted for Dlgh1 and Lat. Dlgh1 protein levels were quantitated by densitometry and normalized to Lat. (C) Experimental conditions were as in A. Control oligos showed no variation in Dlgh1 expression when titrated in a similar manner (not depicted). Dlgh1 mRNA levels were normalized to L32. (D) OT-1 T cells were treated as in A, and IRF7 mRNA levels were quantitated. (E) OT-1 T cells were stimulated, expanded, and transfected and spun onto APCs for 5 or 15 min to stimulate actin polymerization. T cells were washed off of APCs, fixed, and stained with FITC-conjugated phalloidin and analyzed using FACS. Mean intensities from three experiments were averaged and change in actin was calculated (stimulated mean intensity − unstimulated mean intensity). (F–I) OT-1 T cells were transfected as in A and incubated for 25 min on slides with antigen/APCs. Cells were washed and stained with either FITC-CD3 or FITC-labeled CTX for microscopy. Graphs represent clustering in 100 T cell–APC conjugates analyzed, and data are representative of at least five experiments. U, unstimulated cells; S, stimulated; U1, unstimulated, treated with Dlgh1 siRNA1; S1, stimulated and treated with Dlgh1 1 siRNA1. 71% Dlgh1 knockdown was obtained in experiments shown in A, F, and H, whereas 58% knockdown was obtained in experiments shown in E, G, and I.

otide substitutions (control 2). Specific Dlgh1 knockdown was assayed in mRNA collected from T cells on day 6.5 of culture using quantitative PCR. As shown in Fig. 5, Dlgh1 mRNA and protein expression was specifically impaired, whereas L32 (housekeeping) mRNA levels remained constant between samples. A similar degree of Dlgh1 knockdown was observed at the level of both RNA and protein in multiple experiments and ranged from 50–75%. Titration of Dlgh1 siRNA oligos titrates Dlgh1-specific knockdown at both the protein and mRNA level (Fig. 5, A and C). In addition, siRNA treatment does not induce IFN-β response, as indicated by the lack of induction of IFN regulatory factor (IRF)7
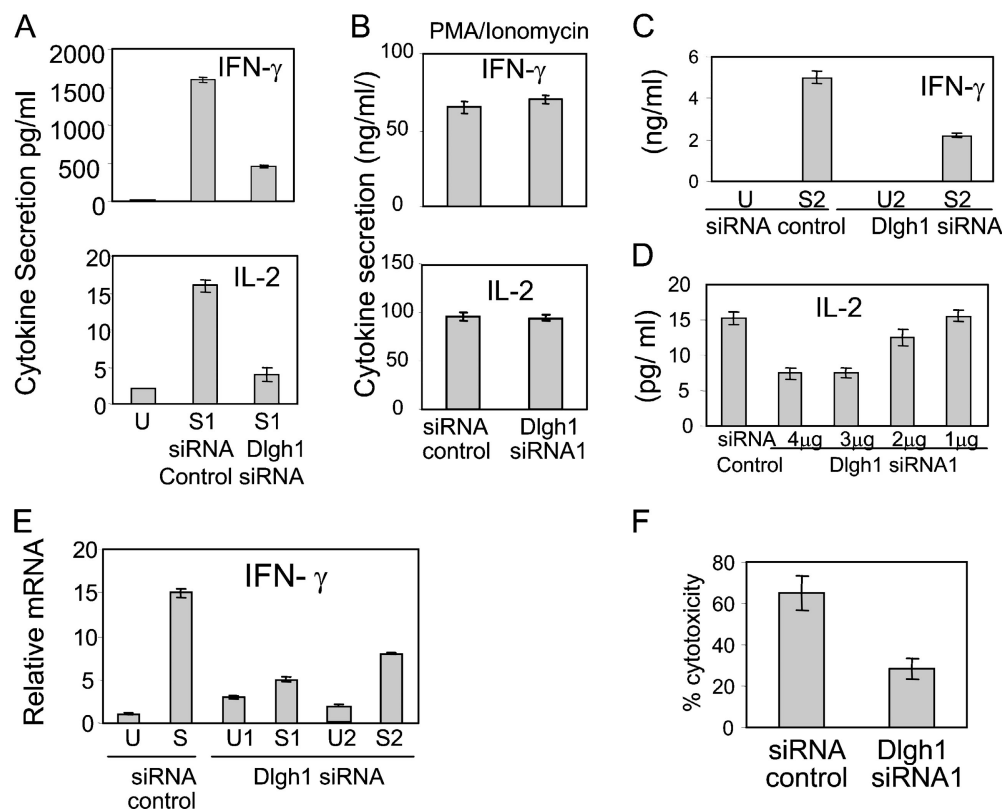
Figure 6. **Loss of Dlgh1 expression leads to impaired antigen–induced cytokine production and cell–mediated cytotoxicity, but continued responsiveness to PMA/ionomycin.** (A) OT-1 T cells transfected with indicated siRNA were restimulated 36 h after transfection for 24 h with antigen/APCs. Supernatants were assayed for IFN-γ or IL-2 using ELISA. Data are representative of at least five experiments. (B) OT-1 T cells were treated as in A, but stimulated with 5 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 24 h. (C) OT-1 T cells were transfected as in A. S1 and S2 refer to independent oligos used for trans-fection. U, unstimulated; S, stimulation with APC/antigen. Supernatants were assayed for IFN-γ using ELISA. (D) OT-1 T cells were transfected with varying amounts of Dlgh1 siRNA for 24 h and restimulated for 24 h. Super-natants were analyzed by ELISA. (E) OT-1 T cells were transfected and stimulated as in A, and IFN-γ levels were determined. (F) OT-1 T cells transfected with indicated siRNA were stimulated with antigen/APCs 36 h after transfection, and percent cytotoxicity was determined. U, unstimu-lated; S, stimulated. 1 and 2 refer to the siRNA oligo used. These data are representative of at least two experiments.

and ISG15 IFN-β response genes (Fig. 5 D and not depicted; reference 34), nor does it effect cell viability (not depicted).

WASp deficiency has been reported to both impair actin polymerization and lipid raft clustering (17). To assess a role for Dlgh1 in orchestrating antigen/APC–induced actin poly-merization, we compared OT–1 T cells treated with control or Dlgh1-specific siRNA for their ability to polymerize actin in response to antigen/APC by quantitating polymerized fil-amentous actin (F-actin). As shown in Fig. 5 E, F-actin lev-els in unstimulated cells are similar in cells treated with siRNA control and Dlgh1 siRNA. Consistent with the liter-ature (15), F-actin levels in OT–1 T cells modestly increase upon antigenic stimulation (Fig. 5 E). Conversely, T cells with diminished Dlgh1 expression do not efficiently increase F-actin content in response to stimulation with antigen/APC (Fig. 5 E). These findings are consistent with a poten-tial role for Dlgh1 in coordinating antigen/APC–induced WASp-mediated actin polymerization.

To determine whether Dlgh1 expression affects synapse assembly, synaptic lipid raft (GM1) and CD3 clustering in re-sponse to specific antigen/APC stimulation were measured.

As shown in Fig. 5, F and H, and consistent with what we observe with freshly isolated OT-1 T cells, activated OT-1 effectors localize CD3 and lipid rafts at the APC–T cell inter-face. Conversely, in Dlgh1 knockdown cells, both CD3 clus-tering and synaptic raft polarization are impaired, whereas surface levels of both CD3 and GM1 remain similar between control and knockdown cells (Fig. 5, G and I).

Dlgh1 is necessary for IFN–γ and IL–2 production and CTL activity

To determine what, if any, effects Dlgh1 knockdown has on CD8+ T cell function, we examined the effects of diminish-ing Dlgh1 levels on induced IFN-γ, IL-2 production, and CTL activity. Treatment of cells with either of two siRNA pairs specific for Dlgh1, but not control oligos, impairs anti-gen-induced IFN-γ and IL-2 production and CTL activity (Fig. 6). Lymphokine production is diminished to the same extent as Dlgh1 expression (70%) by treatment with Dlgh1 siRNA, suggesting a requirement for Dlgh1 in T cell activa-tion. Consistently, affects on cytokine secretion can be ti-trated with decreasing amounts of Dlgh1-specific siRNA

(Fig. 6 D), but not control siRNA (not depicted). However, Dlgh1-impaired T cells retain the ability to produce IFN-γ and IL-2 in response to PMA and ionomycin (Fig. 6 B). These findings indicate that T cells lacking appropriate Dlgh1 expression are capable of producing effector cytokines, but are deficient in coupling antigenic stimulation with signal transduction machinery required for cytokine production. Because PMA and ionomycin are know to mimic T cell activation pharmacologically by directly inducing protein kinase C and Ras activation and release of intracellular Ca^{2+} stores, Dlgh1 likely functions in positively regulating TCR signal transduction upstream of these pathways.

Dlgh1 has been implicated in protein and vesicular trafficking (23, 26). Therefore, defective cytokine production could result from defective polarized TCR-induced cytokine delivery and secretion or defective gene transcription. To distinguish between these we quantitated mRNA expression of IFN-γ. Fig. 6 E shows that siRNA-mediated diminution of Dlgh1 expression affects TCR-induced lymphokine mRNA expression, providing support for a model in which Dlgh1 functions to positively regulate TCR signal transduction required for lymphokine gene expression. However, these data do not rule out an additional role for Dlgh1 in polarized T cell effector molecule delivery.

Overexpression of Dlgh1 enhances TCR–induced NFAT activity and cytokine production

Our results obtained using siRNA implicate Dlgh1 as a positive regulator of cytokine transcription and secretion (Fig. 6). To further investigate this possibility, we used an alternate approach of assessing the effects of overexpressing Dlgh1 in BI-141 hybridoma or primary OT-1 T cells. One mechanism by which Dlgh1 might regulate cytokine transcription could involve enhanced NFAT activity, as WASp, Lck, and Zap70 have each been implicated in NFAT activation (14, 35). Therefore, we overexpressed a full-length Dlgh1 expression plasmid along with an NFAT reporter construct. We found that transfection of modest (0.5 and 1 μg) amounts of Dlgh1 expression plasmid enhances NFAT activity in both BI-141 T cells and 293 T cells (Fig. 7 A). Because gross overexpression of a scaffolding molecule is predicted to have similar effects as too little of the scaffold, we varied the degree of Dlgh1 overexpression. Indeed, in situations where a scaffold functions to bridge signal transduction molecules, an overabundance of the scaffold is predicted to facilitate monovalent binding of a single partner, rather than a bridging of two partners. Although transfection of relatively lower levels (0.5 and 1 μg) of Dlgh1 expression vector facilitates TCR/CD28-mediated NFAT activity, relatively higher levels (5 and 10 μg) are unable to up–regulate NFAT activity (Fig. 7 B).

To further compliment and validate our siRNA studies, we overexpressed Dlgh1 in primary OT-1 T cells and measured the effects on cytokine production. The transfection of NFAT-activating concentrations of Dlgh1 expression vector in primary OT-1 T lymphocytes increases IL-2 and IFN-γ production in response to antigen (Fig. 7 C). These results corroborate our findings that loss of Dlgh1 expression results in decreased cytokine production (Fig. 6) and assigns a positive regulatory role for Dlgh1 in facilitating TCR signal transduction and T cell function.

DISCUSSION

Recently, parallels between molecular mechanisms involved in orchestrating cell polarity and signal transduction within
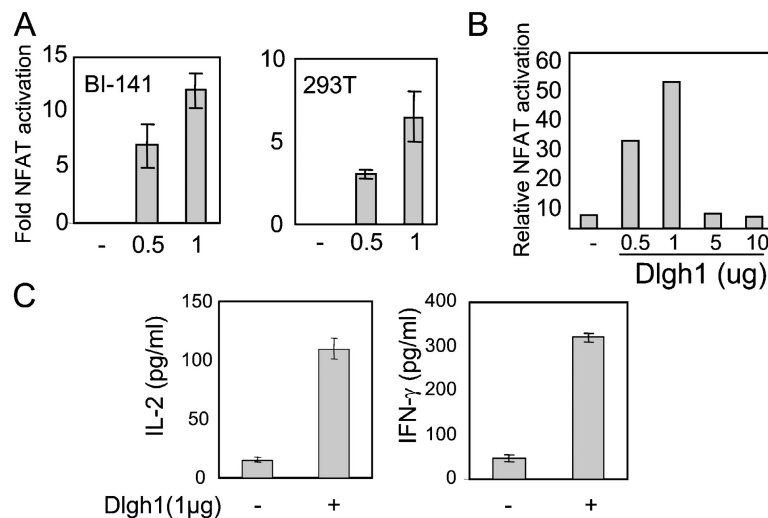

Figure 7. Overexpression of Dlgh1 leads to increased NFAT activity and cytokine production. (A and B) BI-141 T cells or 293 T cells were transfected with indicated amounts of pSport-Dlgh1 vector (0.5–10 μg), pNFAT-TA-Luc reporter, and PRLSV40 Renilla luciferase. Transfected cells were incubated overnight and stimulated with anti-CD3/CD28 overnight. Cell lysates were assayed using the Dual Luciferase Reporter Assay System. Renilla luciferase was used to ensure equal transfection efficiency. (C) Lymphocytes from the spleen and lymph nodes from an OT-1 TCR transgenic mouse were stimulated for 3 d with APC/peptide. Cells were then transferred to 200 U/ml IL-2 media for an additional 2 d. CD8 T cells were then transfected with 1 μg pSport-Dlgh1 vector and cultured in fresh media containing IL-2. 24 h after transfection, cells were washed and restimulated with APC/peptide overnight, and secreted IL-2 and IFN-γ was quantitated using ELISA.

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lymphoid, epithelial, and neuronal synapses have begun to emerge (36–39). In all three cellular systems, lipid raft membrane compartmentalization and MAGUK family members have been implicated in protein and vesicular trafficking, the establishment of cell polarity, and the organization of membrane specializations for optimal signal transduction (24, 39). MAGUKs have been well studied in both neuronal and epithelial cells; however, only recently have they been considered with regard to T cell biology. Several recent studies have identified a role for the lymphocyte-specific MAGUK family member CARMA as an essential scaffold for the activation and lipid raft localization of transducers required for TCR/costimulator-mediated activation of NF-κB (40–42). Here, we establish a role for the MAGUK family member Dlgh1 as a crucial organizer of T cell polarity, actin polymerization, NFAT activity, and cytokine production. Unlike CARMA, Dlgh1 is expressed in multiple tissues and thus, our findings highlight common molecular strategies used for synaptic organization in epithelial, neuronal, and lymphoid systems. Findings that Dlgh1 associates with lymphocyte-specific regulators of TCR signal transduction and actin polymerization illustrate how cells specialize common molecular mechanisms for cell-specific application.

Recent reports have shown that hDLG/Sap97 localizes to epithelial junctions and neuronal synapses (43–45). Here, we show that antigenic stimulation induces a portion of Dlgh1 transport coincident with the TCR- and GM1-containing lipid rafts to the synaptic contact in both freshly isolated and in vitro–expanded stimulated CD8$^+$ OT-1 TCR transgenic T cells (Fig. 1, A and B, and not depicted). Dlgh1 synaptic relocalization requires TCR engagement because APCs lacking expression of MHC/peptide do not induce this translocation. These findings are in keeping with previous reports in which Dlgh1 has been shown to relocalize to the stimulatory contact site of CD3/CD28, CD2, or superantigen-stimulated Jurkat cells or antigen-stimulated, primary in vitro–expanded CD4$^+$ DO11.10 TCR transgenic T cells (22, 23). However, unlike those reports, in which Dlgh1 accumulation was observed readily at 5 min and reported to be transient (absent by 15–25 min after stimulation), we observe a relatively delayed kinetic in CD8$^+$ OT-1 T cells, wherein Dlgh1 accumulation is not apparent at 5 min but can be readily observed by 20–25 min. Different observed recruitment kinetics may reflect differences in stimulatory conditions, antigen affinity, T cell developmental stage (CD4 vs. CD8), and/or degree of transformation. Consistent with our findings, biochemical fractionation reveals that a subset of Dlgh1 resides in the lipid raft compartment and increases its raft partitioning in response to TCR stimulation over the same time frame.

By mapping the Lck–Dlgh1 interaction site to the SH3 domain of Lck, we confirm and extend previous reports identifying Dlgh1–Lck complexes. Furthermore, we demonstrate a role for the Dlgh1–Lck SH3 interaction in Dlgh1 raft membrane association. We also identify WASp and Zap70 as novel constituents of Dlgh1 molecular complexes and demonstrate that Dlgh1-associated Zap70 becomes tyrosine phosphorylated in response to TCR/CD28 costimulation.

In keeping with suggestions that Dlgh1 functions as a scaffold-enabling juxtaposition and activation of associated transducers, reduction of Dlgh1 expression levels using siRNA interferes with TCR/CD28-mediated Lck–Zap70 and Lck–WASp complex formation. Furthermore, we demonstrate a requirement for Dlgh1 in antigen-induced actin polymerization, synaptic lipid raft and TCR clustering, NFAT activity, and lymphokine production. Each of these functions is known to require WASp activity and is defective in mice and humans lacking expression of functional WASp (13, 14). Similarly, each of these activities requires Lck tyrosine kinase and or/SH3 binding activity (1, 3, 11, 46, 47). Furthermore, Vav, a known WASp activator, has been recently identified in Dlgh1 immunoprecipitates, although the molecular basis and functional significance of this association has yet to be elucidated (22). Additionally, Dlgh1 was shown to become associated with T cell membrane actin in response to activation (22). Our findings that modest Dlgh1 overexpression enhances TCR NFAT activity and T cell function are consistent with our findings that Dlgh1 diminution impairs actin reorganization and T cell function. Experiments demonstrating that high levels of Dlgh1 overexpression are unable to enhance NFAT activity may explain a recent contradictory report in which Dlgh1 overexpression is shown to modestly inhibit NFAT activity in Jurkat cells (22). Alternatively, differences may reflect T cell subset–specific roles for Dlgh1 in T cells. Given that the functional outcome of Dlgh1 overexpression was not assessed in that report, it is difficult to fully account for potential differences observed (22). In toto, the available data provide support for a potential role for Dlgh1 in coordinating Lck-induced, WASp-mediated actin polymerization required for synaptic lipid raft clustering and T cell activation.

Although it remains unclear how Dlgh1 influences WASp actin polymerizing activity, several testable possibilities exist. Indeed, WASp–Dlgh1 interactions could function to (a) mediate WASp synaptic colocalization with its activators, (b) relieve WASp auto-inhibitory interactions, or (c) induce WASp tyrosine phosphorylation. Lck phosphorylation of WASp Y291 is known to increase basal WASp actin polymerizing activity and enable SH2-induced WASp activation (16). Previous studies elucidating mechanisms of WASp regulation provide support for each of these potential modes of Dlgh1 regulation of WASp activity (14, 16, 48–50). Future studies characterizing the molecular basis of Lck–Dlgh1–Zap70–WASp interactions and activation should aid in testing these possibilities.

Despite progress in identifying early intermediates involved in TCR signal transduction, how these signal transducers are translated into processive TCR signals required for T cell activation and lymphokine gene expression remain incompletely understood. Recent data indicate that the early peak in tyrosine kinase signaling precedes central clustering of the TCR within the synaptic contact in CD4 T cells (51). Therefore, TCR molecular patterning within the synapse

cannot be responsible for initiating or amplifying this early wave of protein tyrosine phosphorylation. Nonetheless, continued TCR engagement and signal transduction extending well beyond the time frame of initial synapse assembly are essential for T cell commitment to activation and lymphokine gene expression (1, 10, 11, 46, 52). Therefore, reorganization of the actin cytoskeleton and lipid rafts within the synaptic contact remain among the few known physical correlates of processive TCR signaling (3, 11, 17, 19, 30, 53–55). In addition to the organization afforded through membrane partitioning accompanying raft clustering, a synaptic actin cytoskeletal scaffold may also function in transducer concentration, complex formation, and costimulatory or adhesion molecule signaling (11, 19, 39).

Recent evidence has alluded to a role for Dlgh1 in LFA1 clustering and transport through association of Dlgh1 with PTA1 and FERM member 4.1 (21). Furthermore, LFA1 adhesiveness depends on lipid raft clustering and actin reorganization and is impaired in cells expressing Lck SH3 mutants (9, 56). We have yet to determine if the described Dlgh1 contributions to T cell function are through impacting LFA1 activation.

Modest Dlgh1 overexpression enhances both NFAT activation and lymphokine production, whereas loss of Dlgh1 interferes with both synapse formation and effector function. Furthermore, previous studies have implicated both Zap70 and WASp in NFAT activity. Therefore, it is possible that the effects on synapse formation reflect a requirement for synaptic organization in processive signaling.

Alternatively, although Dlgh1 coordinates both synapse assembly and lymphokine gene expression, it is possible that these are parallel events. Indeed, Dlgh1 contributions to synapse assembly might be more relevant to polarized delivery of effector molecules and down-regulation of TCR signals, whereas its contributions to lymphokine gene expression could rely on signals generated by Dlgh1 scaffolding activity independent of synaptic raft and/or actin remodeling. Further elucidation of molecular mechanisms by which Dlgh1 impacts TCR signal transduction and synapse formation should aid in understanding the pivotal role Dlgh1 plays in coordinating TCR engagement with functional outcome.

MATERIALS AND METHODS

T cell stimulation and transfections. SigOVA$_{257-264}$ MEC.B7.1 APC stably expressing H2K^b, B7.1, and OVAp (257–264) or M1B cells that lack H2K^b and peptide but retain B7.1 were used for stimulating T cells from OT-1 TCR transgenic mice (57). OT-1 and OT-1 RAG$^{-/-}$ mice were used at 8–12 wk of age. 2×10^5 APCs (final volume of 1 mL) were placed in either a 24-well plate or a chamber slide in media containing 20 µg/ml IFN-γ for 48 h before stimulation to up-regulate MHC levels and washed with PBS. The spleen and lymph nodes from OT-1 transgenic mice and 2×10^6 cells were either stimulated for 25 min in chamber slides for analysis of unprimed populations or expanded for 3 d with antigen/APC in 24-well plates to be used for siRNA experiments.

For transient transfections, T cells were removed from antigen APCs after 3 d and transferred to a new 24-well plate containing supplemented RPMI media containing 200 U/ml IL-2 for an additional 2 d. 2×10^6 T cells were then transfected with 3 µg of double-stranded RNA oligos

(QIAGEN) against one of the following target sequences: Dlgh1 1: TACGGGAGCAGATGATGAAA; Dlgh1 2: AACCCAAATCCATG-GAAAATA or siRNA control 1: AATTCTCCGAACGTGTCACGT or control 2: TACGGGAGC**GT**ATGATGAATA; and control 3: TTGT-GAAAATCCGTCCCCGATAA) or indicated quantities (0.5–10 µg) of pSPORT-Dlgh1 full-length plasmid (American Type Culture Collection) using TransMessenger transfection reagent (QIAGEN) according to the manufacturer's instructions. T lymphocytes were resuspended in unsupplemented media while 3 µg RNA or indicated quantities of Dlg plasmid, 6.4 µl of enhancer R reagent, and 83.6 µl ECR reagent (final volume of 100 µl per 2×10^6 cells) were vortexed and incubated at room temperature for 5 min. 10 µl TransMessenger reagent was added to this mixture and incubated for 10 min. Complexes were added to the T cells and cultured for 4 h. Cells were placed in media with 200 U/ml IL-2 for an additional 36 h. Cells were restimulated with antigen/APCs and assayed for synapse formation or cytokine production. BI-141 cells were transfected similarly.

Confocal microscopy. Chamber slides (Nalge) were coated with 20 ug/ml poly-L-lysine (Sigma-Aldrich) for 15 min at 37°C before the addition of 2×10^5 APCs or control M1B APCs (57) in media with IFN-γ for 48 h. 2×10^6 naive or cells treated with siRNA Dlgh1 or control siRNA were placed in chamber slides, briefly spun, and cocultured at 37°C for 5 or 25 min. Stimulation was stopped with 4% formaldehyde and cells were permeabilized with 0.3% Triton X-100 for 20 min at 37°C. Cells were blocked and then stained with 8 ug/ml FITC-conjugated Cholera Toxin β subunit (C-1655; Sigma-Aldrich), 5 ug/ml anti-Dlg antibody (610874; BD Trans Labs), or mouse IgG$_1$ isotype control (sc-2025; Santa Cruz Biotechnology, Inc.) followed by a 1:300 dilution of Texas red–conjugated anti–mouse IgG secondary antibody (715-075-150; Jackson ImmunoResearch Laboratories). TCR was stained using FITC-conjugated anti-Vβ5.1,5.2 (553189; BD Biosciences). Cells were washed twice with PBS and mounted with Prolong Antifade Reagent (P7481; Molecular Probes). All images represent single sections taken using a Leica TCS-SP spectral confocal inverted microscope equipped with argon (488 nm blue excitation) and krypton (568 nm yellow excitation) lasers (Leica).

Raft fractionations, immunoprecipitations, and GST pull-downs. 4×10^7 BI-141 cells were left unstimulated or stimulated as described previously (11) and lysed on ice for 30 min in TNE buffer (50 mm Tris, pH 6.8, 1% NP-40, and 20 mM EDTA) containing leupeptin, apropotin, PMSF, and sodium vanadate inhibitors. Lysates were either subjected to sucrose density centrifugation as described previously (11) or precleared by centrifugation at 10,000 *g* for 10 min at 4°C and then incubated with 20 µl of protein G–coated beads alone for 1 h at 4°C. Lysates were then immunoprecipitated with 2 µg of indicated antibodies overnight at 4°C. The following antibodies were used: anti-Zap70 (610239; BD Trans Labs), anti-WASp (sc-13139; Santa Cruz Biotechnology, Inc.), anti–phospho-tyrosine (4G10), and anti-Lck (11). For solubilization of lipid rafts, 60 mM octylglucoside was used instead of TNE buffer. Blots were quantitated using Scion Image software and normalized to indicated loading controls.

Lck and other GST fusions have been described (11, 31). Full-length Dlgh1 was amplified by PCR using primers F′ GAATTCATGCCGGT-CCGGAAGCAAG and R′ GTCGACCGTGGTCAAGAAGAATATG. Dlg was placed into pGEX4T1 vector (Promega) using ECORI and SalI sites. GST fusions were purified according to the manufacturer's instructions. Equal amounts of GST fusions bound to glutathione beads were incubated with BI-141 lysates for 4 h at 4°C.

Cytoxicity assay. Cell-mediated cytotoxicity was determined using the Cytoscan-LDH Cytotoxicity Assay Kit (Genotech) according to the manufacturer's instructions. In brief, T lymphocytes transfected with Dlgh1 siRNA or control siRNA were incubated with 2×10^3 antigen APCs at an effector to target ratio of 15:1 for 4 h. Plates were briefly spun and supernatants were transferred to a new 96-well plate. Substrate mix was added and plates were incubated at room temperature for 30 min. Stop solution was

added and absorbance at 490 nm was recorded. Percent cytotoxicity is calculated as: (experimental − effector spontaneous–target spontaneous release/target maximum–target spontaneous release) × 100.

Quantitative PCR. RNA was collected from T cells 36 h after transfection using TRIzol reagent. Primers used for quantitation of Dlgh1 and IFN-γ are as follows: Dlgh1: F 5′-3′ GCAGGCCAGAAAAACA-TTTGAG R 5′-3′ TCTCCCTGGACAATAGCTGTGAA and IFN-γ: F 5′-3′ GTCAACAACCCACAGGTCCAG R 5′-3′ CCTTTTCCGCT-TCCTGAGG. ISG15 and IRF7 are IFN-β target genes, and primers have been described (34). Quantitative PCR was performed as described previously (34).

FACS: actin polymerization assays, CD3, and GM1. 36 h after transfection with Dlgh1 siRNA or control siRNA, lymphocytes were briefly spun onto SigOVA$_{257–264}$ • MEC.B7.1 APCs. Lymphocytes were gently washed off and either stained with 8 ug/ml FITC-conjugated cholera toxin or CD3 and then fixed in 2% formaldehyde. Alternatively, cells were immediately fixed and permeabilized with 200 μl per sample of BD Cytofix/Cytoperm (51-2090KZ; BD Biosciences) overnight at 4°C for actin polymerization assays. Cells were washed in wash buffer (1× PBS, 3% FCS, and 0.1% sodium azide) and stained for 1 h with 5 μg/ml FITC-conjugated phalloidin (P-5282; Sigma-Aldrich), washed, and analyzed using the FACSCalibur (Becton Dickinson).

Luciferase assays. BI-141 T lymphocytes were electroporated (300 mV, 960 mFd) with indicated quantities of pSPORT-Dlgh1 plasmid, 200 ng NFAT reporter plasmid (provided by K. Siminovitch, University of Toronto, Toronto, Canada), 500 ng pRLSV40 plasmid (Promega) for normalization, and pCDNA3 (to ensure equal DNA content). 24 h after transfection, cells were stimulated with anti-CD3/CD28 overnight. Cells were lysed using Passive Lysis Buffer and 50 μl lysates was used for the Dual Luciferase Assay System according to the manufacture's instructions (Promega).

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