GAKIN, a Novel Kinesin-like Protein Associates with the Human Homologue of the Drosophila Discs Large Tumor Suppressor in T Lymphocytes

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Reorganization of the cortical cytoskeleton is a hallmark of T lymphocyte activation. Upon binding to antigen presenting cells, the T cells rapidly undergo cytoskeletal re-organization thus forming a cap at the cell-cell contact site leading to receptor clustering, protein segregation, and cellular polarization. Previously, we reported cloning of the human lymphocyte homologue of the Drosophila Discs Large tumor suppressor protein (hDlg). Here we show that a novel protein termed GAKIN binds to the guanylate kinase-like domain of hDlg. Affinity protein purification, peptide sequencing, and cloning of GAKIN cDNA from Jurkat J77 lymphocytes identified GAKIN as a novel member of the kinesin superfamily of motor proteins. GAKIN mRNA is ubiquitously expressed, and the predicted amino acid sequence shares significant sequence similarity with the Drosophila kinesin-73 motor protein. GAKIN sequence contains a motor domain at the NH2 terminus, a central stalk domain, and a putative microtubule-interacting sequence called the CAP-Gly domain at the COOH terminus. Among the MAGUK superfamily of proteins examined, GAKIN binds to the guanylate kinase-like domain of PSD-95 but not of p55. The hDlg and GAKIN are localized mainly in the cytoplasm of resting T lymphocytes, however, upon CD2 receptor cross-linking the hDlg can translocate to the lymphocyte cap. We propose that the GAKIN-hDlg interaction lays the foundation for a general paradigm of coupling MAGUKs to the microtubule-based cytoskeleton, and that this interaction may be functionally important for the intracellular trafficking of MAGUKs and associated protein complexes in vivo.

Dlg protein encoded by the Drosophila tumor suppressor gene lethal (1)/discs large-1 (dlg) is located at the cytoplasmic surface of septate junctions between epithelial cells. Loss of function mutations ofdlg result in the neoplastic overgrowth of imaginal discs and larval lethality (1). Dlg is also present at the synaptic junctions of neurons and is required for the development of synaptic structure at neuromuscular junctions (2). Dlg is a prototypical member of a growing family of proteins termed membrane-associated guanylate kinase homologues (MAGUKs).1 MAGUKs are peripheral membrane proteins composed of either one or three PDZ domains, a single SH3 domain, and a carboxyl-terminal domain that is homologous to guanylate kinases (3, 4). Growing evidence indicates that MAGUKs function as scaffolding proteins necessary for the assembly and organization of protein complexes at specialized membrane sites (3–5). For example, the p55 MAGUK links actin-spectrin-protein 4.1 complexes to the cytoplasmic face of the erythrocyte plasma membrane (6). The adaptor function of p55 is mediated via the interaction of its single PDZ domain and HOOK domain to the cytoplasmic terminus of glucocorticoid C and the FERM domain of protein 4.1, respectively (7–9). A similar paradigm has been established for other MAGUKs although a detailed understanding of the scaffolding lattice requires isolation and identification of individual components of the MAGUK-associated protein complex in mammalian cells (8–11).

MAGUKs can be broadly classified into several subgroups based on their domain organization: Dlg-like, p55-like, Lin-2 like, and ZO-1-like. Mammalian members of the Dlg subgroup include the PSD-95 (SAP90), Chapsyn-110 (PSD-93), NE-Dlg (SAP102), and hDlg (SAP97) (12–15). A hallmark of MAGUKs is the presence of a guanylate kinase-like domain of ~190 residues (16). Both prokaryotic and eukaryotic guanylate kinases catalyze phosphorylation of GMP to GDP by transferring a phosphate from ATP (16). Initial recognition of the guanylate kinase-like (GUK) domain in MAGUKs raised an interesting possibility that this domain may couple the guanine nucleotide metabolism to the Ras signal transduction pathway (1). However, subsequent studies revealed that the GUK domain of hDlg is enzymatically inactive consistent with the three-amino acid deletion found within its ATP binding A-motif (17, 18). The catalytically inactive GUK domain of SAP97, a rat homologue of hDlg, binds GMP/GDP in vitro but cannot rescue mutations in the yeast guanylate kinase (18). These observations suggest that the GUK domain of the Dlg subfamily of MAGUKs may have evolved to perform a specialized non-enzymatic function in vivo.

1 The abbreviations used are: MAGUKs, membrane-associated guanylate kinase homologues; GUK domain, guanylate kinase-like domain; hDlg, human homologue of discs large protein; GAKIN, guanylate kinase associated kinesin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; aa, amino acid(s); PAGE, polyacrylamide gel electrophoresis; EST, expressed sequence tag; PCR, polymerase chain reaction; kb, kilobase pair(s); RACE, rapid amplification of cDNA ends; mAb, monoclonal antibody.

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The first evidence showing GUK domains as specialized protein recognition modules came from the observation that the GUK domains of PSD-95/SAP90 andDlg/SAP97 associate with a family of proteins termed SAPAPs/GKAP/DAP-1 (19–21). Transfection studies indicate that the GUK domains of the GUK family of human p55 (aa 266–466) was cloned into pGEX2T plasmid and expressed as GST fusion proteins in DH5α (23). Because the GUK domains of MAGUKs as expressed preferentially in the brain, the proteins that bind to the GUK domain of Dlg/SAP97 in non-neuronal cells remain to be identified. It is noteworthy that the GUK domain of PSD-93/Chapsyn-110 binds to the microtubule-associated protein 1A, and the PSD-93/Chapsyn-110 localizes to the dendritic microtubules of cerebellar Purkinje neurons (24). Since the binding affinity of full-length PSD-93 to microtubule-associated protein 1A is relatively weak, it was suggested that the accessibility of the GUK domain might be blocked by its intramolecular interactions. The direct association of Dlg with a ubiquitously expressed kinesin-like motor may reveal new aspects of Dlg function in neuronal as well as non-neuronal cells.

**MATERIALS AND METHODS**

*T Cell Line and Monoclonal Antibody—* Jurkat J77 cell line was maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Sigma), 2.0 mM glutamine (Sigma), 50 units/ml penicillin (Sigma), and 50 μg/ml streptomycin (Sigma). The monoclonal anti-Dlg antibody was produced using NH₂-terminal guanylate kinase domain-associated protein as identified that binds to the GUK domain of PSD-93/SAP97 (26). The physiological function of BEGAIN is not yet known. Together, these studies suggest that the GUK domains of MAGUKs function as specific protein-binding modules.

To define the physiological function of the GUK domain in non-neuronal cells, it is necessary to identify and characterize molecules that specifically associate with the GUK domain of MAGUKs. This information is likely to be critical for understanding the role of MAGUKs in subcellular targeting, assembly of signaling complexes, and the regulation of proliferation pathways. In this paper, we demonstrate that a novel kinesin-like motor protein termed GAKIN (guanylate kinase associated kinesin) binds to the GUK domain of Dlg in T lymphocytes. The direct association of Dlg with a ubiquitously expressed kinesin-like motor may reveal new aspects of Dlg function in neuronal as well as non-neuronal cells.

**Association between MAGUKs and Kinesin-like Motor Protein**

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2 A. H. Chishti, unpublished data.
proteins (nucleotides 1674–3920 of GAKIN cDNA) was used. The probe was radiolabeled using DECAprime II kit (Ambion) and purified using Sephacryl S-200HR column (Amersham Pharmacia Biotech). Hybridization reaction was performed at 65 °C using ExpressHyb Hybridization Solution (CLONTECH Inc.).

Antibody Production—Two polyclonal antibodies were raised against the NH$_2$ and COOH termini of GAKIN. For the NH$_2$-terminal antibody, a GST fusion protein encoding 53–487 amino acids of GAKIN was expressed in bacteria. Inclusion bodies containing GST fusion protein were recovered from bacterial lysate and analyzed by SDS-PAGE. The corresponding protein band was visualized by staining with 4,000 molar solubility and purified from the gel, and used to immunize rabbits. For affinity purification of antibodies, the GST fusion protein was solubilized in 6.0 M urea and renatured by dialyzing in decreasing concentrations of urea. The renatured fusion protein was coupled to CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech). To generate polyclonal antibodies against the COOH terminus of GAKIN, the GST fusion protein encoding 1414–1926 amino acids of GAKIN was expressed in bacteria and affinity purified using glutathione-Sepharose beads. The GST portion of the fusion protein was cleaved by digestion with thrombin. Purified protein without GST was used to immunize rabbits. The GST fusion protein was coupled to CNBr-activated beads, and used to affinity purify antibodies from whole serum.

Immunoprecipitation, GST Pull-down, and Immunoblot Assay—J77 cells were lysed in lysis buffer and the cell lysate was precleared as described before. Anti-GAKIN (COOH terminus) serum (10 μl) or an equivalent amount of preimmune serum was incubated with the J77 lysate for 4 h at 4 °C. Immune complexes were recovered by centrifugation after incubation with Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) beads for 1 h. Beads were washed extensively with lysis buffer and solubilized by boiling in SDS buffer. The GST fusion protein pull-down assay was performed by incubating the cell lysate with 10 μl of glutathione-Sepharose beads that were saturated with GST fusion proteins for 4 h at 4 °C. Beads were recovered by centrifugation, washed extensively, and analyzed by SDS-PAGE. Western blotting was carried out using an affinity purified polyclonal antibody against the NH$_2$ terminus of GAKIN and a monoclonal antibody against hDlg. Blots were developed using LumiGLO kit (Kirkegaard and Perry Laboratories, Inc.).

In Vitro Binding Assay—Defined segments of GAKIN cDNA were cloned into pCITE-2a vector and in vitro transcription and translation reactions were performed using Single Tube Protein System 3 (Novagen). This system utilizes rabbit reticulocyte lysate for in vitro protein synthesis. Protein products were radiolabeled by the addition of [35S]methionine (NEG Life Science Products Inc.) in the translation reaction. After the completion of protein synthesis, 1.0 μg of RNase A was added to each sample and incubated for 5 min at room temperature. The mixture containing translated polypeptides was diluted 10-fold with PBS, 1.0% Triton X-100, divided into two tubes, and incubated with either GST or GST-GUK protein fusion bound to glutathione-Sepharose beads for 2.0 h at 4 °C. Beads were recovered by centrifugation and analyzed by SDS-PAGE. The gel was stained with Coomassie Blue, incubated in Amplify solution (Amersham Pharmacia Biotech) for 15 min, and radioabeled proteins were detected by fluorography.

Immunofluorescence Analysis—To examine the distribution pattern of COOH terminus domain of hDlg in T lymphocytes by GST pull-down and in vitro kinase assays, we detected a prominent phosphorylated protein of 250 kDa associated with the GST-hDlg protein complex. GST fusion proteins of hDlg lacking either its COOH terminus (GST-NT) or the NH$_2$ terminus (GST-ACT) were immobilized on glutathione-Sepharose beads and incubated with J77 cell lysate (Fig. 1). Protein complexes bound to the beads were collected, washed extensively, and incubated with [γ-32P]ATP to examine for the presence of protein kinase activity associated with the protein complex. Previously we have shown that the NH$_2$ terminus domain of hDlg binds to p56lck tyrosine kinase in T cells (30). Consistent with this observation, the GST-ACT fusion protein of hDlg was phosphorylated by the associated protein kinase (Fig. 2A). In contrast, the GST-NT fusion protein that has similar mobility on SDS gels to that of GST-ACT protein but lacks the NH$_2$-terminal p56lck-binding domain was not phosphorylated under these conditions (Fig. 2A). Instead, a prominent phosphorylated band of 250 kDa (pp250) was detected in the lane containing the GST-NT protein complex (Fig. 2A). Based on these results, we concluded that a phosphorylated protein of ~250 kDa specifically associated with the COOH terminus of hDlg in T lymphocytes. To determine the pp250-binding domain within hDlg, a series of fusion protein constructs of hDlg were made (Fig. 1). Only those fusion proteins that included the GUK domain of hDlg bound to pp250 as tested by the GST pull-down and in vitro kinase assays (Fig. 2B). These results indicate that the pp250 interacts with the GUK domain of hDlg and may be a substrate of the protein kinase activity associated with the protein complex. Since the mobility of pp250 was similar to spectrin, filamin, CBP, P300, mTOR, and ankyrin, proteins that are likely to be involved in hDlg cytoskeletal and signal transduction pathways, we attempted to establish the identity of pp250 by Western blotting. Specific antibodies against the protein tagged antibody abolished the antibody, suggesting that the pp250 might be a novel protein (data not shown).

Isolation of pp250—To purify sufficient quantities of pp250 for biochemical analysis, we used an affinity column of GST-GUK fusion protein of hDlg to isolate pp250 from J77 cell lysate. In vitro protein kinase assays confirmed the presence of pp250 in the GST-GUK protein complex (data not shown). The Coomassie
Blue-stained band of pp250 was excised and processed for peptide sequences as described under “Materials and Methods.” Two peptide sequences that were derived from pp250 matched with a cDNA clone in the human EST data base. A combination of polymerase chain reaction, conventional cDNA library screening, and 5’-, 3’-RACE protocols identified an open reading frame of a novel protein of 1826 amino acids (Fig. 3B) (see “Materials and Methods” for details). To confirm the identity of the cDNA clone, additional peptide sequences were obtained from the tryptic digest of pp250 (Table I). More than 30 peptide sequences matched completely with the predicted amino acid sequence of pp250 cDNA (Table I). Sequence comparison analysis of the pp250 cDNA revealed that it encodes a novel member of the kinesin superfamily of motor proteins. We therefore renamed pp250 as GAKIN (guanylate kinase associated kinesin) to highlight its unique association with the guanylate kinase-like domain of hDlg and PSD-95.

Properties of GAKIN—

GAKIN encodes a protein of 1826 amino acids with a calculated molecular mass of 202,650 Da (Fig. 2). A schematic diagram of the domain organization of GAKIN is shown in Fig. 3A. Two major domains are predicted: a motor domain and a stalk. The motor domain is a conserved motif of the kinesin superfamily (boxed and shaded). The CAP-Gly domain located at the COOH-terminal end is underlined.

Fig. 3. Primary structure and sequence analysis of pp250 (GAKIN). A, a schematic diagram of the domain organization of GAKIN. B, deduced amino acid sequence of GAKIN. The motor domain is a conserved motif of the kinesin superfamily (boxed and shaded). The CAP-Gly domain located at the COOH-terminal end is underlined. C, protein matrix alignment analysis of human GAKIN with Drosophila kinesin-73 protein (MacVector 6.5). Note that the sequence identity extends essentially throughout the length of the two proteins except near the end of the COOH terminus but retains the conserved CAP-Gly domain. The nucleotide sequence of GAKIN has been deposited with the EBI/GenBank™ data libraries with the accession number AF279865.
and an isoelectric point of 5.44. A consensus ATP/GTP binding motif (GQGTSGKS) is located within the NH$_2$-terminal motor domain (Fig. 3). There is no detectable repeat structure in the amino acid sequence of GAKIN with the KIF1A/Unc104 motor protein family of kinesin-like motor proteins (36). A comparison of the amino acid sequence of only the motor domain of mouse KIF13B has been published to date (31). GAKIN also contains a recognizable motif called the CAP-Gly domain at its COOH terminus (Fig. 3). The CAP-Gly domain is found in proteins such as CLIP170/restin, dynactin, and kinesin-73, components of the cytoskeleton. It is possible that the GAKIN may function as a monomeric motor in vivo.

Northern blot analysis indicated ubiquitous expression of GAKIN mRNA with relative abundance in kidney, pancreas, brain, and testis (Fig. 4). A single mRNA band of ~8.5 kb was detected in all tissues. Consistent with the abundant expression of GAKIN in T cells, the Jurkat cells also showed a single band of ~8.5 kb by Northern blot analysis. The ubiquitous expression of GAKIN is consistent with the ubiquitous expression profile of hDlg (13) thus implying a functionally conserved interaction between hDlg-GAKIN in these tissues.

Specificity of Interaction between GAKIN and hDlg—A polyclonal antibody was raised against the NH$_2$-terminal domain of GAKIN and affinity purified against the immobilized antigen (see “Materials and Methods”). The affinity purified antibody detected a single ~250-kDa band in the lysate of Jurkat cells (Fig. 5A). This affinity purified antibody was used to detect GAKIN in protein complexes pulled down from J77 lysate by glutathione-Sepharose beads with immobilized GST fusion proteins (Fig. 5B). GAKIN specifically associated with beads containing either the ΔNT fusion protein or the GUK domain of hDlg (Fig. 5B, lanes 2 and 5). In contrast, the GAKIN failed to associate with beads containing either the ΔCT fusion protein or PDZ domains of hDlg (Fig. 5, lanes 3 and 4). It is noteworthy here that the binding of GAKIN to the GUK domain is relatively more efficient than the ΔNT fusion protein of hDlg presumably because of the better folded conformation of the GUK domain, alternatively, the steric hindrance via intradomain interactions within hDlg could account for the reduced binding under these conditions. The specificity of binding between GAKIN and the GUK domain of hDlg was further demonstrated by the observation that the GUK domain of p55 MAGUK failed to associate with hDlg under identical binding conditions (Fig. 5B, lane 1). Unlike hDlg, the GUK domain of p55 is prototypical of the GUK domains of MAGUKs that retain residues necessary for ATP binding (16). To further establish an association between hDlg and GAKIN in vivo, GAKIN was immunoprecipitated from J77 cell lysate using a monoclonal antibody against GAKIN as described earlier (Fig. 5C). Successful immunoprecipitation of GAKIN was confirmed by probing the same blot using a monoclonal antibody raised against the COOH-terminal domain of GAKIN (Fig. 5, lanes 3 and 4). It is noteworthy here that the binding of GAKIN to the GUK domain is relatively more efficient than the ΔNT fusion protein of hDlg presumably because of the better folded conformation of the GUK domain, alternatively, the steric hindrance via intradomain interactions within hDlg could account for the reduced binding under these conditions. The specificity of binding between GAKIN and the GUK domain of hDlg was further demonstrated by the observation that the GUK domain of p55 MAGUK failed to associate with hDlg under identical binding conditions (Fig. 5B, lane 1). Unlike hDlg, the GUK domain of p55 is prototypical of the GUK domains of MAGUKs that retain residues necessary for ATP binding (16). To further establish an association between hDlg and GAKIN in vivo, GAKIN was immunoprecipitated from J77 cell lysate using a monoclonal antibody against GAKIN as described earlier (Fig. 5C). Successful immunoprecipitation of GAKIN was confirmed by probing the same blot using a monoclonal antibody against GAKIN as described earlier (Fig. 5C). These results demonstrate the existence of hDlg-GAKIN complex in human T lymphocytes and suggest that the GUK domain of hDlg mediates this interaction.

Mapping of the Binding Domains within GAKIN and hDlg—Binding between hDlg and GAKIN was investigated by expressing defined segments of GAKIN in vitro using the rabbit reticuloocyte lysate transcription/translation system (Fig. 6). The longest GAKIN construct (Gakin 1–1335) containing the entire motor domain and most of the stalk domain bound to the GST-GUK protein of hDlg but not to control GST (Fig. 6C). This observation suggested that the interaction between hDlg and GAKIN might be direct. However, we cannot exclude the possibility that an undefined protein present in the reticulocyte lysate may facilitate this interaction. The hDlg-binding site was further mapped using smaller segments of GAKIN, and a segment of the stalk domain in close proximity to the motor domain emerged as the most likely binding site within GAKIN (Fig. 6C). This segment of the stalk domain of GAKIN shares sequence similarity with the KIF1A/unc104 subfamily of kinesin-like motor proteins. However, it remains to be ascertained whether other MAGUKs bind to this family of motor proteins.

### Table I

| Residue number | Peptide sequence |
|----------------|------------------|
| 35–46          | VILNPVNITLNSK    |
| 73–81          | YAQQDIDFK        |
| 126–133        | LC5GLFER         |
| 149–157        | VEVSYMIEYNK      |
| 249–259        | LSLLVLAGGER      |
| 279–297        | SLLTTLGIVSALDQGSK |
| 307–315        | DSVTLLWIK        |
| 342–442        | QLESLGISLQSSGIK  |
| 448–468        | CTVLNLNDPALNELLIVYK |
| 513–531        | TVYNGGEVSVSPTQLHHDR |
| 573–593        | VDGSDDVSSEVSNFNYEYAQ |
| 600–623        | AGLSNPDMSQILNSLEQHEEKEK |
| 676–685        | EATLNNLSIR      |
| 715–728        | VTQIPASSLDNRR    |
| 731–742        | GSSLSEPAQVR      |
| 765–781        | DLQYQMKECEEDNPVIR |
| 772–781        | ECEEDNPVIR       |
| 833–839        | LRVEMR          |
| 874–891        | INQATESDPQQLHLSVFVCK |
| 961–974        | KWNALMDQIIAQK    |
| 988–1013       | KLEFQWQLEQNGENEGYCPVEISAK |
| 1095–1101      | WLNALTIK        |
| 1102–1112      | RQYELDQQQK       |
| 1125–1141      | LITLTEER        |
| 1223–1239      | AEASWDSVHAVGCPQLGR |
| 1309–1331      | PEDAQQVKEERELAAKNEV |
| 1346–1358      | SVLAVNLLTLDR    |
| 1359–1366      | LRQEVAVK        |
| 1406–1421      | GRMEQQDVQGTTVSVR |
| 1422–1455      | GIAPAPALSVSPQNHSPDPQGSLNAASYLNPK |
| 1479–1490      | RPSLPAHQFPVR    |
| 1491–1501      | IMQVQASDPDR      |
| 1703–1713      | EGEFVTGAKH      |
| 1719–1741      | YVGPFDQPETWVGVDELPSKG |
| 1791–1810      | SATLSSGGATNALASITAALAK |
proteins such as GKAP/SAPAP/DAP1 and BEGAIN. These observations suggest that a novel protein domain may mediate interaction of Garkin with hDlg and other members of the MAGUK superfamily.

We have also examined whether the Garkin can interact with the GUK domains of other MAGUKs. We used the GUK domain of human PSD-95, which shares significant sequence homology with the GUK domain of hDlg (78% identity), and a relatively distant GUK domain of p55 (27% identity). As shown in Fig. 7, the GUK domain of human PSD-95 binds to the Garkin segment expressed in vitro using the rabbit reticulocyte transcription/translation system. Consistent with the results of the GST pull-down assay (Fig. 5), the GUK domain of p55 failed to associate with Garkin (Fig. 7). Recently, a functional role has been assigned to the COOH-terminal GUK domain of PSD-95. This domain has been implicated in the subcellular targeting of PSD-95 to synaptic sites in neuronal cells (39). It was suggested that the GUK domain of PSD-95 harbor a protein-protein interaction site that may be required for the correct targeting of PSD-95 in vivo (39). Because the GUK domains of PSD-95 and hDlg share significant sequence identity, a possibility exists that the GUK domain of PSD-95 may bind to the same site within hDlg and PSD-95. Our initial mapping results show that the last 11 amino acids of hDlg do not participate in the binding of Garkin since the GST-GUK domain construct of hDlg lacking the last 11 amino acids is able to bind Garkin (Fig. 6B). We attempted to introduce sequential deletions from the COOH terminus of hDlg but the corresponding fusion proteins were completely insoluble as GST fusion proteins in bacteria (data not shown). We also constructed a GST fusion protein encoding the last 25 amino acids of hDlg. This fusion protein did not bind to Garkin in vitro (data not shown). This result suggests that the folding of the binding site either may require an additional portion of the GUK domain or the binding site lies elsewhere within the GUK domain of hDlg. Precise in vitro mutagenesis, peptide scanning, and crystallographic approaches will be needed to identify critical residues for the Garkin-binding site within the GUK domain of hDlg.

Translocation of hDlg to Lymphocyte Cap upon CD2 Cross-linking—Our earlier results indicate that hDlg is localized at the cell-cell contact sites in confluent epithelial cells (13). However, its subcellular localization in non-adherent cells like lymphocytes has yet to be studied. In resting T lymphoma cells (Jurkat J77), the hDlg localization showed a largely diffused pattern throughout the cytoplasm (Fig. 8B). T lymphocytes form cell-cell contacts with their cognate interactors cells whether they be target cells, in the case of cytotoxic T lymphocytes, or antigen presenting cells in the case of helper T lymphocytes. Upon activation through the T cell receptor, additional membrane and intracellular protein alterations occur, resulting in the formation of what some refer to as an “immunological synapse” (40). To examine the possibility that lymphocyte hDlg translocates to a cell-cell contact site upon T cell activation, we cross-linked the cell surface CD2 molecules to mimic the contact made between antigen-presenting cells with T lymphocytes (41). The CD2 molecules distributed uniformly on the plasma membrane in non-cross-linked T cells (Fig. 8C) but formed a distinct patch-like structure after cross-linking with a mitogenic pair of anti-CD2 monoclonal antibodies (Fig. 8G, note that CD2 molecule is clustered in 3 out of 4 Jurkat J77 cells). Similar clustering is observed during T cell conjugate formation with CD58 expressing antigen presenting cells (41–42). Interestingly, immunolocalization studies indicated a significant translocation of hDlg molecules to sites where CD2 molecules clustered into patches (Fig. 8F). Although hDlg partially co-localized with CD2 (Fig. 8H), we failed to coprecipitate the CD2-hDlg complex from J77 cells after cross-linking of the CD2 receptor (data not shown). These results suggest that the translocation of hDlg to immune synapse is not likely to be mediated by the direct interaction between hDlg and CD2 cell surface receptor. Since the T cell surface cross-linking via CD2 adhesion receptor causes polarization and reorganization of microtubule structures (43), we speculate that the translocation of hDlg to immune synapse is mediated by the microtubule-based transport mechanism which requires participation of Garkin.

Localization of Garkin in T Lymphocytes—We used an affinity purified polyclonal antibody against the COOH terminus portion of Garkin to localize Garkin in resting T lymphocytes. This antibody detected a single Garkin band of ~250 kDa in the Jurkat cell lysate (Fig. 5A). The Garkin was localized in the cytoplasm of resting J77 cells (Fig. 9D) consistent with the localization pattern of hDlg in resting T cells (Fig. 8B). A finer microscopic examination of Garkin distribution in resting T cells indicated that it is localized as distinct punctate foci or vesicles of unknown identity (Fig. 9F). This vesicular pattern of Garkin disappeared upon preabsorption of the affinity purified antibody with an immobilized GST fusion protein encoding the COOH terminus segment of Garkin, confirming the specificity of the signal for Garkin. We then attempted to visualize the Garkin redistribution in J77 cells after cross-linking with anti-CD2 monoclonal antibodies as shown for hDlg. These attempts proved unsuccessful because of nonspecific cross-reactivity between the secondary goat anti-rabbit IgG used to detect Garkin and the anti-CD2 monoclonal antibodies used to induce receptor cross-linking (data not shown). Currently we are developing a Garkin-specific monoclonal antibody that will be directly conjugated to an appropriate fluorescent probe. The develop-
ment of fluorescent antibody against GAKIN will allow us to visualize GAKIN distribution upon T cell activation in the context of hDlg targeting to the immune synapse as well as with other known markers of T cells.

**DISCUSSION**

Binding studies have been successful in predicting a scaffolding function of hDlg/SAP97, however, its precise physiological function in vivo remains unknown. Yeast two-hybrid experiments have been successful in predicting a scaffolding function of hDlg/SAP97, however, its precise physiological function in vivo remains unknown. Yeast two-hybrid experiments have been successful in predicting a scaffolding function of hDlg/SAP97, however, its precise physiological function in vivo remains unknown.

**Specific association of GAKIN with hDlg.** A, characterization of anti-GAKIN antibodies. Rabbit polyclonal antisera against the NH2 and COOH termini of GAKIN (see “Materials and Methods”) were affinity purified using immobilized recombinant proteins, respectively. Western blot of Jurkat cell lysate was probed with an anti-COOH terminus antibody. Similar results were obtained with an anti-NH2 terminus antibody (not shown). Note that a single band of GAKIN was detected in the lysate of Jurkat J77 cells. B, immobilized GST fusion proteins were used to pull-down GAKIN from J77 cell lysate. GST fusion proteins containing defined segments of hDlg and p55 were incubated with J77 lysate, and the presence of GAKIN in the pull-down complexes was examined by immunoblotting using an affinity purified antibody against the NH2-terminal domain of GAKIN. Note that the GST-GUK domain fusion protein of hDlg was significantly more efficient in pulling down GAKIN. C, co-immunoprecipitation of hDlg with GAKIN. GAKIN was immunoprecipitated from J77 cells using a polyclonal antibody raised against the COOH-terminal domain of GAKIN or with the preimmune serum (PI). Co-precipitation of hDlg was detected by immunoblotting using a monoclonal antibody against hDlg. Successful immunoprecipitation of GAKIN was confirmed by immunoblotting of the same membrane with an affinity purified antibody raised against the NH2-terminal domain of GAKIN.

**DISCUSSION**

Binding studies have been successful in predicting a scaffolding function of hDlg/SAP97, however, its precise physiological function in vivo remains unknown. Yeast two-hybrid experiments revealed that hDlg/SAP97 binds to COOH termini of Shaker-type K+ channels (44). This interaction conforms to the general binding paradigm of PDZ domains to the COOH termini of transmembrane channels and receptors (45–47). Subsequent transfection studies have indicated that the co-expression of SAP97 (rat homologue of hDlg) and Kv1.4 potassium channels in COS-7 epithelial cells results in the formation of large intracellular aggregates (14, 48). More recent evidence indicates that the hDlg/SAP97 protein also clusters NR2 subunits of N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptors at synaptic sites (49). The differential clustering activity of hDlg/SAP97 and PSD-95 MAGUKs may reflect their specialized roles in transfected epithelial cells, and is consistent with their distinct expression pattern in vivo. For example, the PSD-95 expression is restricted to the neuronal cells and the protein is localized to the postsynaptic density of...
FIG. 8. Translocation of hDlg upon CD2 cross-linking of Jurkat T lymphocytes. Panels A-D represent J77 cells that were not cross-linked with anti-CD2 monoclonal antibodies, and panels E-H represent J77 cells that were cross-linked via CD2 as described under “Materials and Methods.” Panels D and H represent merged images of photographs shown in panels B/C and F/G, respectively. The hDlg protein was visualized using a monoclonal antibody specific for the NH2-terminal domain of hDlg (see “Materials and Methods”). Note that the clustering of hDlg occurred at sites of CD2 cross-linking (panels F/H).

FIG. 9. Immunofluorescent localization of GAKIN in the cytoplasm of Jurkat T lymphocytes. GAKIN was visualized in J77 cells using an anti-GAKIN polyclonal antibody (COOH terminus). Panel A, phase-contrast picture of J77 cells (×60 magnification). Panel B, immunofluorescence image of the same field stained with the goat anti-rabbit fluorescein isothiocyanate antibody alone. Similar results were obtained with an anti-GAKIN antibody that was pre-absorbed with the COOH terminus fusion protein of GAKIN. Panels C and D are phase and immunofluorescence images of J77 cells which were incubated with an affinity purified polyclonal antibody against the COOH terminus of GAKIN. Panels E and F correspond to a magnified view of panels C and D, respectively. Note that the GAKIN is localized throughout the cytoplasm of J77 cells in a punctate/vesicular pattern.
forebrain synapses (12, 50, 51). In contrast, hDlg/SAP97 is distributed widely including cells of neuronal, epithelial, and hematopoietic origin (13, 52). In neuronal cells, SAP97 is localized predominantly at the axonal and presynaptic regions (53). In confluent epithelial cells, hDlg/SAP97 localizes at the basolateral membrane, however, it is found in the cytoplasm of nonconfluent epithelial cells (13, 52, 54). Together, these results suggest that the subcellular targeting of hDlg/SAP97 in neuronal as well as non-neuronal cells may be modulated by interactions mediated by specific motifs present in the hDlg/SAP97.

The PDZ domains of MAGUKs have been extensively characterized (45, 46, 55), however, the biochemical basis of GUK domains remains unclear. The first evidence for the physiological function of the GUK domain came from genetic studies of Dlg tumor suppressor protein in Drosophila. Woods and Bryant (1, 56) identified several mutant alleles affecting the GUK domain of Dlg tumor suppressor: dlg<sup>sw</sup>, dlg<sup>1P20</sup>, dlg<sup>99</sup>, and dlg<sup>Cl-2</sup> alleles result in the progressive deletion of the GUK of Dlg causing tissue overgrowth and suggesting a role in tumor suppression and signal transduction in Drosophila (1, 56). Alternatively, the proliferation defect observed in the GUK domain mutants of Dlg tumor suppressor could be a secondary consequence of altered subcellular targeting of Dlg and a breakdown of septate junction structure (56). Recessive alleles that completely truncate the GUK domain of Dlg produce a full tumorous phenotype in imaginal discs, suggesting a requirement of the GUK domain in cell proliferation events in this tissue. Hough et al. (57) demonstrated that a Dlg derivative without the GUK domain is localized normally in a dlg<sup>−/−</sup> background, and can rescue epithelial structure and growth control phenotypes in a dlg<sup>−/−</sup> background. The most likely explanation for this observation is that in a dlg<sup>−/−</sup> background the Δ-GUK derivative is able to localize normally through multifurcation of PDZ domains with the endogenous full-length Dlg. In the dlg<sup>−/−</sup> background, the Δ-GUK derivative is able to rescue the mutant phenotype because it is expressed at very high levels, which can provide normal genetic function even without normal localization and transport requirements. More recent evidence indicates that the GUK domain of Drosophila Dlg plays an essential role in the development of bouton postsynaptic morphology (58). Mammalian SAP97/hDlg can suppress the tumorigenic growth in dlg<sup>−/−</sup> flies and substitutes for Dlg at the neuromuscular junctions thus implying that the mammalian SAP97/hDlg may perform a similar function in vivo (59).

Further evidence for the protein binding function of the GUK domain of MAGUKs comes from studies of the LIN-2 protein in Caenorhabditis elegans. A minigene of lin-2 was engineered that lacked residues essential for the enzymatic function of the GUK domain of LIN-2 (60). This mutant lin-2 allele can rescue the lin-2 Vul phenotype in C. elegans thus indicating that the classical enzyme function of the GUK domain of LIN-2 is not required for vulval induction (60). Together, these results support the hypothesis that the protein binding properties of the GUK domain of LIN-2 may be sufficient to restore its signaling function in vulval induction.

The central objective of the present investigation was to elucidate protein-binding interactions of the GUK domain of hDlg in human T lymphocytes. Here we report identification, cloning, and partial characterization of a novel kinesin-like motor protein (GAKIN) that interacts with the GUK domain of hDlg. To our knowledge, this is the first report of a biochemical association between a kinesin-like motor protein and the GUK domain of MAGUKs. Kinesin-like motor proteins play critical roles in the anterograde transport (movement toward the plus end of microtubules) of synaptic vesicle precursors, mitochondria transport, spindle function, and chromosome segregation (61–64). The membrane and secreted proteins are sorted into several types of vesicles, which then are translocated along axonal microtubules to their target sites (37). Sequence comparison analysis of GAKIN with the kinesin superfamily suggests that it may play a role in the anterograde transport of synaptic vesicle precursors in vivo. The closest homologue of GAKIN in Drosophila is kinesin-73 (34). Although mutant analysis of kinesin-73 has not been conducted yet, the genetics of a similar kinesin-like gene (unc104) has been investigated in C. elegans. The unc104 mutants show fewer synaptic vesicles and make few small synapses (65). Interestingly, a large number of vesicles are found tethered together in the cytoplasm of cell bodies indicating that Unc104 protein is an anterograde motor for translocation of synaptic vesicles along axonal microtubules. It is relevant to mention here that the unc104 gene of C. elegans is not the closest homologue of Drosophila kinesin-73. In fact, another gene termed CeF58E3.3 has been recently identified during the C. elegans genome-sequencing project which appears to be the true homologue of human GAKIN and Drosophila kinesin-73. Therefore, the functional consequences of genetic inactivation of the closest homologue of GAKIN in C. elegans and Drosophila remain to be elucidated.

A conserved feature of the kinesin-like proteins is the presence of a motor domain that confers microtubule binding and ATP hydrolysis (36). The GAKIN sequence contains an NH2-terminal motor domain and a single copy of the CAP-Gly domain at the COOH terminus. The CAP-Gly domain has been previously identified in Drosophila kinesin-73 (34), CLIP170/Restin (33, 66), Drosophila Glued (67), rat dynactin (32), human tubulin folding cofactors B and E (68, 69), and Alp11 (fission yeast homologue of cofactor B) (70). Functionally, the CAP-Gly domain mediates direct binding of CLIP170 to microtubules and presumably serves as a linking module to endocytic vesicles (33, 66). The identification of CAP-Gly domains in proteins important for microtubule-dependent processes suggest that its presence at the COOH termini of GAKIN and Drosophila kinesin-73 may signify a similar role in microtubule-based functions in vivo. The predicted motor domains of GAKIN and kinesin-73 are homologous to the motor domain of KIF1A/Unc104 subfamily of kinesin-like motor proteins (37, 65). The KIF1A/Unc104 subfamily of kinesin-like motor proteins mediates specific motile events and functions as organelle and vesicle transporters (65). GAKIN also shares sequence similarity with the KIF1A/Unc104 near a segment of the stalk domain that is proximal to the motor domain. Interestingly, the hDlg-binding site in GAKIN was localized within this region (Fig. 6) indicating that the interaction between MAGUKs and KIF1A/Unc104 subfamily of motor proteins may be conserved in vivo. Our results also demonstrate that the GUK domains of hDlg and PSD-95 bind to GAKIN whereas the GUK domain of p55 does not (Figs. 5 and 7). Based on these observations, we speculate that the KIF1A/Unc104 subfamily of motor proteins may bind to the p55 subfamily of MAGUKs. Similarly, the GUK-binding domain of GAKIN shares sequence identity with the corresponding domain in kinesin-73 suggesting that the mammalian GAKIN-hDlg interaction may be evolutionary conserved in Drosophila.

In addition to their function in intracellular transport, some kinesin-like motor proteins are also involved in mitotic spindle formation, chromosome segregation, and control of cell division (62, 71). For example, Drosophila KLP38B which is homologous to KIF1A/Unc104 is known to play a role in mitosis (72, 73). Therefore, a functional role of the GAKIN-hDlg complex in cell division cannot be excluded at this stage. In fact, several
hDlg-interacting proteins are known to interact with components of the cell cycle. For example, the HTLV Tax oncoprotein that binds to the PDZ domain of hDlg (74) interacts with the human mitotic checkpoint protein MAD1 and abrogates the mitotic checkpoint (75). Adenomatous polyposis coli (APC) colon tumor suppressor protein binds to the PDZ domains of hDlg (76) and blocks progression from G2-M to S phase of the cell cycle (77, 78). Similarly, protein 4.1 that binds to the HOOK domain of hDlg (10, 13) has been localized in the centrosome, nucleus, and mitotic spindles (79, 80). Protein 4.1 also associates with the nuclear mitotic apparatus (NuMA) protein (81) and is believed to play a role in cell division. Whether the hDlg-GAKIN complex interacts with these proteins in vivo and plays a role in the regulation of cell division is an issue of considerable significance for future studies.

The differential expression pattern of hDlg/SAP97 in confluent and non-confluent epithelial cells indicates that an active targeting mechanism must exist for the correct localization of hDlg/SAP97 in vivo. Recent studies on synaptic targeting of PSD-95 revealed a 12-amino acid sequence located within the GUK domain, which plays a critical role in the subcellular targeting of PSD-95 in vivo (39). In contrast, the deletion of the GUK domain of SAP97 only slightly reduced its targeting efficiency to the lateral plasma membrane in CACO-2 colon carcinoma epithelial cells (52). A limitation of these studies is that the CACO-2 cells contain a significant amount of endogenous hDlg that complicates the interpretation due to potential oligomerization between transfected and endogenous molecules. Indeed, we have recently shown that a unique amino-terminal domain mediates multimerization of hDlg into dimeric and tetrameric species in solution (82). Another important issue is the characterization of the protein kinase activity associated with the GAKIN-hDlg complex. Initially, we identified GAKIN as a prominent phosphorylated protein by in vitro kinase assays (Fig. 2). The GAKIN sequence has no putative protein kinase domain, hence it is likely that a distinct protein kinase is associated with the GAKIN-hDlg complex. GAKIN is the only protein in the complex that is phosphorylated, therefore, the associated protein kinase has strict substrate specificity for GAKIN. Several kinesin-like proteins are subject to regulation by phosphorylation. For example, phosphorylation of kinesin by camp-dependent protein kinase reduces its binding to synaptic vesicles (83), and phosphorylation by cdc2 kinase regulates spindle association of mitotic kinesins, HsEg5 and CENP-E (84, 85). Another mitotic kinesin, CHO1/MKLP-1, interacts with a protein kinase Plk and is phosphorylated (86). These observations suggest that the protein kinase associated with the GAKIN-hDlg complex may be a specific regulator of GAKIN function and microtubule-based cellular dynamics in vivo. Identification of the protein kinase activity and other components of the GAKIN-hDlg complex will be essential for defining the physiological basis of MAGUKs in cellular transport and motile functions.

The rapid translocation of hDlg from T cell cytoplasm to the site of CD2 receptor cross-linking is consistent with the role of MAGUKs as modulators of signaling pathways that exist between membrane receptors and cytoskeleton. CD2 is a transmembrane receptor expressed in T cells, and binds to the CD58 ligand expressed on antigen presenting cells (87). Upon binding to CD58, the CD2 receptor facilitates cell-cell contact between T lymphocytes (both helper and cytotoxic) and CD58-bearing antigen presenting cell. This in turn optimizes the ability of T cell receptor to recognize the specific antigen bound to major histocompatibility complex molecules (88, 89). The T lymphocyte-antigen presenting cell contact site, a specialized junction often called an “immunological synapse,” contains T cell recep-

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