The LFA-1-associated Molecule PTA-1 (CD226) on T Cells Forms a Dynamic Molecular Complex with Protein 4.1G and Human Discs Large*

Kylie J. Ralston‡, Samantha L. Hird‡, Xinhai Zhang‡, Judith L. Scott‡, Boquan Jin§, Rick F. Thorne¶, Michael C. Berndt¶, Andrew W. Boyd**, and Gordon F. Burns‡‡

From the ‡Cancer Research Unit, School of Biomedical Sciences, The University of Newcastle, University Drive, Callaghan, New South Wales 2308, Australia, the ¶Department of Immunology, The 4th Military Medical University, Xi’an 710032, People’s Republic of China, the ‡Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3168, Australia, and the **Division of Cancer and Cell Biology, Queensland Institute of Medical Research, Royal Brisbane Hospital PO, Brisbane, Queensland 4029, Australia

Clustering of the T cell integrin, LFA-1, at specialized regions of intercellular contact initiates integrin-mediated adhesive responses and downstream signaling, events that are necessary for a successful immunological response. But how clustering is achieved and sustained is not known. Here we establish that an LFA-1-associated molecule, PTA-1, is localized to membrane rafts and binds the carboxyl-terminal domain of isoforms of the actin-binding protein 4.1G. Protein 4.1 is known to associate with the membrane-associated guanylate kinase homologue, human discs large. We show that the carboxyl-terminal peptide of PTA-1 also can bind human discs large and that the presence or absence of this peptide greatly influences binding between PTA-1 and different isoforms of 4.1G. T cell stimulation with phorbol ester or PTA-1 cross-linking induces PTA-1 and 4.1G to associate tightly with the cytoskeleton, and the PTA-1 from such activated cells now can bind to the amino-terminal region of 4.1G. We propose that these dynamic associations provide the structural basis for a regulated molecular adhesive complex that serves to cluster and transport LFA-1 and associated molecules.

A successful immunological response requires the activation, proliferation, and differentiation of T cell subsets into effector cells. These events are initiated by signals generated by sustained interaction between T cell receptors (TCR)¹ and antigen-presenting cells (APC), together with the engagement of accessory counter receptors between the T cell and APC. Without the coordinated engagement of accessory receptors, engagement of the TCR can result in T cell anergy (1). Receptor engagement and signaling appear to be coordinated, both temporally and spatially, at a specialized region known as the immunological synapse (2, 3) increasingly recognized as containing structural elements shared with classical neuronal synapses (4, 5).

Crucial to the formation of the immunological synapse are specialized membrane microdomains known as rafts (6) and also the rearrangement of elements of the cytoskeleton. Both of these essential components are influenced by receptor engagement at the synapse, and, in turn, both orchestrate molecular distribution and signal transduction during the interaction (7–9). An initiating event in synapse formation may be the migration of intercellular adhesion molecule 3 (ICAM-3) to sites of cell-cell contact with the APC, where it engages its counter receptor as part of the “scanning” process (10). Engagement of ICAM-3 results in an increase in intracellular calcium concentration, the activation of tyrosine kinases, and activation of the integrin LFA-1 (10, 11). The mechanisms involved in such “inside-out” activation of leukocyte integrins are complex and not fully understood (12, 13), but changes in avidity and affinity of LFA-1 involve clustering in membrane rafts (14–16) and remodeling of the actin (9, 17) and microtubule (18, 19) cytoskeleton. Increased intracellular calcium concentration activates the small GTPase Rap1, emerging as a major stimulus for LFA-1 clustering and ligand binding (20–23). Calcium also activates calpain (24), the proteolytic enzyme that cleaves talin, a process required for the linkage between integrins and the actin cytoskeleton that contributes to their clustering and activation (24–27). Recently, Ginsberg’s group demonstrated that a phosphotyrosine motif within a β turn of the integrin β subunit binds to the four point 1/ezrin/radixin/moesin (FERM) (28) domain contained in the talin head region and exposed by cleavage from the remainder of the molecule (27, 29).

Subsequently, the TCR and small accessory adhesion receptors migrate to the core of the synapse to form the central supramolecular activating complex, and larger molecules, including LFA-1 and associated talin, are displaced to an outer adhesive ring, the peripheral supramolecular activating complex (2, 30). These movements may be regulated in part by the greater numbers of small molecules simply displacing LFA-1*

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¹ The abbreviations used are: TCR, T cell receptor(s); APC, antigen-presenting cell; FERM, four point one ezrin/radixin/moesin; ERZ, ezrin, radixin, moesin; MAGUK, membrane-associated guanylate kinase homologue; hDlg, human discs large; GEM, glycolipid-enriched membranes; GST, glutathione S-transferase; CTD, carboxyl-terminal domain; ATD, amino-terminal domain; ICAM, intercellular adhesion molecule; GARIN, guanylate kinase-associated kinesin; mAb, monoclonal antibody; aa, amino acid(s); CHO, Chinese hamster ovary; RAM, rabbit anti-mouse immunoglobulin(s); MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate.
and other larger molecules within rafts (30). However, larger adhesion molecules such as CD43, the presence of which might hinder TCR engagement on physical grounds, are actively excluded from the synapse by association with ERM proteins (31). The ERM proteins themselves participate in a number of processes essential to T cell activation, including the movement of adhesion receptors, membrane and cytoskeletal redistribution, and signal transduction (32–35). Members of an extended protein 4.1 superfamily, these proteins are characterized by their possession of a FERM domain toward the amino terminus and an F actin-binding segment at the carboxyl terminus: in the resting state, these regions form an intramolecular association that masks other associations, but following activation by threonine phosphorylation and binding of phosphoinositides, the tail region is able to extend to bind actin, and the FERM domain is exposed to bind the juxtamembrane region of any of several adhesion molecules, thereby providing a transmembrane linkage to the actin cytoskeleton (36–39).

The FERM domain of the prototypic protein 4.1R shares about 30% identity with that of the ERM family members (28) and shares the capacity to bind transmembrane proteins (40). In addition, however, this domain of protein 4.1 can bind to another class of proteins, the membrane-associated guanylate kinase homologues (MAGUKs) (41). MAGUK family members contain a number of protein-protein interactive domains, including Src homology 3 and PDZ domains, and serve to mediate the tight clustering of both transmembrane receptors and ion channels at sites of cell-cell communication such as neuronal synapses (42). Since the sequence of amino acids in the FERM domain of 4.1 that binds to transmembrane receptors can differ from that bound by MAGUKs, these molecules potentially can form a protein 4.1-transmembrane protein–MAGUK ternary complex to provide a functional unit at the plasma membrane with links to the cytoskeleton, and there are now several examples of such a clustering complex (41, 43). Furthermore, linkages between these complexes and the cytoskeleton may be more than passive, since several regulators of small GTPases contain residues conserved in the 4.1-MAGUK binding site (41, 44, 45).

Recent work from Chishti’s laboratory has raised the exciting possibility that such a MAGUK complex might play a role in the formation of physical contacts between T cells and APC and in the regulation of T cell activation, thus extending the commonalities between the immunological synapse and classical neuronal synapses (46). This group first identified that in T cells the MAGUK, human discs large (hDlg), interacts with both the tyrosine kinase Lck and the potassium ion channel, Kv1.3 (47). Both Lck and Kv1.3 are known regulators of T cell signaling and adhesion that may localize to the immunological synapse (48–50), and hDlg translocates to the lymphocyte cap upon cross-linking of the CD2 receptor (46). hDlg also associates with a kinesin-like motor protein termed guanylate kinase-associated kinesin (GARKIN), an association with the potential to drive the microtubule-based trafficking of the complex to the plasma membrane (46, 51, 52). Moreover, in different cell types, both MAGUKs and members of the 4.1 family are able to bind transmembrane proteins, thus contributing to the scaffolding complex (41). In the present study, we identify a binding partner for the protein 4.1 paralogue, 4.1G, in T cells, the transmembrane protein PTA-1 (CD226). In addition, we demonstrate that PTA-1 can bind to hDlg in a process requiring its carboxyl-terminal peptide residues, which contain a PDZ-binding motif. We show that PTA-1 binds to the carboxyl-terminal domain of 4.1G, and the proteins associate under resting conditions, but, upon stimulation of Jurkat cells, PTA-1 now can bind to the amino-terminal region of 4.1G and the complex associates with the cytoskeleton.

PTA-1 (also known as CD226, TLiSA-1, and DNAM-1) is a member of the immunoglobulin superfamily, the expression of which is regulated by phorbol ester and calcium (53, 54). Initially identified as a T cell activation antigen, antibodies and F(ab)\(^2\) fragments against PTA-1 inhibit the development of cytotoxic T cells and T cell clones from their precursors (55–57). PTA-1 was also identified on platelets where it may engage in signal transduction, since anti-PTA-1 monoclonal antibodies (mAbs) induce platelet activation and aggregation in a process that requires the Fc receptor (58). Recently Shibuya et al. (59) showed that PTA-1 was physically and functionally associated with LFA-1 in NK cells and activated T cells in a process requiring phosphorylation of a serine residue in the PTA-1 cytoplasmic tail. In addition, cross-linking of LFA-1 induced tyrosine phosphorylation of PTA-1, possibly mediated by Fyn with which PTA-1 also associates. Circumstantially, these findings place PTA-1 at the peripheral supramolecular activating complex of the immunological synapse during T cell activation and suggest that it may play a role in LFA-1 activation and downstream signaling. In this study, we additionally show that PTA-1 associates with Rap-1 in membrane fragments from activated T cells and that the complex of protein 4.1G, hDlg, and PTA-1 is contained within membrane rafts.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The LeoA1 (55) and FMU4 (60) mAbs are directed against PTA-1. Rabbit polyclonal antibodies to 4.1G were raised against GST fusion proteins containing the amino-terminal domain, 4.1G-Head (aa 1–115) and the carboxyl-terminal domain, 4.1G-Tail (aa 830–1005). Affinity-purified IgG was obtained using either GST-4.1G-Head or GST-4.1G-Tail conjugated to a 1.1 mixture of Aff-gel 10 and 15 (Bio-Rad) (1 mg of protein/10 ml of resin). Pooled antisera from multiple bleedings were loaded through the relevant column, and bound IgG was eluted with 0.1 M glycine, pH 2.4, immediately reneutralized with one-fifth volume of 1 M Tris, pH 8.0, and dialyzed against Tris saline buffer, pH 7.4. This affinity-purified antibody was then passed through a GST-Aff gel 10/15 column to remove IgG against the GST portion of the fusion protein. The mAb directed against hDlg (2D11) was a gift from Dr. M. Lutcheman (Tufts University School of Medicine, Boston, MA). The V8IA7 anti-DAF mAb was a gift from Dr. D. Shafren (Department of Microbiology and Immunology, University of Newcastle). The Rap1 and calnexin mAbs were purchased from BD Transduction Laboratories (Lexington, KY). The anti-CD3 mAb (OKT3) was purchased from ATCC.

**Cell Lines and Establishment of Permanent Transfectants**—The Jurkat T cell line (ATCC) was maintained in RPMI 1640 medium (Thermo Trace, Melbourne, Australia) containing 10% fetal bovine serum (CSL, Melbourne, Australia). PTA-1 cDNA was subcloned into the high expression level eukaryotic vector, pEF-BOS, as described previously (54). The permanent Jurkat-PTA-1 cell line was established using the LipofectAMINE™2000 reagent (Invitrogen) to introduce a 10:1 excess of the pEF-BOS-PTA-1 vector (which lacks a eukaryotic selection marker) to preP9 (which encodes neomycin phosphotransferase; Invitrogen), followed by selection with 750 μg/ml G-418 (Invitrogen). The PTA-1-positive population was verified by flow cytometry and further enriched by fluorescence-activated cell sorting with the LeoA1 mAb.

The Chinese hamster ovary (CHO) cell line permanently transfected with PTA-1 (CHO-PTA-1) or DAF (CHO-DAF) were established as described above and maintained in Dulbecco’s modified Eagle’s medium (CSL, Melbourne, Australia) with 10% fetal bovine serum. The CHO-DAF cells were kindly provided by Dr. D. Shafren.

**Preparation of Glycosped-nine-derived Membranes (GEMs)**—These were prepared as described previously (61). Briefly, Jurkat-PTA-1 cells (~7 × 10⁶ cells/treatment) were treated with TPA (Sigma) at 50 ng/ml at 37 °C for varying times, as indicated, or cross-linked with either LeoA1 or control mAbs followed by rabbit anti-mouse immunoglobulins (RAM) (as described below). Whole cell lysates were solubilized in 1% (w/v) Triton X-100 in MES-buffered saline, pH 7.0, and adjusted to 40% sucrose and applied under a discontinuous 5–30% sucrose gradient. Following overnight ultracentrifugation, the GEMs separated as a low density light-reflective band. The soluble fraction and GEMs were collected; GEMs were washed and dissolved in MES-buffered...
saline, and the insoluble pellet was further processed by washing with 100 μl of MES-buffered saline and centrifuged at 14,000 × g for 15 min at 4 °C, and then reducing Laemmli sample buffer was added to the pellet and boiled. Equal protein quantities (15 μg) of the GEM and soluble fractions, along with an equal proportion of the insoluble pellets, were separated on 7.5% SDS-PAGE and electrotransferred to nitrocellulose (Sartorius, Germany) using 25 μl Tris, 192 μM glycine, and 20% methanol transfer buffer. To visually determine equal protein loading, the nitrocellulose membrane was stained with Pronase (S.05% Pronase S, 1% glacial acetic acid) and then washed with TBS-T, followed by immunoblot analysis.

Immunoblotting—Nitrocellulose membranes were blocked in 5% skim milk in TBS-T (25 mM Tris-HCl, pH 8.0, 144 mM NaCl, 0.05% Tween-20) at room temperature for 30 min and then incubated with primary antibodies for 2 h at room temperature. The nitrocellulose membranes were washed with PBS and then 0.1% SDS, 50 mM NaPO₄, pH 6.5, 5% non-fat dry milk, followed by 2× SSC. Membranes were then washed with 0.1% SDS and then in 0.2× SSC, 1× SSC, 0.1% SDS and then in 0.2× SSC, 1% SDS for 60 min at 55 °C, followed by autoradiography. Positive plaques were isolated from the original plate, and the bacteriophage eluted in PSB (0.1M NaCl, 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, 0.05% gelatin) overnight at 4 °C. Secondary and tertiary screens were performed to isolate the interaction of a single positive clone. The bacteriophage particles were purified from the PSB eluate, and the DNA was isolated according to standard procedures. The cDNA insert was released from the phage vector by EcoRI restriction, subcloned to pBluescript vector (Promega Corp., Madison, WI), followed by automated DNA sequencing.

Northern Blot Analysis—Northern blotting was performed as previously described (54), using total RNA isolated from either resting Jurkat cells or Jurkat cells treated with TPA at 50 ng/ml overnight to induce PTA-1 expression. The DNA probe consisted of the 4.1G cDNA insert isolated from the yeast two-hybrid library screen, labeled using a high prime DNA labeling kit (Roche Applied Science). Hybridizations were performed in prehybridization hybridization solution (50% formamide, 5× SSC, 5× Denhardt’s solution, 0.1% SDS, 50 mM NaPO₄, pH 6.3, 5 μg of herring sperm DNA) for 2 h at 42 °C prior to overnight hybridization with the 32P-labeled 4.1G probe at 42 °C. The filters were washed at room temperature in 0.1× SSC, 0.1% SDS and then in 0.2× SSC, 1% SDS for 60 min at 55 °C, followed by autoradiography. Positive plaques were isolated from the original plate, and the bacteriophage eluted in PSB (0.1 mM NaCl, 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, 0.05% gelatin) overnight at 4 °C. Secondary and tertiary screens were performed to isolate the isolation of a single positive clone. The bacteriophage particles were purified from the PSB eluate, and the DNA was isolated according to standard procedures. The cDNA insert was released from the phage vector by EcoRI restriction, subcloned to pBluescript vector (Promega Corp., Madison, WI), followed by automated DNA sequencing.

Immunofluorescent Cell Staining and Confocal Microscopy—CHO-PTA-1 cells were grown on glass coverslips overnight and then fixed and permeabilized with cold methanol for 10 min at −20 °C. Dual color immunostaining was performed with Alexa 488-conjugated goat anti-rabbit immunoglobulins (Molecular Probes, Inc., Eugene, OR). The stained coverslips were mounted onto glass slides with Prolong™ Antifade reagent (Molecular Probes), and the fluorescently labeled cells were visualized on a Zeiss LSM510 confocal microscope.

For the cytchalasin D experiments, CHO-PTA-1 and CHO-DAF cells were grown on glass coverslips overnight and then fixed and permeabilized with cold methanol for 10 min at −20 °C. Dual color immunostaining was performed with Alexa 488-conjugated goat anti-rabbit immunoglobulins (Molecular Probes, Inc., Eugene, OR). The stained coverslips were mounted onto glass slides with Prolong™ Antifade reagent (Molecular Probes), and the fluorescently labeled cells were visualized on a Zeiss LSM510 confocal microscope.

The method described by Harder and Kuhn (62) was used with slight modifications. Briefly, LeoA1 (anti-PTA-1) and OKT3 (anti-CD3) mAbs were coupled to M-450 sheep anti-mouse magnetic beads (Dynal), according to the manufacturer’s instructions. Jurkat-PTA-1 cells (3 × 10⁶ cells/treatment) were incubated with either the PTA-1- or CD3-coupled beads for 10 min at 4 °C. The bead-cell conjugates were washed in H buffer (10 mM Hepes, pH 7.2, 250 mM NaCl, 10 mM NaF, 0.1% SDS, 0.2 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mg/ml Complete™ protease inhibitors) and then resuspended in 1 ml of H buffer containing 0.2 mM pervanadate prior to homogenization of the cells on ice with a glass Dounce homogenizer. The homogenates were washed with cold H buffer, and the beads were isolated with a magnet (Dynal), and this was repeated three times. After the final wash, the proteins were eluted from the beads with reducing Laemmli sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting.

Northern blot analysis was performed as previously described (54), using total RNA isolated from either resting Jurkat cells or Jurkat cells treated with TPA at 50 ng/ml overnight to induce PTA-1 expression. The DNA probe consisted of the 4.1G cDNA insert isolated from the yeast two-hybrid library screen, labeled using a high prime DNA labeling kit (Roche Applied Science). Hybridizations were performed in prehybridization hybridization solution (50% formamide, 5× SSC, 5× Denhardt’s solution, 0.1% SDS, 50 mM NaPO₄, pH 6.3, 5 μg of herring sperm DNA) for 2 h at 42 °C prior to overnight hybridization with the 32P-labeled 4.1G probe at 42 °C. The filters were washed at room temperature in 0.1× SSC, 0.1% SDS and then in 0.2× SSC, 1% SDS for 60 min at 55 °C, followed by autoradiography. Positive plaques were isolated from the original plate, and the bacteriophage eluted in PSB (0.1 mM NaCl, 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, 0.05% gelatin) overnight at 4 °C. Secondary and tertiary screens were performed to isolate the isolation of a single positive clone. The bacteriophage particles were purified from the PSB eluate, and the DNA was isolated according to standard procedures. The cDNA insert was released from the phage vector by EcoRI restriction, subcloned to pBluescript vector (Promega Corp., Madison, WI), followed by automated DNA sequencing.

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For the cytchalasin D experiments, CHO-PTA-1 and CHO-DAF cells were grown on glass coverslips overnight and then fixed and permeabilized with cold methanol for 10 min at −20 °C. Dual color immunostaining was performed with Alexa 488-conjugated goat anti-rabbit immunoglobulins (Molecular Probes, Inc., Eugene, OR). The stained coverslips were mounted onto glass slides with Prolong™ Antifade reagent (Molecular Probes), and the fluorescently labeled cells were visualized on a Zeiss LSM510 confocal microscope.

For the cytchalasin D experiments, CHO-PTA-1 and CHO-DAF cells were grown on glass coverslips overnight and then fixed and permeabilized with cold methanol for 10 min at −20 °C. Dual color immunostaining was performed with Alexa 488-conjugated goat anti-rabbit immunoglobulins (Molecular Probes, Inc., Eugene, OR). The stained coverslips were mounted onto glass slides with Prolong™ Antifade reagent (Molecular Probes), and the fluorescently labeled cells were visualized on a Zeiss LSM510 confocal microscope.

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individual images using Adobe Photoshop™ software.

Construction, Expression, and Purification of GST Fusion Proteins—
The GST-PTA-1 construct containing the PTA-1 carboxyl terminus and part of the transmembrane region (aa 266–336) was generated by subcloning the EcoRI restriction fragment from pGBT9LaCZ-PTA-1 to pGEX-2T (Amersham Pharmacia Biotech). The GST-PTA-1-TRV construct also contains the same region of PTA-1 but lacks the three carboxyl-terminal residues Thr, Arg, and Val (aa 286–333), subcloned to pGEX-4T-3 using EcoRI/NotI restriction sites (provided by Dr. P. Sherrington). The GST-4.1-G-Tail (GST-4.1-G-CTD) construct containing the 175 carboxyl-terminal residues (aa 830–1005) was prepared from EcoRI restriction of pGAD10–4.1-G subcloned to pGEX-2T. The GST-4.1G-Head construct comprises the 67 amino-terminal residues (aa 1–115) of PTA-1 and is complemented with GST protein expression was induced with 0.1 mM isopropyl-1-thio-

BL21 (DE3) cells (Stratagene) were transformed with the various GST constructs, cultured to an A600 of 0.6–1.0, and recombinant GST fusion protein expression was induced with 0.1 mM isopropyl-1-thio-

Triton X-100 was added (1% final) and mixed for 10 min at 4°C, and the soluble and insoluble proteins were separated by centrifugation at 3000 × g for 10 min. The recombinant GST proteins were purified from the soluble supernatant by incubation with PBS-washed glutathione-Sepharose 4B beads (Amersham Biosciences) at 4°C for 2 h. The GST-head complexes were then washed three times with PBS. For the pull-down experiments, the GST-head complexes were further washed with lysis buffer prior to incubation with cell lysates. For recombinant GST protein purification, the GST proteins were washed four times with bed volumes of glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8, supplemented with Complete™ protease inhibitors) at 4°C, and the beads were removed by centrifugation. The eluted proteins were evaluated for purity and integrity by SDS-PAGE and quantified by spectrophotometry.

GST Pull-down Assay—For some experiments, Jurkat cells were biosynthetically labeled with [35S]cysteine/methionine prior to lysis. The cells were pelleted and washed with PBS prior to digitonin (Sigma) lysis buffer and then eluted by boiling with reducing Laemmli sample buffer. Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and then analyzed by immunoblotting.

RESULTS

PTA1 Is Recruited into Membrane Rafts and Associates with Rap1 and the Cytoskeleton upon Cross-linking—A physical and functional association between PTA-1 and LFA-1 integrin suggested that PTA-1 may be implicated in the adhesive processes that occur during T cell activation. Many of the molecules that engage in the T cell signaling cascade are found to localize in or be recruited to lipid rafts during the activation process (8). To examine whether PTA-1 might be contained within rafts, we used the biochemical approach of isolating the cold Triton X-100-insoluble material known as GEMs or detergent-insoluble glycolipid structures that may represent raft material (Fig. 1A). Resting T lymphocytes and Jurkat T cells express very little PTA-1 protein (54); therefore, to facilitate protein detection, we utilized Jurkat cells stably transfected with PTA-1 for these assays. However, similar results were obtained with non-transfected Jurkat cells that had been prestimulated for 30 h with TPA to induce PTA-1 protein expression and then rested overnight before restimulation (data not shown; see below). In these assays, a proportion of PTA-1 from resting cells was found to localize to the GEM fraction (G in Fig. 1A), indicating raft-resident status; however, cross-linking of the PTA-1 antigen with anti-PTA-1 mAb followed by rabbit anti-mouse antibody (PTA-1 + RAM) (Fig. 1A) or stimulation of the cells with TPA (Fig. 1B) induced an increased proportion of the PTA-1 to locate to the GEM fraction. The endoplasmic reticulum-resident protein, calnexin, was used as a control in these experiments and was found predominantly in the soluble fraction (S) as expected; its distribution was not altered by cell stimulation (Fig. 1B). In these experiments, it was also found that cross-linking of PTA-1 resulted in an accumulation of this antigen in the washed cytoskeletal pellet that had precipitated through the sucrose gradient (I; Fig. 1A).

Upon cross-linking or cell stimulation with TPA or anti-CD3, PTA-1 becomes phosphorylated on a serine residue and associates with LFA-1 (58, 59). Subsequent LFA-1 engagement and cross-linking results in the tyrosine phosphorylation of PTA-1 and its association with Lyn PTK (59). These data implicate PTA-1 in both the regulation of LFA-1 function and further downstream signaling. Recent work has strongly implicated Rap1 in the regulation of LFA-1 function (22), and in the transfected Jurkat cells, we found Rap1 to be a resident of GEMs (Fig. 1B); therefore, we considered whether PTA-1 might associate with Rap1. The use of GST-PTA-1 in pull-down experiments did not reveal any direct association (data not shown); therefore, we employed the method developed by Harder and Kuhn (62) to analyze plasma membrane subfragments for associated signaling molecules. Magnetic beads were...
and shown to contain the same sequence identified as identical against a range of controls. The three clones were sequenced.

CD3-coated beads after 5 and 10 min of cross-linking at 37°C were immunoblotted for PTA-1, calnexin, and Rap1.

**A.** The GEM fractions and protein equivalents of the soluble fractions of the total protein content; therefore, because of equal protein loading, I). Note that the soluble fraction contains over 90% of the total protein content; therefore, because of equal protein loading, PTA-1 contained in this fraction is greatly diluted and yields a low signal. Note also that PTA-1 is represented by the diffuse band at around 64 kDa; the tight band below (*) is a nonspecific band that was sometimes observed in immunoblots for PTA-1. As a control, identical samples were immunoblotted for the endoplasmic reticulum protein calnexin. B, PTA-1-transfected Jurkat cells were stimulated with TPA as indicated, and cell lysates were separated on sucrose gradients as in A. The GEM fractions and protein equivalents of the soluble fractions were immunoblotted for PTA-1, calnexin, and Rap1. C, immunoblotting for Rap1 shows association with the plasma membrane fragments immunopurified with anti-PTA-1-coated magnetic beads but not with anti-CD3-coated beads after 5 and 10 min of cross-linking at 37°C.

coated with the anti-PTA-1 mAb, LeaA1, or, as a control, with anti-CD3. These were mixed with PTA-1-transfected Jurkat cells and warmed at 37°C for 5 or 10 min before homogenization of the cells and immunopurification of the bound membrane fragments. Analyzed by immunoblotting, the CD3 fragments co-purified with several signaling molecules after 5 and 10 min of cell stimulation (62) (data not shown) but not with Rap1 (Fig. 1C). In contrast, stimulation and immunoprecipitation with the anti-PTA-1 (LeaA1)-coated beads revealed that Rap1 co-assembles with PTA-1 in membrane fragments isolated from the transfected Jurkat cells after PTA-1 cross-linking (Fig. 1C). This result places Rap1 in the vicinity of PTA-1 and LFA-1 at the plasma membrane of these cells; other signaling molecules associated with the complex will be reported elsewhere.

**The Cytoplasmic Domain of PTA-1 Binds the Carboxy-terminal Domain (CTD) of Protein 4.1G—**Yeast two-hybrid analysis was then used to identify PTA-1 binding partners that might contribute to complex formation. The entire cytoplasmic region and part of the transmembrane domain of PTA-1 in the bait vector (Fig. 2A) was used to screen a Jurkat T cell cDNA library. The entire library of 2 × 10⁶ independent clones was screened, and three positive clones were isolated. All three clones were confirmed as true binding partners when tested against a range of controls. The three clones were sequenced and shown to contain the same sequence identified as identical to the CTD of protein 4.1G (Fig. 2B), a ubiquitous member of the protein 4.1 family (65). The relative strength of binding between PTA-1 and the CTD of 4.1G was measured in a liquid β-galactosidase assay, and the well-characterized CD4-Lck interaction was measured as a comparative control; CD4-Lck interaction produced 2.9 β-galactosidase units, and the PTA-1–4.1G produced 91.4 units, indicating strong binding in the yeast two-hybrid assay. To further validate this result and to identify additional binding partners, we used the same region of PTA-1 to screen a second cDNA library. A human brain library was selected, because PTA-1 is also expressed in certain brain tissues,2 and 11 positive clones were isolated from screening 5 × 10⁶ clones. Validation tests confirmed only one of these clones to be a true positive, and this was found to encode exactly the same protein as those isolated from the Jurkat library. Isolation of this same clone validates the interaction between PTA-1 and the CTD of 4.1G in this assay, since the same protein was obtained from four separate clones and two different libraries.

Parra et al. (65) record that protein 4.1G is generally expressed in a wide range of tissues but is relatively less abundant than 4.1R in hematopoietic tissues and only weakly expressed in peripheral leukocytes; therefore, we examined the expression of 4.1G mRNA in Jurkat cells by Northern blot analysis. Fig. 2C shows that 4.1G mRNA in these cells appears as three major transcripts, at approximately 4.1, 4.6, and 7.3 kb. This is very similar to the pattern reported for human brain but quite different from that obtained in seven other human tissues where a single prominent transcript at around 5 kb was observed (65). The multiple brain transcripts were attributed to alternative splicing and/or differential use of polyadenylation sites (65), and in support of alternative splicing in Jurkat cells we isolated a cDNA from this cell line that showed apparent differential exon usage from the published sequence of 4.1G assembled from various EST clones (65) (GenBank™ accession number A5126600). Since the expression of PTA-1 in Jurkat cells is induced by TPA at the transcriptional level (65), we also examined the influence of TPA on the isoforms of 4.1G expressed in these cells. Fig. 2C illustrates that this treatment resulted in the down-regulation of the larger transcripts, leaving only the 4.1-kb transcript.

In order to examine 4.1 protein expression, rabbit antibodies ("tail" antibodies) were raised and affinity-purified against the CTD of 4.1G in the form of a GST fusion protein and used for Western blot analyses. These analyses confirmed the presence of 4.1G protein in the Jurkat cells and also in the CHO cells used for some immunofluorescent staining experiments (Fig. 2D). The major products identified from the Jurkat cells were at around 170 kDa, and a lesser band was seen at around 111 kDa. The largest band approximates in size to the major product identified from transfected COS cells by Parra et al. (65) and is larger than the size of ~113 kDa predicted from the cDNA. These different products possibly could exhibit some post-translational processing, and a smaller band at ~110 kDa was also illustrated by Parra et al. (65) in their transfected COS cell analysis. Fig. 2D from the Jurkat cell lysate also reveals the presence of a series of polypeptides ranging from 30 to 173 kDa; whether these represent alternatively spliced products or proteolytic products is not known, but this pattern was highly reproducible in numerous experiments. Similar results were obtained with CHO cell lysates analyzed in the same way (Fig. 2D), although some of the bands differed in Mr from those of the Jurkat cells. This wide range in polypeptide size was not entirely unexpected, since 4.1 proteins are reported to display

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2 G. F. Burns, unpublished data.
extensive diversity in molecular weight, with one Western blot study revealing 4.1 immunoreactive proteins ranging in size from 30 to 210 kDa (66). Nevertheless, because the CTD of 4.1G bound by PTA-1 in the yeast two-hybrid system that was also used for immunization shares some 60% identity with the same region of 4.1R, it was important to establish that each of the immunoreactive bands represented 4.1G and not cross-reactive 4.1R or another 4.1 family member. To accomplish this, rabbit antibodies were raised and affinity-purified against a GST fusion protein incorporating only the first 115 aa residues that are unique to 4.1G ("head" antibody). Used directly for Western blotting of Jurkat (Fig. 2E) or CHO (data not shown) cell lysates or in reciprocal immunoprecipitation and blotting experiments, both the "head" and the "tail" antibodies yielded similar results, thereby validating the status of the multiple 4.1G polypeptides (Fig. 2E).

We then utilized these antibodies to seek to identify a physical in vivo association between PTA-1 and 4.1G. CHO cells stably transfected with PTA-1 were used in these experiments, and good co-localization was seen at the plasma membrane between PTA-1 and 4.1G detected with either "head" or "tail" antibodies (Fig. 3A). Protein 4.1 contains a spectrin/actin-binding domain and might be expected to associate with actin, particularly at the submembranous skeleton at the plasma membrane. To visualize this, permeabilized transfected CHO cells were incubated with Alexa 488-phalloidin to illuminate F-actin and also stained for 4.1G (Fig. 3B). Some degree of co-localization was obtained, but this was much more evident after the actin cables were disrupted by treatment with cytochalasin D causing the fibers to collapse into a pronounced lumpy appearance. After such treatment, 4.1G staining was found to be coincident with the areas of condensed actin (Fig. 3B).

It was noted above that upon antibody-mediated cross-linking of PTA-1 on Jurkat cells, a proportion of the PTA-1 was found to precipitate with the cytoskeleton (see Fig. 1A). Therefore, we questioned whether this might also occur in the transfected CHO cells and, if so, whether this distribution of 4.1G...
was also influenced by such cross-linking. PTA-1-transfected CHO cells were treated with anti-PTA-1 mAb (or control) and then cross-linked with RAM before lysis and centrifugation of the postnuclear supernatant to isolate the cytoskeletal pellet. Immunoblotting for PTA-1 showed that after specific cross-linking a large proportion of the PTA-1 antigen now pelleted with the cytoskeleton, indicating a tight association (Fig. 3C). Reblotting the same samples for 4.1G revealed that a proportion of this protein pelleted with the cytoskeleton in the resting CHO cells; however, this proportion was increased following cross-linking of PTA-1 (Fig. 3C). These results show that cross-linking of PTA-1 on the cell surface can influence the distribution of 4.1G, further substantiating an association between the molecules.

The Carboxyl-terminal Peptide of PTA-1 Binds the MAGUK, Human Discs Large—As we documented in the Introduction, members of the protein 4.1 family are found to associate with MAGUKs, which play an important role in assembling signal transduction complexes at the interface of the membrane cytoskeleton (67). MAGUKs contain one or more PDZ domains that engage in protein-protein interactions, including binding to the carboxyl-terminal peptides of some transmembrane proteins. The carboxyl terminus of PTA-1 terminates in the sequence Lys-Thr-Arg-Val (KTRV), a recognized PDZ-binding motif; therefore, we determined whether this region of PTA-1 engaged in protein binding. For this, we prepared GST fusion proteins encoding the entire cytoplasmic tail of PTA-1 or the PTA-1 tail lacking the three terminal residues, TRV (PTA-1TRV). These were used in pull-down experiments with lysates of resting or TPA-stimulated Jurkat cells that had been prelabeled with [35S]Cys/Met. The fluorographs obtained revealed several polypeptides that bound to GST-PTA-1 but not to GST alone (Fig. 4A), indicating specific binding. Comparison of the specific bands precipitated by PTA-1 versus PTA-1TRV identified several differences, including one polypeptide at around 21 kDa that, intriguingly, was more prominent in the PTA-1TRV precipitate (Fig. 4A). The identity of this band is not known, but this result indicates that the carboxyl-terminal peptides can influence PTA-1-protein binding, possibly either by direct binding to a PDZ-containing protein that blocks binding to another site or by virtue of a particular conformation. Two bands in particular were prominent in the PTA-1 pull-downs that were negligible or absent in the pull-downs with PTA-1TRV; notably, these bands were also more prominent from lysates of Jurkat cells that had been stimulated with TPA-1 (Fig. 4A).

Either of these two bands might represent binding to a PDZ-containing protein, and we focused on the band at around 110 kDa (Fig. 4A, arrow) because this approximates the migration of hDlg in SDS-PAGE, and hDlg has been shown to associate with protein 4.1R in epithelial cells (52). Further pull-down experiments were therefore carried out with the PTA-1 and PTA-1TRV proteins and tested by immunoblotting for hDlg; in addition, since PKC-mediated phosphorylation of PTA-1 has been implicated in its function (68), we also tested binding to the GST-PTA-1 protein that had been subjected to PKC-mediated phosphorylation in vitro (+PKC). The results obtained are shown in Fig. 4B, where it is apparent that GST-PTA-1 can bind to hDlg. Binding is modestly increased after phosphorylation of the GST-PTA-1 by PKC, but removal of the terminal TRV peptide residues from PTA-1 almost abolishes binding of hDlg, with the small residual binding perhaps being attributable to a ternary complex (Fig. 4B). To confirm that PTA-1 associates with hDlg in vivo, we carried out co-precipitation analyses from TPA-stimulated Jurkat PTA cells analyzed by Western blotting and established that precipitation of PTA-1 co-precipitates hDlg; however, the co-association signal was very faint (data not shown), possibly because the associated molecules were being lost by prior precipitation with the cytoskeleton. In support of this, when co-immunoprecipitations were carried out after pretreatment of the cells with cytochalasin D to disrupt the actin cytoskeleton, clear co-association was obtained (Fig. 4C).

Next we examined whether hDlg was localized in the GEM fraction representing membrane rafts and whether its distribution in the transfected Jurkat cells was altered following TPA treatment or cross-linking of PTA-1 (Fig. 5). PTA-1-transfected Jurkat cells were left untreated or stimulated with TPA for 3 or 30 min before cell lysis and centrifugation through sucrose gradients to isolate the GEM fraction. The isolated GEM fractions were run in SDS-PAGE together with equal protein loading from the soluble fractions (Fig. 5A) and then immunoblotted for hDlg and also 4.1G (head), PTA-1, and, as a control, calnexin. The results (Fig. 5B) indicated that hDlg is a resident of rafts; the proportion of the protein found in the GEM fraction did not increase upon treatment of the cells with TPA but actually appeared to decrease without material becoming apparent in the soluble fraction, probably indicating...
loss of hDlg to the pelleted cytoskeleton. In these assays, both 4.1G and PTA-1 were identified predominantly in the GEM fractions, whereas calcinein located to the soluble fractions (Fig. 5B). Experiments using PTA-1 cross-linking in which the cytoskeletal pellet also was analyzed showed that hDlg was prominent in this fraction as well as the GEM fraction, although the relative distribution of hDlg was not altered by cross-linking (data not shown).

The PDZ-binding Peptide at the Carboxyl Terminus of PTA-1 Influences Binding to Multiple Isoforms of 4.1G—As we noted above, the cytoplasmic tail of PTA-1 can be phosphorylated by PKC after treatment of Jurkat cells or platelets with TPA. The residue that is phosphorylated in this way was identified by Shibuya et al. (59) as serine 329 toward the carboxyl terminal of PTA-1, within the last 8 residues (FS329RRPKTRV) and adjacent to the putative PDZ-binding peptide identified by us. Shibuya et al. (59) mutated this serine residue to phenylalanine (Ser → Phe) and recorded that this mutation abrogated PTA-1 phosphorylation in response to TPA treatment of transfected murine thymoma cells and also that these cells were no longer able to mediate PTA-1-mediated cell adhesion; the authors concluded from these data that phosphorylation of Ser329 plays a critical role in PTA-1-mediated adhesion and signaling.

We therefore tested whether Ser329 might be implicated in PTA-1 binding to 4.1G. Table I shows a representative yeast two-hybrid binding analysis of the interaction between the CTD of protein 4.1G, the cytoplasmic region of PTA-1, and mutations of PTA-1 in which Ser329 was mutated to either phenylalanine (Ser → Phe) or alanine (Ser → Ala). As shown in Table I, measured in β-galactosidase units, it is apparent that substitution of the serine at 329 with phenylalanine reduced the strength of binding in this assay but still permitted strong binding to occur. Substitution of this serine residue with alanine did not significantly reduce the PTA-1–4.1G interaction. From these analyses, we conclude that whereas Ser329 within PTA-1 may be implicated in binding to the CTD of protein 4.1G, such involvement is more likely to result from the conformation of PTA-1 in this region rather than an involvement of serine phosphorylation, since the bulky phenylalanine residue caused more disruption than alanine as a substitute. In support of this interpretation, we found that substitution of Ser329 with aspartate (Ser → Asp) to mimic phosphoserine did not alter the strength of PTA-1–4.1G interaction in the β-galactosidase assay (data not shown). It is interesting to note that the Ser → Phe substitution that still binds to CTD of 4.1G in the context of the PTA-1 cytoplasmic tail now has the sequence FFRR, similar to the FFKR sequence within the α subunit of LFA-1 that is central to the regulation of integrin adhesive function (69, 70); should this region be directly implicated in the PTA-1–4.1G binding interaction, then it is possible that LFA-1 also could bind to the CTD of 4.1G.
of erythrocytes, between 4.1R, the MAGUK, p55, and the transmembrane protein, glycoprotein C, is interdependent and dynamic; for example, the affinity of binding between glycophorin C and p55 is increased by an order of magnitude by 4.1R in *in vitro* binding assays (43). Therefore, we utilized the GST fusion proteins PTA-1 and PTA-1ΔTRV and also *in vitro* phosphorylated GST-PTA-1 in pull-down assays for 4.1G to determine whether the putative PTA-1-hDiG-4.1G complex might be similarly interdependent (Fig. 6).

For these experiments, we used lysates from Jurkat cells that had been left untreated or stimulated for 3 min, 10 min, 2 h, or overnight with TPA. Initially, we concentrated on examining immunoblots of the higher molecular weight material, around the major 4.1G 170-kDa isoform identified in whole cell lysates. An example of these blots is shown in Fig. 6A, and similar results were obtained by immunoblotting with the “tail” antibody from lysates from both unstimulated and TPA-stimulated Jurkat cells (data not shown). It is apparent that whereas there is a degree of specific pull-down by the PTA-1 fusion proteins compared with GST alone, there are no differences between PTA-1 and PTA-1ΔTRV; nor is there any relative enrichment of this 4.1G isoform in the pull-downs compared with the total lysate.

A totally different profile was obtained when we examined the pull-downs of lower molecular weight isoforms of 4.1G (Fig. 6B). Several bands were prominent in the PTA-1 fusion protein pull-downs that were absent in the GST-alone track, and some were apparently greatly enriched compared with the whole lysate blot. The *arrowheads* in Fig. 6B point to bands that were consistently seen to be enriched in the PTA-1 fusion protein pull-downs; these bands were identified with both the “tail” antibody (Fig. 6B) and the “head” antibody (data not shown), indicating that they represent 4.1G isoforms and not proteolytic breakdown products, and each of the identified isoforms appeared to be equally represented in lysates from unstimulated or GST-stimulated Jurkat cells (Fig. 6B, a and b). Of particular interest were the bands identified at around 55 and 35 kDa (*asterisk* on *arrowhead* in Fig. 6B). The isoform at ~55 kDa was poorly represented in the total cell lysates and strongly precipitated by each of the PTA-1-GST fusion proteins, but the pull-down with PTA-1ΔTRV yielded more of this product than native or phosphorylated PTA-1 (Fig. 6B). This result perhaps indicates that interaction between PTA-1 and a PDZ-containing protein reduces the association between PTA-1 and the 4.1G isoforms of 4.1G. The opposite was the case with the 35-kDa isoform pulled down by native and phosphorylated PTA-1 but not by PTA-1ΔTRV. In this instance, the binding of

| Sample   | β-Galactosidase units |
|----------|-----------------------|
| PTA1 alone| 0.99 ± 0.22           |
| Ser → Phe alone | 0.62 ± 0.40         |
| Ser → Ala alone  | 0.29 ± 0.07           |
| PTA1 + 4.1G -LT  | 85.41 ± 13.33         |
| Ser → Phe + 4.1G -LT | 23.72 ± 3.30         |
| Ser → Ala + 4.1G -LT | 70.95 ± 3.81         |
| PTA1 + 4.1G -LTH | 216.56 ± 76.49        |
| Ser → Phe + 4.1G -LTH | 97.16 ± 12.97        |
| Ser → Ala + 4.1G -LTH | 168.01 ± 26.44       |
| PTA1 + pTD1    | 3.34 ± 1.72           |
| Ser → Phe + pTD1 | 10.18 ± 6.03          |
| Ser → Ala + pTD1 | 1.24 ± 0.51           |

**TABLE I**

Quantitative yeast two-hybrid analysis of the interaction between mutations of PTA-1 and the CTD of 4.1G

*S. cerevisiae* HF7c cells were co-transformed as described under “Experimental Procedures.” Protein interactions were tested by growth in selective liquid SD medium lacking Trp for the single transformants, Leu and Trp (−13) for the double transformants, or Leu, Trp, and His with the inclusion of 3-aminotriazole (−LTH) for higher stringency and then quantified using a liquid β-galactosidase assay. The strong interaction between the cytoplasmic tail of PTA-1 and the 4.1G CTD under activation domain pTD1 plasmid. The expression of these blots is shown in Fig. 6A, around the major 4.1G 170-kDa isoform identified in whole cell lysates. An example of these blots is shown in Fig. 6A, and similar results were obtained by immunoblotting with the “tail” antibody from lysates from both unstimulated and TPA-stimulated Jurkat cells (data not shown). It is apparent that whereas there is a degree of specific pull-down by the PTA-1 fusion proteins compared with GST alone, there are no differences between PTA-1 and PTA-1ΔTRV; nor is there any relative enrichment of this 4.1G isoform in the pull-downs compared with the total lysate.

A totally different profile was obtained when we examined the pull-downs of lower molecular weight isoforms of 4.1G (Fig. 6B). Several bands were prominent in the PTA-1 fusion protein pull-downs that were absent in the GST-alone track, and some were apparently greatly enriched compared with the whole lysate blot. The *arrowheads* in Fig. 6B point to bands that were consistently seen to be enriched in the PTA-1 fusion protein pull-downs; these bands were identified with both the “tail” antibody (Fig. 6B) and the “head” antibody (data not shown), indicating that they represent 4.1G isoforms and not proteolytic breakdown products, and each of the identified isoforms appeared to be equally represented in lysates from unstimulated or GST-stimulated Jurkat cells (Fig. 6B, a and b). Of particular interest were the bands identified at around 55 and 35 kDa (*asterisk* on *arrowhead* in Fig. 6B). The isoform at ~55 kDa was poorly represented in the total cell lysates and strongly precipitated by each of the PTA-1-GST fusion proteins, but the pull-down with PTA-1ΔTRV yielded more of this product than native or phosphorylated PTA-1 (Fig. 6B). This result perhaps indicates that interaction between PTA-1 and a PDZ-containing protein reduces the association between PTA-1 and the 4.1G isoforms of 4.1G. The opposite was the case with the 35-kDa isoform pulled down by native and phosphorylated PTA-1 but not by PTA-1ΔTRV. In this instance, the binding of
a PDZ-containing protein may be required for binding to this small isoform of protein 4.1G. Note that in Fig. 6B, the immunoblot of the whole lysate appears to identify a prominent band in the same position as the 35-kDa isoform pulled down by PTA-1. However, this band, which actually migrates slightly slower than the PTA-1-associated band, is not the same polypeptide, since this band is not illuminated with the "head" antibody, whereas the associated band is identified by both the "head" and "tail" antibodies (Fig. 6C). Therefore, this isoform too represents a minor 4.1G product that is greatly enriched by binding to PTA-1 in pull-down experiments.

The Amino-terminal Region of 4.1G Binds to PTA-1 from Activated Cell Lysates—Despite the association between PTA-1 and 4.1G documented in numerous assays above, in repeated attempts we were unable to demonstrate an association between PTA-1 from cell lysates and the 4.1G CTD used as a GST fusion protein. The reasons for this are not clear, but it was not readily attributable to interference by another binding protein, since we obtained no detectable direct binding between GST-PTA-1 and GST-4.1G CTD in surface plasmon resonance experiments (data not shown). In further pull-down experiments with GST fusion proteins, we also sought to identify an association between PTA-1 and activated Rap1 by pull-down with the Rap binding protein (20), followed by immunoblotting for PTA-1. These also gave a negative result, but for these experiments we had used the GST-4.1G-Head protein as an additional control and obtained the unexpected result that this unique domain of 4.1G was able to pull down PTA-1 from lysates of activated but not resting Jurkat cells (Fig. 7). The example shown in Fig. 7A shows the pull-down of PTA-1 obtained from Jurkat cells that had been either pretreated with TPA for 30 h to induce PTA-1 protein expression and then rested overnight (Nil); treated with the anti-PTA-1 mAb (LeoA1) alone, with anti-PTA-1 cross-linked with RAM antibody, or with TPA to induce activation. Western blotting for PTA-1 shows that this protein was precipitated by the GST-4.1G "head" protein after the Jurkat cells had been stimulated by antibody-mediated cross-linking (Fig. 7A, track 10) or by treatment with TPA (Fig. 7A, track 14). These results indicate that upon activation of the Jurkat cells, PTA-1 undergoes some change enabling it to bind the amino-terminal region of 4.1G; this change could be in consequence of a direct conformational change in PTA-1, perhaps mediated by phosphorylation or lipid association, or by an association between PTA-1 and another molecule such as hDlg. Alternatively, activation of the Jurkat cells could cause a conformational change to a PTA-1-binding protein such that it is no longer able to bind, thus freeing PTA-1 to bind to the head region of 4.1G. To begin to discriminate between these different possibilities, we carried out direct binding experiments between GST-PTA-1 and GST-4.1G "head" proteins by surface plasmon resonance. The results obtained from these BIACore experiments (Fig. 7B) show that these two GST-fusion proteins can interact directly. From this, we can conclude that the cytoplasmic tail of PTA-1 can bind to the "head" region of 4.1G without the requirement for amino acid modification or binding to an intermediary protein.

Taken together, we believe that the simplest interpretation of the different 4.1 binding data obtained in this report is that in the resting cell, PTA-1 constitutively associates with the CTD of 4.1G. Because of the relative excess of 4.1G protein over PTA-1 in these cells (witnessed in immunofluorescent staining and immunoprecipitation experiments (data not shown)), PTA-1 in the cell lysate is unable to bind exogenously presented 4.1G CTD in the form of a GST fusion protein. There are likely also to be conformational constraints, since GST-PTA-1 and GST-4.1G CTD did not bind directly in surface plasmon resonance experiments (data not shown); but direct binding was indicated within the context of yeast cells, and GST-PTA-1 was able to precipitate multiple isoforms of 4.1G from lysates of resting Jurkat cells (Fig. 6). Upon cell activation, we propose, there is induced a conformational change in 4.1G, perhaps analogous to that which occurs in ERM proteins (38), that alters the conformation of the CTD of 4.1G and exposes a PTA-1 binding region in the head domain. These dynamic associations, together with an association with hDlg, provide a structural basis for a regulated protein complex at the T cell surface with links to the cytoskeleton.

**DISCUSSION**

Here we present evidence for a direct physical association between a T cell activation antigen, PTA-1, a protein 4.1 family member, 4.1G, and a MAGUK, human discs large. Since the related protein 4.1R also binds hDlg (71), it is possible that PTA-1 forms a ternary complex with 4.1G and hDlg, redolent of other 4.1-transmembrane protein-MAGUK ternary complexes that form important functional units serving as a clustering apparatus at sites of cell-cell communication (41). The reported physical and functional association between PTA-1 and LFA-1 (59), together with its involvement in the development of cytotoxic T cells from their precursors (55), suggests that such a
complex may play an important functional role at the immunological synapse. A model incorporating this putative complex is given in Fig. 8.

There are, however, substantial differences between the molecular interactions described for the other known ternary complexes involving a transmembrane protein in association with 4.1 proteins and that described here for PTA-1. Thus, in the best characterized example, involving the transmembrane protein glycoporphin C, the MAGUK, p55, and protein 4.1R, both the cytoplasmic domain of glycoporphin C and the HOOK domain of p55 bind to the FERM domain of 4.1R at adjacent sites (72). By contrast, the cytoplasmic domain of PTA-1 binds to the cytoplasmic tail of protein 4.1G, as determined in yeast two-hybrid assays, and to the amino-terminal domain (ATD) of 4.1G, at least in activated cells, as shown in GST fusion protein pull-down assays and by surface plasmon resonance. Note that the ATD used in these assays incorporated only the first 115 residues of 4.1G, a region that is unique to this protein 4.1 family member and does not incorporate any of the downstream FERM domain (see Fig. 2B). The function of these domains of 4.1 family members is unknown. The CTD of protein 4.1G has been shown to bind to the immunophilin-binding protein, FKBP13, that may function as a molecular chaperone to catalyze the folding and/or assembly of proteins in the endoplasmic reticulum (73), and to an intracellular loop of the A1 adenosine receptor (74). In addition, the CTD of nuclear forms of the paralogues 4.1N and 4.1R have been demonstrated to bind to and to be implicated in the functioning of a nuclear GTPase that regulates phosphatidylinositol 3-kinase kinase activity and the nuclear mitotic apparatus protein, respectively (75, 76). The ATD of 4.1R contains a high affinity calcium-dependent calmodulin binding domain (77); however, this region of 4.1G shares only 23% identity over the 17 residues involved (73); therefore, it is unclear whether such a functional domain is present in 4.1G.

To our knowledge, PTA-1 is the first protein identified as binding the unique head region of 4.1G.

We show that PTA-1 also binds the MAGUK, hDlg, probably through binding to a PDZ domain. This finding potentially places PTA-1 as a membrane anchor for a large multimeric complex incorporating a diversity of functional entities, each with the capacity to modify the immunological response. hDlg comprises a series of modules that bind to a bewildering number of proteins. The protein additionally undergoes multimerization that could contribute to clustering (67). The proline-rich amino-terminal domain of hDlg interacts with Lck protein tyrosine kinase. This is followed by three PDZ domains known to bind the potassium ion channel, Kv1.3, and the tumor suppressor PTEN among others (47, 78); a Src homology 3 domain; and then a HOOK domain able to bind the FERM domain of 4.1R. Whether this interaction also occurs between hDlg and 4.1H has not been established, but this is likely to be the case, since the FERM domains of these paralogues are highly homologous, and, at least in epithelial cells, this interaction plays a critical role in recruiting hDlg to the lateral membrane (52). At the carboxyl terminus of hDlg is a guanylate kinase-like domain, the hallmark of MAGUK family members. This domain is enzymically inactive and seems to have evolved as a specialized protein recognition module. The guanylate kinase domain of hDlg has been shown to bind to the Rap1 GTPase-activating protein known as SPA-1, SPAL, or SPAR that plays a role in regulating cell adhesion (44, 45, 79). This domain also binds to the kinesin-like motor protein known as GAKIN, and in T lymphocytes (Jurkat T cells) the hDlg-GAKIN complex moves from the cytoplasm to the lymphocyte cap, presumably along microtubules, upon stimulation by antibody-mediated cross-linking to induce cell activation (46). GAKIN-mediated transport of hDlg is dependent upon its molecular motor domain, and Asaba et al. (51) have proposed that hDlg is a cargo molecule of GAKIN that is directed to specialized sites of cell-cell contact by the microtubule plus end-directed motor protein. Adding to the potential size of the complex is the range of partners known to associate with protein 4.1 family members by binding to the FERM domain; these include Ca2+-calmodulin, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate and transmembrane proteins such as CD44 (41), and it has been suggested that this domain is likely also to bind several GTPase protein regulators (41, 80).

The FERM domains of 4.1G and 4.1R share 76% identity (65); therefore, it is likely that 4.1G also has several binding partners in this region. Whether any of these 4.1/hDlg binding molecules also co-assemble with a PTA-1 complex is not known, but since several can be located to rafts at the immunological synapse, it seems possible that such multimeric complexes will be formed. This possibility raises the interesting questions of where the complex(es) are formed and how the existence of such a PTA-1-associated complex might influence LFA-1 binding and signaling.

We favor the hypothesis that the 4.1G-associated complexes are formed at recycling rafts found in the Golgi region (81). Parra et al. (65) demonstrated that 4.1G is concentrated in the perinuclear region in transfected COS cells, and we show here that 4.1G, hDlg, and PTA-1 are concentrated in the GEM fraction that represents raft material. The composition of the...
raft material would promote clustering and protein-protein associations, and, in association with growing microtubules, the complex could be delivered to specific areas at the leading edge (82), perhaps driven by GAKIN. Alonso and Millán (83) have recently provided a detailed consideration of the role of rafts in the assembly and trafficking of signaling complexes in T cells; they note that in these cells, the uropod is rich in rafts that might provide a reservoir for subsequent delivery of raft proteins and lipids to the immunological synapse. Notably, TCR engagement drives the transport of gangliosides and Lck (which is known to associate with hDlg) (47) from an intracellular store to the plasma membrane and the translocation of Lck-associated PKCθ to rafts at the immunological synapse. Of interest, ICAM-3 that accumulated in the uropod of polarized T cells after delivery by binding to the FERM domain of moesin (34) is subsequently targeted to sites of immune cell-cell contact by an unknown mechanism (10). The similarities between the FERM domains among 4.1 superfamily members might allow ICAM-3 at the uropod to interact with 4.1G for transport back to the leading edge. Similarly, the FERM domain of 4.1G potentially could interact with the β subunit of LFA-1 (29), thereby providing a mechanism for the documented association with PTA-1 (59). However, Shibuya et al. (59) noted that PTA-1 associated specifically with LFA-1 after T cell activation rather than with all β2 integrins as would be the case if β2 interacted with the 4.1G FERM domain. In this regard, the recent work of Katagiri et al. (84) may be instructive. These authors identified a Rap1 effector termed RAPL that binds to Rap1 and also to subunit of LFA-1 (29), thereby providing a mechanism for the documented association with PTA-1 (59). However, Shibuya et al. (59) noted that PTA-1 associated specifically with LFA-1 after T cell activation rather than with all β2 integrins as would be the case if β2 interacted with the 4.1G FERM domain. In this regard, the recent work of Katagiri et al. (84) may be instructive. These authors identified a Rap1 effector termed RAPL that binds to Rap1 and also to 23. Tohyama, Y., Katagiri, K., Pardi, R., Lu, C., Springer, T. A., and Kinashi, T. (2002) J. Biol. Chem. 277, 1151–1158 24. Leverkus, M., Rey-Ladino, J., Dreolini, L., Shaw, D., and Takei, F. (2003) J. Cell Biol. 159, 215–222 25. Porter, J. C., Bracke, M. M., Smith, A. M., Davies, D., and Hogg, N. (2002) J. Immunol. 168, 6330–6335 26. Katagiri, K., Hattori, M., Minato, N., and Kinashi, T. (2002) Mol. Biol. Cell. 13, 2957–2968 27. Stewart, M. 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The LFA-1-associated Molecule PTA-1 (CD226) on T Cells Forms a Dynamic Molecular Complex with Protein 4.1G and Human Discs Large

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