CADM1 Interacts with Tiam1 and Promotes Invasive Phenotype of Human T-cell Leukemia Virus Type I-transformed Cells and Adult T-cell Leukemia Cells

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CADM1 encodes a multifunctional immunoglobulin-like cell adhesion molecule whose cytoplasmic domain contains a type II PDZ95/Dlg/ZO-1 (PDZ)-binding motif (BM) for associating with other intracellular proteins. Although CADM1 lacks expression in T lymphocytes of healthy individuals, it is overexpressed in adult T-cell leukemia-lymphoma (ATL) cells. It has been suggested that the expression of CADM1 protein promotes infiltration of leukemic cells into various organs and tissues, which is one of the frequent clinical manifestations of ATL.

Amino acid sequence alignment revealed that Tiam1 (T-lymphoma invasion and metastasis 1), a Rac-specific guanine nucleotide exchange factor, has a type II PDZ domain similar to those of membrane-associated guanylate kinase homologs (MAGUKs) that are known to bind to the PDZ-BM of CADM1. In this study, we demonstrated that the cytoplasmic domain of CADM1 directly interacted with the PDZ domain of Tiam1 and induced formation of lamellipodia through Rac activation in HTLV-I-transformed cell lines as well as ATL cell lines. Our results indicate that Tiam1 integrates signals from CADM1 to regulate the actin cytoskeleton through Rac activation, which may lead to tissue infiltration of leukemic cells in ATL patients.

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3 The abbreviations used are: ATL, adult T-cell leukemia/lymphoma; HTLV-I, human T-cell leukemia virus type I; GEF, guanine nucleotide exchange factor; PDZ, PSD95/Dlg/ZO-1; DH, Dbl homology; GST, glutathione S-transferase; MAGUK, membrane-associated guanylate kinase homolog; BM, binding motif; Dox, doxycycline; mAb, monoclonal antibody; pAb, polyclonal antibody; HEK, human embryonic kidney; NHD, normal human dermal fibroblast(s); HMVEC, human adult dermal microvascular endothelial cell(s); BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol.
expression of CADM1 accelerates the tissue infiltration of ATL cells (8).

The cytoplasmic domain of CADM1 contains two conserved protein-interaction modules (1). One is the submembranous protein 4.1-binding motif (protein 4.1-BM) in which members of the protein 4.1 family bind and link CADM1 to the actin cytoskeleton (14). The other is the C-terminal EYFI sequence called the type II PDZ-binding motif (PDZ-BM), in which membrane-associated guanylate kinase homologs (MAGUKs) interact through their PDZ (PSD-95, Discs large and ZO-1) domains (6, 15). PDZ domains are composed of ~90 amino acids and bind to the C-terminal PDZ-binding motif of target protein. Type I, II, and III PDZ domains recognize E(S/T)X(V/I), ΦXΦ, and XDXV, respectively, where Φ is any amino acid and Φ is a hydrophobic amino acid residue (16, 17). Proteins harboring PDZ-BM interact with PDZ domain-containing proteins and induce various cellular functions. One well-known example is the Tax oncoprotein encoded by HTLV-I, a key player of ATL leukemogenesis, which has type I PDZ-BM, ETEV, at the C terminus. Tax exerts transforming activities by binding with several intracellular PDZ domain-containing proteins (18, 19), which are believed to be involved in ATL leukemogenesis. Bioinformatic analysis of the amino acid sequence revealed that Tiam1 (T-lymphoma invasion and metastasis 1) has a type II PDZ domain that shares significant similarities with those of MAGUKs. Tiam1 was originally identified as an invasion- and metastasis-inducing gene in murine T-lymphoma cells that encodes a guanine nucleotide exchange factor (GEF) specific for Rac, a member of the Rho GTPases (20, 21). Rho GTPases, including Rho, Rac, and Cdc42, act as molecular switches by cycling between active (GTP-bound) and inactive (GDP-bound) states to regulate actin dynamics that are involved in diverse cellular responses, including cell adhesion and motility (22). The activation of Rho GTPases is mediated by specific GEFs that catalyze the exchange of GDP for GTP. In their active state, Rho GTPases bind to their effectors with high affinity, thereby eliciting downstream responses (22). It has been well documented that reorganization of the actin cytoskeleton by Rho GTPases is the primary mechanism of cell motility and is essential for most types of cell migration. Among the Rho GTPases, Rac has long been known to induce the formation of actin-rich membrane ruffles or lamellipodia at the leading edge of motile cells that are required for forward movement of migratory cells (22–24). Overexpression of Tiam1 is known to increase invasion of T lymphoma cells into a fibroblast monolayer (21) as well as to induce formation of lamellipodia and cell spreading through activation of Rac. There is a growing body of evidence indicating that the interaction between GEFs and other proteins through PDZ motifs is a general mechanism for controlling the exchange activity of GEFs (25). No scaffold protein or integral membrane protein has as yet been reported to associate with the PDZ domain of Tiam1, although this seems highly probable. We hypothesized, therefore, that in addition to its role as an anchor of the actin cytoskeleton to the cell membrane through protein 4.1, CADM1 is able to recruit Rac-specific GEF Tiam1 to the cell membrane and induce reorganization of the cortical actin in ATL cells, thereby rendering ATL cells motile. This scenario could explain the invasive nature of ATL cells overexpressing CADM1.

In the present study, we have demonstrated that the cytoplasmic domain of CADM1 directly associates with Tiam1 through the PDZ domain of Tiam1 and induces formation of lamellipodia through Rac activation in both HTLV-I-transformed cell lines and ATL cell lines. This interaction between CADM1 and Tiam1, therefore, could play a role in infiltration of leukemic cells into various organs and tissues in ATL patients.

**EXPERIMENTAL PROCEDURES**

**Cells**—Jurkat, Molt-4, CCRF-CEM, T-all, and CEM/C2 cell lines derived from acute lymphoblastic T-cell leukemia patients and H9 and Hut78 derived from cutaneous T-cell lymphoma were obtained from the American Type Culture Collection (ATCC). The Jurkat Tet-Off cell line was purchased from Takara Bio. HTLV-I-transformed cell lines used in this study, MT-2, MT-4, C91/PL, and C8166–45, were supplied from the National Institutes of Health AIDS Research and Reference Reagent Program. ATL-1K, TL-Om1, and ATL-43Tb (−) cell lines were leukemic T-cell lines derived from ATL patients and provided by Masanao Miwa (Nagahama Institute of Bio-science and Technology, Nagahama, Japan), Kazuo Sugamura (Tohoku University, Sendai, Japan), and Michiyuki Maeda (Kyoto University, Kyoto, Japan), respectively. The ATL-31 cell line was described previously (26). All T cell lines described above were maintained in RPMI medium (Sigma) supplemented with 10% Tet system-approved fetal bovine serum (Takara Bio), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Mouse NIH3T3 fibroblasts and human embryonic kidney (HEK) 293 cells were from the ATCC and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. Normal human dermal fibroblasts (NHDF) and human adult dermal microvascular endothelial cells (HMVEC) were purchased from Lonza Walkersville Inc. and maintained in culture media specified by the manufacturer’s instructions.

**Antibodies and Reagents**—Mouse monoclonal antibodies (mAbs) specific to V5 (R960-25), CD44 (C26), Talin (8D4), and α-tubulin (sc-8035) were obtained from Invitrogen, BD Biosciences, Sigma, and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. CADM1 antibodies used in this study were rabbit polyclonal antibodies (pAbs) against the cytoplasmic domain, CC2 (7), and number 6 (27); a rabbit pAb against the ectodomain, EC (28); and a chicken mAb against the ectodomain, 3E1 (29). A rabbit pAb to Tiam1 (C16) was purchased from Santa Cruz Biotechnology, Inc. Secondary antibodies used for immunoblot analysis were from GE Healthcare. For immunofluorescent staining, all fluorophore-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. Alexa Fluor 568-phalloidin or Alexa Fluor 633-phalloidin (Invitrogen) was used to visualize the actin cytoskeleton.

**Construction of Expression Vectors**—Full-length human Tiam1 cDNA was generated from adult human brain poly(A) RNA (Takara Bio) by RT-PCR using the Superscript first strand synthesis system (Invitrogen) and the Expand high fidelity PCR system.
Interaction of CADM1 with Tiam1 in ATL Cells

Immunoblot Analysis and Immunoprecipitation—Preparation of cell lysates and immunoblot analysis were described previously (7). A rabbit anti-CADM1 pAb, CC2, was used for detecting CADM1 by immunoblot analysis. For immunoprecipitation, cell lysates containing 500 μg of protein were first precleared by incubation with protein A-Sepharose or protein G-Sepharose (GE Healthcare) for 3 h at 4 °C. The precleared lysates were then incubated with a rabbit anti-CADM1 pAb, EC1, or a mouse anti-5 mA b overnight at 4 °C. A rabbit IgG or mouse IgG was used as a negative control. The protein-antibody conjugates were precipitated with protein A-Sepharose or protein G-Sepharose for 1 h at 4 °C. Immunoprecipitates were rinsed three times with the lysis buffer, fractionated either in a 4–12% gradient Nupage gel (Invitrogen) or a 3–5% Tris acetate vol% acrylamide gel, and stained with Coomassie blue. The bands were visualized by chemiluminescence (Roche Applied Science). The bands were quantified by densitometry using Quantity One software (Bio-Rad Laboratories). The mean optical density (OD) of the bands was calculated and compared with the control using GraphPad Prism 3.02 software (GraphPad Software). The bands without significant differences (p > 0.05) were considered to be statistically equivalent.

RESULTS

Expression of CADM1 and Tiam1 in HTLV-I-transformed Cell Lines and ATL Cell Lines—We first examined localization of CADM1 in HTLV-I-transformed cell lines and ATL cell lines. When cultured in media, these cells tend to grow in aggregates as observed in HTLV-I-transformed MT-2 cells (Fig. 1A). CADM1 was concentrated at cell-cell contact sites (Fig. 1A), indicating that homophilic trans-interaction of CADM1 may have promoted the formation of these cell aggregates. In epithelia, homophilic trans-interaction of CADM1 mediates adhesion of apposing cells (30), and the PDZ-BM of CADM1 associates with MAGUKs, such as MPP3 (15), Pals2 (6), and CASK (5). It has been suggested that the interaction of CADM1 and MAGUKs plays a role in maintaining epithelial morphology (33). In an attempt to elucidate the role of CADM1 in ATL cells, we performed data base homology searches of all NCBI sequences looking for cytoplasmic proteins with type II PDZ domains similar to the PDZ domains of MAGUKs and found T-lymphoma invasion and metastasis 1 (Tiam1), which is a Rac-specific GEF. A GST pull-down assay was performed as described previously (14).
Interaction of CADM1 with Tiam1 in ATL Cells

A.

MT-2

B.

C.

FIGURE 1. Expression of CADM1 and Tiam1 in HTLV-I-transformed cell lines and ATL cell lines. A, expression of CADM1 in the HTLV-I-transformed cell line. MT-2 cells were immunostained for CADM1 (green) and α-tubulin (red) with a rabbit anti-CADM1 pAb (CC2) and a mouse anti-α-tubulin mAb and examined by confocal microscopy. Scale bar, 10 μm. Note that CADM1 is concentrated at the cell-cell attachment sites of the MT-2 cells. B, schematic representation of the domain structure of Tiam1 and sequence alignment of type II PDZ domains of selected MAGUKs and Tiam1. Tiam1 contains myristoylation signal (myr), Pleckstrin region (P), N-terminal pleckstrin homology domain (PHn), Ras-binding domain (RBD), type II PDZ (PSD-95/Dlg/ZO-1) domain (PDZ), Dbl homology domain (the catalytic domain) (DH), and C-terminal pleckstrin homology domain (PHc). Sequences were aligned using ClustalW. Conserved residues have been highlighted in red, and semiconserved residues are shown in green (very similar) and yellow (similar). The secondary structure elements, β-sheet and α-helix, are indicated as β and α. The GenBankTM data base accession numbers for the nucleotide sequences that encode human MPP2, MPP6, CASK, and Tiam1 are BC030287, NM016447, AF032119, and NM003253, respectively. C, immunoblot analysis for the expression of CADM1 and Tiam1 in acute lymphatic leukemia (ALL) cell lines, HTLV-I-transformed cell lines, and ATL cell lines. Five μg of cell lysates were fractionated in a 4–12% gradient NuPAGE Norfix BisTris gel, followed by immunoblot analysis as described under “Experimental Procedures.” The antibodies used were a rabbit anti-Tiam1 pAb (C16) and a rabbit anti-CADM1 pAb (CC2). A mouse anti-α-tubulin mAb was used to show a loading control.

Interaction of CADM1 with Tiam1 in HTLV-I-Transformed Cell Lines and ATL Cell Lines—We initially tested the physiological association of CADM1 with Tiam1 in a co-immunoprecipitation assay using an HTLV-I-transformed MT-2 cell line. When endogenous CADM1 was immunoprecipitated with a rabbit anti-CADM1 pAb (EC) from the MT-2 cell lysate, co-immunoprecipitation of endogenous Tiam1 was detected (Fig. 2A). Conversely, CADM1 was specifically detected in Tiam1 immunoprecipitate (Fig. 2A). The molecular weight of CADM1 co-precipitated with Tiam1 was slightly higher than the CADM1 signal detected in the MT-2 whole cell lysate (Fig. 2A), which may suggest that Tiam1 selectively interacts with heavily glycosylated CADM1 species. Reciprocal co-precipitation of CADM1 and Tiam1 was also observed in another HTLV-I-transformed cell line, C91/PL, as well as in an ATL cell line, ATL-3I (data not shown), proving that CADM1 was indeed associated with Tiam1 in those cells. N-terminal truncation of Tiam1 (C1199) (Fig. 2C, left) has been known to enhance its in vitro GEF activity and also has been suggested to increase the stability of Tiam1 due to the loss of PEST domains (34). We examined, therefore, whether this active form of Tiam1, C1199, could associate with CADM1. A plasmid encoding either the full-length Tiam1 tagged with V5 (Tiam1-V5) or C1199 tagged with V5 (C1199-V5) was transfected into HEK293 cells, and both Tiam1-V5 and C1199-V5 were immunoprecipitated with a mouse anti-V5 mAb. Endogenous CADM1 was co-immunoprecipitated with either Tiam1 or ATL.

Cytoplasmic Domain of CADM1 Interacts with Tiam1 in HTLV-I-Transformed Cell Lines and ATL Cell Lines—We initially tested the physiological association of CADM1 with Tiam1 in a co-immunoprecipitation assay using an HTLV-I-transformed MT-2 cell line. When endogenous CADM1 was immunoprecipitated with a rabbit anti-CADM1 pAb (EC) from the MT-2 cell lysate, co-immunoprecipitation of endogenous Tiam1 was detected (Fig. 2A). Conversely, CADM1 was specifically detected in Tiam1 immunoprecipitate (Fig. 2A). The molecular weight of CADM1 co-precipitated with Tiam1 was slightly higher than the CADM1 signal detected in the MT-2 whole cell lysate (Fig. 2A), which may suggest that Tiam1 selectively interacts with heavily glycosylated CADM1 species. Reciprocal co-precipitation of CADM1 and Tiam1 was also observed in another HTLV-I-transformed cell line, C91/PL, as well as in an ATL cell line, ATL-3I (data not shown), proving that CADM1 was indeed associated with Tiam1 in those cells. N-terminal truncation of Tiam1 (C1199) (Fig. 2C, left) has been known to enhance its in vitro GEF activity and also has been suggested to increase the stability of Tiam1 due to the loss of PEST domains (34). We examined, therefore, whether this active form of Tiam1, C1199, could associate with CADM1. A plasmid encoding either the full-length Tiam1 tagged with V5 (Tiam1-V5) or C1199 tagged with V5 (C1199-V5) was transfected into HEK293 cells, and both Tiam1-V5 and C1199-V5 were immunoprecipitated with a mouse anti-V5 mAb. Endogenous CADM1 was co-immunoprecipitated with either Tiam1 or ATL.

Cytomembrane Domain of CADM1 Interacts with Tiam1 in HTLV-I-Transformed Cell Lines and ATL Cell Lines—We initially tested the physiological association of CADM1 with Tiam1 in a co-immunoprecipitation assay using an HTLV-I-transformed MT-2 cell line. When endogenous CADM1 was immunoprecipitated with a rabbit anti-CADM1 pAb (EC) from the MT-2 cell lysate, co-immunoprecipitation of endogenous Tiam1 was detected (Fig. 2A). Conversely, CADM1 was specifically detected in Tiam1 immunoprecipitate (Fig. 2A). The molecular weight of CADM1 co-precipitated with Tiam1 was slightly higher than the CADM1 signal detected in the MT-2 whole cell lysate (Fig. 2A), which may suggest that Tiam1 selectively interacts with heavily glycosylated CADM1 species. Reciprocal co-precipitation of CADM1 and Tiam1 was also observed in another HTLV-I-transformed cell line, C91/PL, as well as in an ATL cell line, ATL-3I (data not shown), proving that CADM1 was indeed associated with Tiam1 in those cells. N-terminal truncation of Tiam1 (C1199) (Fig. 2C, left) has been known to enhance its in vitro GEF activity and also has been suggested to increase the stability of Tiam1 due to the loss of PEST domains (34). We examined, therefore, whether this active form of Tiam1, C1199, could associate with CADM1. A plasmid encoding either the full-length Tiam1 tagged with V5 (Tiam1-V5) or C1199 tagged with V5 (C1199-V5) was transfected into HEK293 cells, and both Tiam1-V5 and C1199-V5 were immunoprecipitated with a mouse anti-V5 mAb. Endogenous CADM1 was co-immunoprecipitated with either Tiam1 or ATL.

Interaction of CADM1 with Tiam1 in ATL Cells

Expression of Tiam1 and CADM1 in HTLV-I-transformed cell lines and ATL cell lines. A, expression of CADM1 in the HTLV-I-transformed cell line. MT-2 cells were immunostained for CADM1 (green) and α-tubulin (red) with a rabbit anti-CADM1 pAb (CC2) and a mouse anti-α-tubulin mAb and examined by confocal microscopy. Scale bar, 10 μm. Note that CADM1 is concentrated at the cell-cell attachment sites of the MT-2 cells. B, schematic representation of the domain structure of Tiam1 and sequence alignment of type II PDZ domains of selected MAGUKs and Tiam1. Tiam1 contains myristoylation signal (myr), Pleckstrin region (P), N-terminal pleckstrin homology domain (PHn), Ras-binding domain (RBD), type II PDZ (PSD-95/Dlg/ZO-1) domain (PDZ), Dbl homology domain (the catalytic domain) (DH), and C-terminal pleckstrin homology domain (PHc). Sequences were aligned using ClustalW. Conserved residues have been highlighted in red, and semiconserved residues are shown in green (very similar) and yellow (similar). The secondary structure elements, β-sheet and α-helix, are indicated as β and α. The GenBankTM data base accession numbers for the nucleotide sequences that encode human MPP2, MPP6, CASK, and Tiam1 are BC030287, NM016447, AF032119, and NM003253, respectively. C, immunoblot analysis for the expression of CADM1 and Tiam1 in acute lymphatic leukemia (ALL) cell lines, HTLV-I-transformed cell lines, and ATL cell lines. Five μg of cell lysates were fractionated in a 4–12% gradient NuPAGE Norfix BisTris gel, followed by immunoblot analysis as described under “Experimental Procedures.” The antibodies used were a rabbit anti-Tiam1 pAb (C16) and a rabbit anti-CADM1 pAb (CC2). A mouse anti-α-tubulin mAb was used to show a loading control.
Interaction of CADM1 with Tiam1 in ATL Cells

Direct interaction of CADM1 with Tiam1. A, reciprocal co-immunoprecipitation of CADM1 with Tiam1 in the HTLV-I-transformed cell line, MT-2. Five hundred μg of cell lysates were immunoprecipitated (IP) with a rabbit anti-CADM1 pAb (EC), a rabbit anti-Tiam1 pAb (C16), or normal rabbit IgG. The precipitates were subjected to immunoblot analysis with the rabbit anti-Tiam1 pAb (top) and a rabbit anti-CADM1 pAb (CC2) (bottom). Red, blue, and black arrows, Tiam1, CADM1, and the rabbit IgG heavy chain, respectively. B, interaction of CADM1 with the active form of Tiam1 (C1199). Five hundred μg of cell lysates from HEK293 cells transiently transfected with pcDNA3.1, pcDNA/Tiam1-V5, or pcDNA/C1199-V5 were immunoprecipitated with a mouse anti-V5 mAb or normal mouse IgG and subjected to immunoblot analysis (WB) with CC2 (top). Five μg of whole cell lysates (WCL) were also analyzed for expression of CADM1 (middle), Tiam1-V5, and C1199-V5 (bottom) by immunoblot analysis with CC2 and anti-V5 mAb, respectively. C, the PDZ domain of Tiam1 is required for binding to the PDZ-BM of CADM1. The domain structure and truncated constructs of Tiam1 are shown on the left. GST, GST fused with the cytoplasmic domain of TSLC1, amino acids 392–442 (GST-CADM1-C), and GST fused with the cytoplasmic domain lacking C-terminal 3 amino acid residues, amino acids 392–439 (GST-CADM1-C3), were expressed in Escherichia coli. [35S]Methionine-labeled full-length and truncated Tiam1 were synthesized in reticulocytes. The size of the in vitro translated (IVT) proteins is shown in the right panel with dots indicating each full-length in vitro translated product. For in vitro binding, labeled proteins were incubated with GST or GST fusion protein immobilized on glutathione-Sepharose beads and subjected to SDS-PAGE. Binding of 35S-labeled proteins was detected by autoradiography (middle column). The entire images of the GST pull-down assay are shown in supplemental Fig. S2.

In an attempt to assess the role of CADM1 in the infiltration of ATL cells into tissue, we established doxycycline (Dox)-inducible acute lymphoblastic T-cell leukemia Jurkat cell clones expressing CADM1 (Jurkat/CADM1). In the isolated Jurkat/CADM1 cell clone, expression of CADM1 was tightly regulated with Dox (Fig. 3A). Expression of CADM1 induced extensive formation of cell aggregates in Jurkat cells (Fig. 3A, bottom right) that were reminiscent of cell aggregates seen in HTLV-I-transformed cell lines, such as MT-2 cells (Fig. 1A) and ATL cell lines indicating that homophilic trans-interaction of CADM1 mediated formation of cell aggregates in Jurkat cells. It appears that homophilic trans-interaction of CADM1, therefore, contributes to formation of aggregates in many of the HTLV-I-transformed cell lines and ATL cell lines. In Jurkat/CADM1 cells, co-immunoprecipitation of endogenous Tiam1 was also confirmed in the precipitate with anti-CADM1 pAb (EC) (Fig. 3B). We next examined localization of endogenous Tiam1 and CADM1 in Jurkat/CADM1 cells co-cultured on a monolayer of mouse NIH3T3 fibroblasts for 7 h. In the absence of Dox, Jurkat cells expressing CADM1 tightly adhered to and spread over the monolayer of fibroblasts, forming lamellipodia where CADM1 and Tiam1 were co-localized (Fig. 3C, middle and bottom). In contrast, Jurkat/CADM1 cells in which CADM1 expression was repressed with Dox neither infiltrated into the monolayer of fibroblasts nor formed membrane ruffles at the cell edge, and Tiam1 was distributed in the cytoplasm of those cells (Fig. 3C, top panels). These findings together demonstrated that CADM1 could induce formation of lamellipodia and recruit Tiam1 at the periphery of the lamellipodia. Such effects by CADM1 required its cytoplasmic domain because the deletion mutant lacking the cytoplasmic domain of CADM1 could not
induce formation of lamellipodia in Jurkat cells co-cultured on the monolayer of fibroblasts (Fig. 3D). In addition, Tiam1 did not accumulate at the cell periphery of these cells, suggesting that the interaction of the cytoplasmic domain of CADM1 and Tiam1 was necessary for the formation of lamellipodia (Fig. 3D). Because Tiam1 is known to be a Rac-specific GEF, we also tested whether activation of Rac was needed for CADM1-induced formation of lamellipodia by introducing a dominant negative mutant of Rac tagged with green fluorescent protein (T17NRac1-GFP) in Jurkat/CADM1 cells. T17NRac1-GFP completely blocked CADM1-induced formation of lamellipodia (Fig. 3E), thereby underscoring the importance of Rac activation presumably through Tiam1 in CADM1-induced formation of lamellipodia.

**FIGURE 3.** The cytoplasmic domain of CADM1 is necessary for formation of lamellipodia in Tet-off Jurkat cells cultured on NIH3T3 fibroblasts. A, inducible expression of CADM1 in Tet-off Jurkat cells. The established Tet-off Jurkat cell clones, Jurkat/vector and Jurkat/CADM1, were cultured in the presence (+) and absence (−) of 100 μg/ml Dox for 5 days. Five μg of cell lysates were subjected to immunoblot analysis with an anti-CADM1 pAb (CC2) and anti-Tiam1 pAb (C16) as shown in the top panel. The red and blue arrows indicate Tiam1 and CADM1, respectively. Representative phase-contrast images of Jurkat/CADM1 cells cultured in media with or without 100 μg/ml Dox for 5 days are shown in the bottom panels. Original magnification was ×100. Note that CADM1-expressing Jurkat/CADM1 cells show extensive aggregates. B, co-immunoprecipitation of CADM1 with Tiam1 in the Tet-off Jurkat/CADM1 cells. The Jurkat/CADM1 cells were cultured in the presence (+) and absence (−) of 100 μg/ml Dox for 5 days. To confirm the expression of Tiam1 and CADM1, 5 μg of whole cell lysates (WCL) were subjected to immunoblot analysis (left halves of panels). Five hundred μg of cell lysates were immunoprecipitated (IP) with an anti-CADM1 pAb (EC) and subjected to immunoblot analysis for Tiam1 with C16 (right half of top panel) and for CADM1 with CC2 (right half of the bottom panel). Co-precipitated Tiam1 is indicated with a black arrowhead. The red and blue arrows indicate Tiam1 and CADM1, respectively. C, CADM1 is necessary for formation of lamellipodia in Jurkat cells cultured on NIH3T3 fibroblasts. Jurkat/CADM1 cells were co-cultured on a monolayer of NIH3T3 cells for 17 h. The cells were stained for CADM1 (blue) and Tiam1 (green) with a chicken anti-CADM1 mAb (3E1) and an anti-Tiam1 pAb (C16), respectively. Magnified images show that CADM1 and Tiam1 are detected at membrane ruffling areas (middle and bottom panels). Scale bar, 10 μm. D, cytoplasmic domain of CADM1 induces formation of lamellipodia. Jurkat/CADM1 and Jurkat/ΔC-HA were seeded on NIH3T3 monolayers and incubated for 17 h. Cells were triple-stained for CADM1 (green), Tiam1 (red), and actin (blue). Actin filaments are shown in the merged panels. Note that stress fibers of NIH3T3 cells are seen in the top merged panel, indicating that Jurkat/CADM1 cells infiltrated into the NIH3T3 monolayer. Scale bar, 10 μm. E, a dominant negative mutant of Rac1, T17NRac1, blocked CADM1-induced formation of lamellipodia. Jurkat/CADM1 cells cultured in the presence (+) or absence (−) of 100 μg/ml Dox for 5 days were transiently transfected with pEGFP or pEGFP-T17NRac1. The next day, the transfected cells were seeded on NIH3T3 monolayers and incubated for 17 h. Cells were stained for CADM1 (blue). Scale bar, 10 μm.

**CADM1 and Tiam1 Co-localizes at the Leading Edge of Migrating ATL-3I Cells**—Infiltration of ATL cells into various kinds of tissue like skin involves several key steps: 1) adhesion to endothelial cells; 2) transmigration of vessel walls; 3) crawling into dermis where fibroblasts are abundant; and 4) epidermal localization. We looked, therefore, into the localization of CADM1 and Tiam1 in ATL cell lines co-cultured on either HMVEC or NHDF. After a 17-h co-culture of the ATL cell line, ATL-3I, on top of the monolayer of HMVEC, the cells firmly adhered to HMVEC and formed lamellipodia where CADM1 and Tiam1 were intensely co-accumulated (Fig. 4A and supplemental Fig. S3). ATL-3I cells adhered to a NHDF monolayer in shorter 6-h co-culture (Fig. 4, C and D) and exhibited lamellipodia in most of the cells adhered to NHDF where co-localization of CADM1 and Tiam1 was seen (Fig. 4D). As observed in Jurkat/CADM1 cells overlaid on NIH3T3 cells, a dominant negative T17NRac1 mutant also inhibited the formation of lamellipodia in ATL-3I cells on HMVEC, suggesting that Rac activity was required for ATL-3I cells to tightly attach to the monolayer of HMVEC and subsequently form lamellipodia.
Interaction of CADM1 with Tiam1 in ATL Cells

A. ATL-3I/HMVEC (17h)

B. GFP CADM1 Actin Merged

C. CADM1 a–b b–c c–d d–e e–f

D. CADM1 Tiam1 CD44 Merged

FIGURE 4. CADM1 and Tiam1 are co-localized at the leading edge of ATL-3I cells. A, co-localization of CADM1 with Tiam1 at the peripheral margin of ATL-3I cells. Cells were seeded on a monolayer of HMVEC and cultured for 17 h. Cells were double-stained for CADM1 (green) and Tiam1 (red) with a chicken anti-CADM1 mAb (3E1) and a rabbit anti-Tiam1 pAb (C16), respectively, and then examined by confocal microscopy. Scale bar, 10 μm. A line scan graph of CADM1 (green) and Tiam1 (red) fluorescence intensities is shown. Fluorescence intensities were measured along a white dotted line from a to b in the merged panel. The arrows in the graph indicate the position of the cell edges. The patterns of CADM1 and Tiam1 intensities are similar and high at the edges of the cell. B, a dominant negative mutant of Rac1, T17N Rac1, blocked CADM1-induced formation of lamellipodia. ATL-3I cells transfected with pEGFP or pEGFP-T17N Rac1 were co-cultured on an HMVEC monolayer for 17 h and stained for CADM1 (blue) and actin (red). Scale bar, 10 μm. C, CADM1 co-localized with the leading edge marker Talin but not with the trailing edge marker CD44 at the invasive front of ATL3I cells. a–f, xz sections of ATL3I cells adhered to NHDF. CADM1 was co-localized with Talin (a–c) but not with CD44 (d–f) at the invasive front of ATL3I cells. ATL-3I cells co-cultured on a NHDF monolayer for 6 h were double-stained for CADM1 (green) and Talin (red in b and c) or CD44 (red in e and f) with 3E1 and a mouse anti-Talin mAb or mouse anti-CD44 mAb (C26), respectively. Note that intense staining of CADM1 and Talin but not CD44 is seen at the adhesion sites of ATL-3I cells to NHDF. Illustrations of the cells are depicted above the top panels (a and e) with red arrows that indicate the direction of cell migration. Scale bar, 10 μm. D, CADM1 and Tiam1 co-localized at the invasive front of ATL3I cells. a–h, xz sections of ATL3I cells adhered to the NHDF monolayer. Cells were triple-stained for CADM1 (green), Tiam1 (red), and CD44 (blue) with 3E1, C16, and C26, respectively. The white arrows show co-localization of CADM1 and Tiam1 at the ventral surface of ATL3I cells invading the NHDF monolayer (a–d) and at the leading edge of ATL-3I cells adhering to the monolayer (e–h). Illustrations of the cells are depicted above the top panels (a and e) with red arrows that indicate the direction of cell migration. Scale bars, 5 μm.

We realize that the first requirement for a cell to initiate migration is the acquisition of polarized morphology, and establishment of cell polarity occurs during the migration of lymphocytes (35). In ATL-3I cells adhered to NHDF, therefore, we compared the distribution of CADM1 with that of Talin and CD44, which are well-established markers for the leading and trailing edges of chemokine-induced migratory T lymphocytes, respectively. xz cross-section analysis of these cells revealed that intense staining of CADM1 and Talin was detected at the contact zones of ATL-3I cells to NHDF (Fig. 4C, a–c). In contrast, CD44 was not observed in those contact regions (Fig. 4C, d–f), indicative of CADM1 localization at the leading edge of the migrating cells. Triple immunofluorescence staining for CADM1, Tiam1, and CD44 in the xz cross-section of such cells revealed that CADM1 was completely co-localized with Tiam1 at the cell periphery (Fig. 4D, a–d and e–h). At the adhering sites of ATL-3I cells to NHDF, however, CADM1 was co-localized with Tiam1, but not with CD44 (Fig. 4D, a–d and e–h), demonstrating that CADM1 and Tiam1 were distributed at the leading edge of polarized migrating cells. This was further confirmed by quantitative analysis of the xz cross-sections of the adhering area of ATL-3I cells to NHDF monolayers. Thirty-seven of 40 (93%) ATL-3I cells showed CADM1/Tiam1 double-positive and CD44-negative adhering sites.

Both CADM1 and Tiam1 Are Necessary for Lamellipodium Formation of HTLV-I-transformed and ATL Cell Lines—To further substantiate the importance of CADM1 and Tiam1 in CADM1-induced formation of lamellipodia, we introduced either CADM1 or Tiam1 siRNA into HTLV-I-transformed cell lines, C91/PL and MT-4, and an ATL cell line, ATL-3I. Immunoblot analysis of CADM1 and Tiam1 protein revealed that decreases in CADM1 and Tiam1 were most prominent in C91/PL cells (Fig. 5A) due to them having the highest transfection efficiency among the three cell lines (data not shown). Forty-eight h after the introduction of siRNA, the cells in which CADM1 expression had been knocked down lost their aggregate morphology and started growing as single cells (Fig. 5B, upper middle and right panels), whereas siRNA-mediated knockdown of Tiam1 interfered with aggregate formation to a much lesser extent (Fig. 5B, bottom panels). It should be noted that disruption of cell aggregation in ATL3I cells by CADM1 knockdown was not as striking as in MT-4 cells (data not
Interaction of CADM1 with Tiam1 in ATL Cells

A. 

C91/PL

| siRNA  | cont. | CADM1 #1 | CADM1 #2 | cont. | Tiam1 #1 | Tiam1 #2 |
|--------|-------|----------|----------|-------|----------|----------|
| Tiam1  |       |          |          |       |          |          |
| CADM1  |       |          |          |       |          |          |
| α-Tubulin |     |          |          |       |          |          |

B. 

siRNA

| cont. | CADM1 #1 | CADM1 #2 |
|-------|----------|----------|
| Tiam1 #1 |          |          |
| Tiam1 #2 |          |          |

C. 

Graph showing relative cell growth

D. 

Graph showing relative cell adhesion to NHDF

E. 

C91/PL on NHDF (6 hr)

Merged
shown), suggesting that other adhesion molecules are also involved in cell aggregation of HTLV-I-transformed cell lines and ATL cell lines. In assessing whether the reduction in aggregate formation influenced cell growth, we counted the number of living cells 7 days after siRNA introduction and found that knockdown of CADM1 dramatically reduced cell growth of C91/PL cells to ∼30% (Fig. 5C). Although not as strongly as in C91/PL cells, cell growth of MT-4 and ATL-3I cells was also decreased from 50 to 66% upon silencing of CADM1 (Fig. 5C).

In contrast, knockdown of Tiam1 did not affect cell growth at levels comparable with those induced by knockdown of CADM1 (Fig. 5C). Together, these indicate that CADM1 enhanced the cell growth of both HTLV-I-transformed cell lines and an ATL cell line by mediating formation of cell aggregates through homophilic trans-interaction of CADM1, but it is unlikely that Tiam1 was involved in the effect. We further analyzed whether CADM1 or Tiam1 was necessary for adhesion to NHDF and subsequent formation of lamellipodia on NHDF. An adhesion assay of a 2-h co-culture of these ATL cell lines on a NHDF monolayer showed that knockdown of either CADM1 or Tiam1 reduced the level of cell adhesion by a similar amount (50–70%) (Fig. 5D). This result was consistent with the immunofluorescent microscopic observation, which showed that siRNA-mediated knockdown of CADM1 and Tiam1 in C91/PL cells on NHDF repressed formation of lamellipodia and abrogated cell periphery localization of Tiam1 and CADM1, respectively (Fig. 5E). These data together indicate that either Tiam1 or CADM1 are necessary for formation of lamellipodia in HTLV-I-transformed cell lines and ATL cell lines.

Co-localization of CADM1 and Tiam1 in Lymph Node Lesions of ATL Patients.—Finally, we examined the expression of CADM1 and Tiam1 in lymph node lesions from nine ATL patients with lymph node involvement by immunohistochemistry. As representativey shown in Fig. 6A, infiltrating tumor cells demonstrated immunoreactivity for CADM1 in eight of the nine patients tested (four strongly positive, four positive, and one negative). In comparison, Tiam1 was detected in three patients who also were positive for CADM1, indicating that one-third of the specimens from these nine ATL patients were double-positive. Immunohistostaining revealed that CADM1 was detected at the plasma membrane of the tumor cells (Fig. 6A, left column), and aggregate accumulation of CADM1 was seen in some cases as an intense punctate structure (Fig. 6A, bottom left panel). Tiam1 was observed not only at the membrane but also in the cytoplasm of the infiltrating ATL cells (Fig. 6A, right column). In an effort to determine more precisely whether CADM1 and Tiam1 co-localize, we performed immunofluorescence double-staining. Although none of the Tiam1-positive specimens showed a unique pattern of punctate structure-like distribution of CADM1 as seen in Fig. 6A, CADM1

![Image](image_url)

**FIGURE 6.** Co-localization of CADM1 and Tiam1 in lymph node lesions of ATL patients. A, immunohistochemical staining for CADM1 and Tiam1. Tissues were incubated with a rabbit anti-CADM1 pAb (number 6) (left panels) or a rabbit anti-Tiam1 pAb (C16) (right panels) and stained with aminoethylcarbazole. The nuclei were counterstained with hematoxylin. Representative images of the CADM1 /Tiam1 double-positive specimens are shown. Original magnification was ×200 (top panels) and ×400 (bottom panels). B, co-localization of CADM1 and Tiam1 at the cell membrane of infiltrating ATL cells. Immunofluorescence double-staining of sections for CADM1 (green) and Tiam1 (red) was performed with a chicken anti-CADM1 mAb (3E1) and a rabbit anti-Tiam1 pAb (C16). Scale bars, 50 μm.
Interaction of CADM1 with Tiam1 in ATL Cells

and Tiam1 were well co-localized at the cell membrane of ATL cells (Fig. 6B). When considered together, these data support our finding in vitro that both CADM1 and Tiam1 are cooperatively involved in the infiltration of ATL cells.

DISCUSSION

In this study, we identified Rac-specific GEF, Tiam1, as a binding partner of CADM1 in both HTLV-I-transformed and ATL-derived cell lines and demonstrated that the interaction induced formation of lamellipodia, structures seen at the leading edge of motile cells, indicating that CADM1-Tiam1 interaction was involved in the infiltrative propensity of ATL cells. Although the cohort of ATL patients we investigated was not large enough to provide conclusive clinical evidence, CADM1 and Tiam1 were well co-localized at the cell membrane in the Tiam1+/CADM1+ specimens from ATL patients with lymph node involvement. In normal T lymphocytes in which CADM1 is barely detectable (8), Tiam1 regulates chemokine-induced T-cell polarization and chemotaxis by associating with the Par3-Par6-atypical protein kinase C polarity complex (36). In ATL cells, the overexpressed CADM1 interacts with Tiam1 through the type II PDZ-BM of CADM1 and recruits Tiam1 to the intracellular submembranous domain. Rac-specific guanine nucleotide exchange factor activity of Tiam1 is known to depend on the membrane localization of Tiam1, which is mediated by binding to phosphoinositides through the N-terminal pleckstrin homology domain (PHn) of Tiam1 (Fig. 1B) (37). Others have reported, however, that the N-terminal pleckstrin homology domain of Tiam1 possesses a relatively weak affinity and less specificity for phosphoinositide compared with the pleckstrin homology domains of PLC6 and Akt (38), indicating that binding of the N-terminal pleckstrin homology domain with phosphoinositide seems to play an accessory but not a defining role in targeting Tiam1 to cell membranes. It is, therefore, likely that the interaction of Tiam1 with transmembrane proteins facilitates its membrane localization. Furthermore, these transmembrane proteins with which Tiam1 interacts may determine the downstream signals subsequently triggered by such interaction. Accordingly, the association of CADM1 with the PDZ domain of Tiam1 could reinforce tethering of Tiam1 to the membrane and induce the signal specific to CADM1. We have previously shown that CADM1 binds to actin through its protein 4.1-BM (14). CADM1, therefore, seems to recruit actin and a regulator of actin, the Rac-specific guanine nucleotide exchange factor, together to the juxtamembrane region, thereby becoming a powerful driving force for actin reorganization to induce cell motility (Fig. 7). As can be surmised, co-expression of CADM1 and Tiam1 in T lymphocytes appears to be an unwanted combination that leads to the deviated invasive tendencies of ATL cells.

Regardless of its tumor suppressor activities in various cancers (1, 33), our study revealed that CADM1 functions rather as an oncoprotein in ATL. Intriguingly, similar complexity of paradoxical functions has been well illustrated in Tiam1 (39). Besides promoting invasion of T-cell lymphoma cells (20, 40), Tiam1 expression has been demonstrated to correlate with the invasive and metastatic phenotypes of breast and colon cancers (39). It has also been shown that Tiam1−/− mice are resistant to the development of Ras-induced skin tumors, suggesting that Tiam1 contributes to tumorigenicity (41). As opposed to these abilities of Tiam1 to promote invasion and metastasis as well as tumor formation, Tiam1 expression has been demonstrated to be inversely correlated with the invasive potential of renal cell carcinoma cell lines (42). Such contradictory findings are proposed to be due to the effects of Tiam1, which solely depend on the cell type and the Rho GTPase activation status in the particular spatio-temporal context of given cells (39). It is plausible, therefore, that the dualistic effects of CADM1 may be also attributable to the cell type and activation state of Rho GTPs. In fact, we previously demonstrated using epithelial Madin–Darby canine kidney cells that the cytoplasmic domain of CADM1 induced prolonged activation of Rac and reduced activation of Rho, leading to suppression of hepatocyte growth factor-induced epithelial mesenchymal transitions, which is a crucial step for tumor cells to become invasive (30). Coincidentally, ectopic expression of Tiam1 or constitutively active Rac was reported to block hepatocyte growth factor-induced cell scattering of Madin-Darby canine kidney cells with high Rac and low Rho activities (43). It is tempting to speculate, therefore, that CADM1 spatio-temporally associates with Tiam1 even in some epithelial cells where they cooperatively work together as a tumor suppressor by maintaining epithelial integrity.

Although it is becoming increasingly clear that adhesion molecules are involved in transendothelial migration of leukocytes, this may not be the only possible process in which...
Cadmi1 is involved in infiltration of ATL cells into various organs and tissues, such as skin. Skin involvement is one of the most frequent manifestations in ATL patients. The extracellular domain of Cadmi1 is known to interact homophilically with itself (6, 7), as well as heterophilically with Cadmi2 (Necl-3) (44), Cadmi3 (Necl-1) (6), Nectin-3 (6) and CRTAM (45–47). The molecular interactions that facilitate ATL migration to and retention in skin may involve various cells residing in the skin that express those ligands for Cadmi1. Such cells may include mast cells that are immune cell residents of the dermis constitutively expressing Cadmi1 (48). Our study revealed that ATL cells adhered to NHDF with even higher affinity than to HMVEC. NHDF are abundant in epidermis and quite possibly help ATL cells to crawl into the dermal interstitium. We have found that Cadmi1-positive cells tend to show a high affinity with fibroblasts, which are Cadmi1-negative, suggesting that fibroblasts express some of those heterophilic ligands described above or as yet unidentified ligands for Cadmi1. ATL cells often display an affinity for Langerhans cells, immature dermal dendritic cells, and cluster around them in the epidermis forming Pautrier's microabscesses (49). Because Cadmi1 is known to define a certain subset of dendritic cells (45), it is possible that Langerhans cells express Cadmi1, thereby attracting ATL cells to the epidermis. It is noteworthy that the dermis is abundant in sensory nerve fibers that constitute an elaborate network. Other than CRTAM, the Cadmi1 ligands listed above are copiously expressed in nerve cells (5, 44). It seems plausible, therefore, that ATL infiltration into skin is promoted through the interaction between ATL cells and nerve fibers. It remains elusive, however, whether the Cadmi1-Tiam1-Rac cascade is constitutively activated in ATL cells or the activation is triggered by adhesion of ATL cells to the cells expressing those Cadmi1 ligands mentioned above. Further studies to define the mechanism of Cadmi1 activation of Rac through Tiam1 are now ongoing. Because phosphorylation of Tiam1 on its serine/threonine and tyrosine residues was previously reported (34, 39), investigating spatio-temporal phosphorylation of Tiam1 in HTLV-I-transformed and ATL-derived cell lines would provide pivotal information toward understanding the Cadmi1-Tiam1-Rac signaling in ATL cells.

In the present study, we found that Cadmi1 knockdown affected cell growth, suggesting that Cadmi1 may play an auxiliary role in transformation of HTLV-I-infected T cells. During such a transformation stage, Tiam1 may not be the sole binding partner of Cadmi1 because Tiam1 knockdown did not have an effect on cell growth. Tax is reported to be directly associated with small Rho GTPase and therefore is believed to play a role in the infiltrating propensity of ATL cells (18, 50). It is known, however, that Tax expression is frequently lost in ATL cells (51). In contrast, a previous study from others (8) reported that Cadmi1 was expressed in all of the primary ATL cells tested. In such Tax-negative ATL cells, Cadmi1-Tiam1 interaction may become one of the driving forces for cytoskeletal reorganization, thereby contributing to the infiltrating phenotype of ATL cells. In support of this, it has been reported that, compared with ED, the Cadmi1-negative ATL cell line, ED cells overexpressing Cadmi1 caused larger tumor formation and massive infiltration into various organs in NOG mice (52).

Elucidating the molecular mechanisms of the Cadmi1-Tiam1 pathway involved in tissue infiltration of ATL cells may offer alternative approaches to the treatment of ATL, such as specific interference with Cadmi1 using a mAb against the ectodomain of Cadmi1. The use of therapeutic mAb for the treatment of cancer has shown promising results over the past few years, as exemplified by the major success of a mAb against HER2 in treatments for metastatic breast cancer and lung cancer. Our study revealed that Cadmi1 affected both lamellipodium formation and cell growth, thus suggesting that interference with Cadmi1 may inhibit not only tissue infiltration of ATL cells but also the growth of ATL cells. Tiam1 may also become an attractive pharmacological target for developing small molecular inhibitors of the invasive nature of ATL cells by manipulating the Cadmi1-Tiam1-Rac signaling pathways.

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Interaction of CADM1 with Tiam1 in ATL Cells

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