

Close Homolog of L1 Is an Enhancer of Integrin-mediated Cell Migration*

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Close homolog of L1 (CHL1) is a member of the L1 family of cell adhesion molecules expressed by subpopulations of neurons and glia in the central and peripheral nervous system. It promotes neurite outgrowth and neuronal survival *in vitro*. This study describes a novel function for CHL1 in potentiating integrin-dependent cell migration toward extracellular matrix proteins. Expression of CHL1 in HEK293 cells stimulated their haptotactic migration toward collagen I, fibronectin, laminin, and vitronectin substrates in Transwell assays. CHL1-potentiated cell migration to collagen I was dependent on $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, as shown with function blocking antibodies. Potentiated migration relied on the early integrin signaling intermediates c-Src, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase. Enhancement of migration was disrupted by mutation of a potential integrin interaction motif Asp-Gly-Glu-Ala (DGEA) in the sixth immunoglobulin domain of CHL1, suggesting that CHL1 functionally interacts with β_1 integrins through this domain. CHL1 was shown to associate with β_1 integrins on the cell surface by antibody-induced co-capping. Through a cytoplasmic domain sequence containing a conserved tyrosine residue (Phe-Ile-Gly-Ala-Tyr), CHL1 recruited the actin cytoskeletal adapter protein ankyrin to the plasma membrane, and this sequence was necessary for promoting integrin-dependent migration to extracellular matrix proteins. These results support a role for CHL1 in integrin-dependent cell migration that may be physiologically important in regulating cell migration in nerve regeneration and cortical development.

In vertebrates, the L1 family of cell adhesion molecules (CAMs)¹ is comprised of four members: L1/NgCAM, close homolog of L1 (CHL1), neurofascin, and NrCAM, all serving mul-

tle functions in the development and function of the nervous system. L1, the prototype of this family of transmembrane glycoproteins, has been shown to participate in cell migration, axon fasciculation, and guidance, as well as synaptogenesis and adult synaptic plasticity (1). The importance of L1 function in human brain development is revealed by the association of mutations in the L1 gene with a syndromic form of mental retardation, the L1 syndrome, formerly named CRASH (corpus callosum agenesis, mental retardation, adducted thumbs, spasticity, and hydrocephalus) (2–4). L1 knockout mice display nervous system anomalies similar to those seen in human patients, although there appear to be additional genetic modifiers of the disease (5–8).

L1 family members share a structural plan consisting of an extracellular region comprised of six Ig-like domains, four or five fibronectin type III domains, a single transmembrane segment, and a short, conserved cytoplasmic region (9). The extracellular portion of these proteins is highly glycosylated and allows them to participate in both homophilic and heterophilic interactions with a variety of ligands, including other members of the Ig superfamily. The cytoplasmic domain of L1 family members interacts with components of the actin cytoskeleton (10), protein kinases (11–13), and complexes associated with endocytosis and protein trafficking in a lipid raft-associated manner (14, 15). An important binding partner is ankyrin, a protein that binds to the subcortical actin/spectrin cytoskeleton (16, 17). The interaction of L1 with ankyrin occurs through a conserved FIGQY sequence (Phe-Ile-Gly-Gln-Tyr) within the cytoplasmic domain and is proposed to stabilize axonal membranes and/or intercellular connections (18). This idea is supported by the finding that in mice lacking ankyrin B, the axons eventually degenerate (19), although initial axon outgrowth and L1 targeting are relatively normal.

CHL1 is a newly identified member of the L1 family that is expressed in subpopulations of developing neurons in the central and peripheral nervous systems and that persists at low levels in the mature brain in areas of high plasticity (20, 21). CHL1 is also expressed by Schwann cells, astrocytes, and oligodendrocyte precursors (22) and is strikingly up-regulated in Schwann cells and sensory neurons upon nerve crush injury (23). The CALL gene, the human ortholog of the CHL1 gene (24), is closely linked to the 3p- syndrome characterized by mental retardation (25). The human CHL1 gene is mutated in a patient with mental retardation (26). An increased risk for schizophrenia associated with a missense polymorphism has also been reported (27). A recent study (28) showed that CHL1-deficient mice display misguided axons within the hippocampus and olfactory tract and anomalies in behavior. These findings emphasize the importance of CHL1 in the nervous system, although its specific functions are yet unknown.

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¹ The abbreviations used are: CAM, cell adhesion molecule; CHL1, close homolog of L1; GFP, green fluorescent protein; MAP, mitogen-activated protein; MEK, MAP kinase/extracellular signal-regulated kinase kinase; PI, phosphatidylinositol; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HBSS, Hanks' balanced saline solution; TRITC, tetramethylrhodamine isothiocyanate.

CHL1 shares the basic structural plan of L1 family members (22) and has strong neurite outgrowth promoting capacity (20). The sequence of CHL1 reveals ~60% amino acid identity with L1 in the extracellular region and ~40% identity in the cytoplasmic domain. Yet in contrast to L1, CHL1 is not capable of self-associating, nor does it bind heterophilically to L1, and an extracellular domain-binding partner remains to be found (20). The extracellular segment of CHL1 contains a potential integrin-binding motif Arg-Gly-Asp (RGD) in the second Ig-like domain, where it may be masked by the predicted horseshoe conformation of the molecule (29, 30), rather than within the sixth Ig-like domain as in L1. However, the sixth Ig domain of CHL1 contains another potential integrin interaction motif Asp-Gly-Glu-Ala (DGEA), which has been reported to mediate integrin-collagen interactions in platelets (31). Finally, CHL1 is the only L1 family member with an altered sequence (FIGAY) in the presumed ankyrin-binding domain, and it lacks the RSLE motif, which is characteristic of other family members and is involved in endocytosis.

The structural resemblance and the differences between CHL1 and the other members of the L1 family of CAMs suggest that this molecule might have both similar and distinctive functions within cells. Recently it was shown that L1 promotes integrin-mediated haptotactic migration of cultured cells toward extracellular matrix proteins (32–37). Because of potential integrin interaction motifs in CHL1, it was speculated that CHL1 may have a role in integrin-mediated cell migration. Here it is shown that CHL1 promotes haptotactic cell migration toward extracellular matrix proteins, but this function differs from L1 in regard to preference of extracellular matrix substrate and integrin partners. These differences are related to the structural differences between CHL1 and L1 and may be important for differentially regulating cell migration during nerve regeneration and neuronal migration during development.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—The following cDNAs were subcloned into pcDNA3 (Stratagene, La Jolla, CA): wild type mouse CHL1, a CHL1 mutant in which the RGD sequence in the Ig2 domain was mutated to KGE, a CHL1 mutant in which the DGEA sequence in the Ig6 domain was mutated to AGEV, a CHL1 mutant in which the cytoplasmic sequence FIGAY was mutated to FIGAA, and human wild type L1 (+RSLE) (from J. Hemperly, BD Technologies, Research Triangle Park, NC). A plasmid encoding ankyrin G fused to green fluorescent protein (ankyrin-GFP) was provided by Vann Bennett (Duke University, Durham, NC). A rabbit polyclonal antibody was made against mouse CHL1-Fc (22). Mouse monoclonal antibody Neuro4 against an extracellular epitope of human L1 was a gift of J. Hemperly. The following antibodies were obtained from Chemicon (Temecula, CA): anti-human β_1 integrin monoclonal antibody 2253Z (clone 6s6), an activating monoclonal antibody MAB2000 (clone HB1.1) against human β_1 integrin, anti-human α_1 integrin monoclonal antibody 1973Z (clone FB12), and anti-human α_2 integrin monoclonal antibody 1950Z (clone P1E6). Non-immune mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). Human vitronectin, human fibronectin, and murine laminin were from Invitrogen and Peninsula Laboratories (San Carlos, CA). BD Biosciences (Palo Alto, CA) provided type I collagen from rat tail. MEK inhibitor U0126 (Promega, Madison, WI), Src inhibitor PP2 and inactive analog PP3, and PI 3-kinase inhibitor Ly294002 (CalBiochem, San Diego, CA) were dissolved in Me₂SO.

Cell Culture and Haptotactic Migration Assay—HEK293 cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech Cellgro), 4.5 mg/ml glucose, 10% heat-inactivated fetal bovine serum, 50 μ g/ml gentamicin, and 250 μ g/ml kanamycin. Cultures at 80–90% confluence in 60-mm dishes were transfected for transient expression of CHL1 or L1 pcDNA3 plasmids (5 μ g) using LipofectAMINE 2000 (Invitrogen) in Opti-MEM I (Invitrogen). After 18–24 h at 37 °C, the transfected cells were used for haptotactic migration or ankyrin recruitment assays.

Haptotactic migration of HEK293 cells transiently expressing CHL1 or L1 was assayed essentially as previously reported (37), using modi-

fied Boyden chambers with 8- μ m pore filters (Transwells 3422, Corning/Costar, Acton, MA) in serum-free medium (DMEM, 0.4 mM MnCl₂, 50 μ g/ml gentamicin, 250 μ g/ml kanamycin). In the absence of Mn²⁺, CHL1 potentiated haptotactic migration of HEK293 cells toward collagen I (1.9-fold) to nearly the same extent as in the presence of Mn²⁺ (2.3-fold). The bottom sides of the filters were precoated with extracellular matrix proteins (500 μ l of 20 μ g/ml) or 2% bovine serum albumin in PBS at 4 °C overnight and blocked in 2% bovine serum albumin. The cells were detached with 5 mM Na-EDTA in Hanks' balanced saline solution (HBSS) and plated at 20,000 cells/Transwell. In some experiments, the cells were preincubated in serum-free medium with anti-integrin antibodies (1–4 μ g/ml) for 15–30 min at 4 °C prior to plating. The cells were allowed to migrate for 3–8 h at 37 °C in a CO₂ incubator. After migration cultures were fixed in 4% paraformaldehyde, rinsed in PBS, and then treated with blocking solution (10% goat serum, 0.2% fish skin gelatin, PBS) for either 1 h at room temperature or overnight at 4 °C.

To score migration, cells from the upper or lower sides of filters were removed, and cells on the opposite side were stained by indirect immunofluorescence with purified CHL1 polyclonal antibodies (20 μ g/ml) or L1 monoclonal antibody Neuro4 (5 μ g/ml) in blocking solution for 4 h at room temperature, followed by fluorescein isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) diluted 1:75 to 1:100 in blocking solution. The cells were counterstained with 10 μ M bis-benzimide (Hoechst 33258; Molecular Probes, Eugene, OR). The filters were mounted with Vectashield (Vector Laboratories, Burlingame, CA) on glass slides, and the cells were scored on both top and bottom surfaces of filters under epifluorescence illumination. For each filter, at least 150 cells were scored from six or more randomly selected fields using a 20 \times objective. To obtain the total number of cells on each side of a filter, the mean number of cells/field was determined and multiplied by a factor based on the number and size of fields and a filter diameter of 6.5 mm. The percentage of CHL1-immunoreactive cells that transmigrated was calculated as the ratio of CHL1-positive cells on the bottom of filters to total (top and bottom) CHL1-positive cells. The percentage of CHL1-negative bis-benzimide-positive cells that transmigrated was determined similarly. The percentage of cells transmigrated was converted to the total number of cells migrated per Transwell by multiplying the percentage by the number of cells plated. The experiments were performed in duplicate or triplicate, and the results of each condition were averaged. The means and standard errors were determined for each condition. Significant differences between experimental groups was evaluated by Student's *t* test (*p* < 0.05, one-tailed). Transfection efficiency of HEK293 cells was determined as the number of fluorescein isothiocyanate-labeled cells (transfected) divided by the number of bis-benzimide-labeled cells (transfected plus nontransfected) and was 50–70%. There was no deleterious effect of integrin antibodies or inhibitors on cell adhesion, which was evaluated by counting the total number of cells recovered after each assay. Similarly, inhibitors used at concentrations indicated in the signaling experiments (PP2, PP3, Ly294002, and U0126) did not affect the viability or cell recovery.

Co-capping Experiments—HEK293 cells transfected with the pcDNA3-CHL1 plasmid were dissociated in 5 mM EDTA in HBSS, washed with 10% fetal bovine serum in DMEM, and resuspended in DMEM. The cells (30,000 cells/100 μ l) were incubated with anti-integrin β_1 mouse monoclonal antibody MAB2000 (20 μ g/ml) at 4 °C for 20 min. The cells were washed with ice-cold HBSS, resuspended in DMEM containing 5 μ g/ml goat anti-mouse IgG (Fc γ fragment-specific), and incubated for 20 min at 4 °C. After washing in ice-cold HBSS, the cells were resuspended in 10% fetal bovine serum in DMEM, plated onto collagen-coated MatTek plates (MatTek Corp., Ashland, MA), and incubated for 1 h at 37 °C to allow integrins to cluster. The cells were then fixed in 4% paraformaldehyde in PBS for 15 min, washed, and blocked with 10% donkey serum in PBS for 1 h at room temperature. To label CHL1, the cells were incubated with CHL1 rabbit polyclonal antibody (20 μ g/ml in blocking buffer) for 2 h at room temperature, washed with PBS, and then incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG and TRITC-conjugated donkey anti-goat IgG diluted 1:100 in blocking buffer. Finally, the cells were rinsed, mounted in Vectashield, and examined using an Olympus FV500 laser confocal microscope at the Microscopy Service Laboratory (Dr. Robert Bagnell, Department of Pathology, University of North Carolina-Chapel Hill) using appropriate filter sets.

Assay for Ankyrin Recruitment—Ankyrin recruitment to CHL1 in the plasma membrane was assayed essentially as previously described for L1 (38) with the following modifications. HEK293 cells on poly-D-lysine-coated MatTek dishes were transfected with plasmid pEGFP-N1 ex-

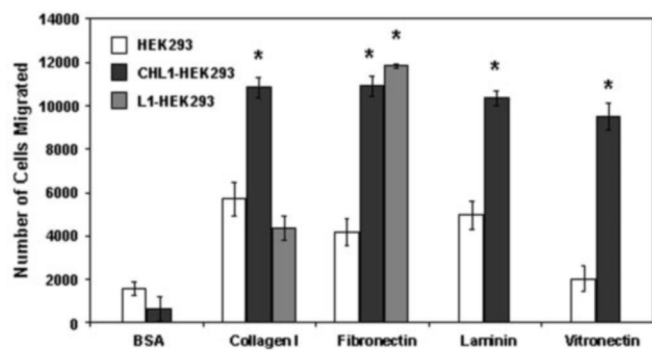


FIG. 1. **CHL1 potentiates haptotactic migration of HEK293 cells to extracellular matrix proteins.** Haptotactic cell migration toward extracellular matrix proteins or random migration toward bovine serum albumin was measured in HEK293 cells transiently expressing CHL1, L1, or expressing no L1 family adhesion molecule for 8 h. The samples were assayed in triplicate, and the experiments were repeated at least twice with similar results. *, statistically significant differences in means of transfected (expressing CHL1 or L1) versus nontransfected cells using the *t* test ($p < 0.05$).

pressing a fusion protein between ankyrin G and green fluorescent protein (ankyrin-GFP) (0.05 μ g) with or without co-transfection of pcDNA3-CHL1 (0.1 μ g) using LipofectAMINE 2000. After 24 h, the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 15 min, washed, and incubated with blocking buffer (10% normal goat serum in PBS) for 30 min. To label CHL1 on the cell surface, the cells were incubated for 4 h at room temperature with rabbit polyclonal antibody against CHL1 (20 μ g/ml in blocking buffer), then washed, and incubated for 2 h with TRITC-conjugated goat anti-rabbit IgG diluted 1:200 in blocking buffer. The cells were washed and mounted in Vectashield. GFP/immunofluorescence images were recorded on an Olympus FV500 laser confocal microscope. The ankyrin-GFP fluorescence was recorded using the 488-nm excitation line of the laser, whereas the TRITC-labeled CHL1 was examined using the 543-nm excitation line of the laser and the appropriate band pass filters. Each experiment was repeated four times.

RESULTS

CHL1 Potentiates Haptotactic Cell Migration to Extracellular Matrix Proteins through β_1 Integrins—Extracellular matrix proteins are important in mediating cell migration and neuronal process growth in the developing nervous system (39) and are implicated in nerve response to injury (40, 41). The ability of CHL1 to promote haptotactic migration toward extracellular matrix proteins was studied in Transwell assays in which cells were allowed to migrate from top to bottom chambers through filters coated on the underside with purified matrix molecules (37). The human embryonic kidney cell line HEK293 was used for these studies, because it expresses defined integrin subunits including α_1 , α_2 , α_3 , α_5 , α_v , and β_1 , which can serve as extracellular matrix protein receptors (42, 43), and because it does not express detectable levels of cell adhesion molecules of the L1 family (38). HEK293 cells were transfected for transient expression with pcDNA3 plasmids encoding CHL1 or L1, and haptotactic migration was assayed toward purified matrix proteins. Nontransfected HEK293 cells displayed greater migration toward collagen type I, fibronectin, and laminin compared with random migration toward bovine serum albumin but did not migrate significantly toward vitronectin (Fig. 1). Expression of CHL1 significantly enhanced haptotactic migration toward each of these substrates as well as to vitronectin (Fig. 1). In contrast to CHL1, L1 was clearly not capable of enhancing HEK293 cell migration to collagen I, similar to previous findings in rat B35 neuroblastoma cells (37), although L1 was as effective as CHL1 at potentiating cell migration to fibronectin (Fig. 1). These results showed that CHL1 can promote haptotactic cell migration toward a range of ECM substrates and suggested that CHL1 might functionally interact with integrins.

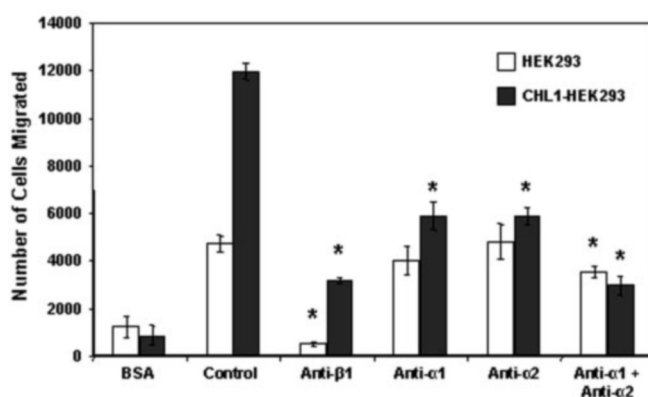


FIG. 2. **CHL1 potentiates migration of HEK293 cells through β_1 integrins.** Haptotactic migration of CHL1-expressing or nonexpressing HEK293 cells was measured for 3 h toward collagen I. Cells migrating toward collagen I were pretreated with nonimmune mouse IgG (Control; 30 μ g/ml), integrin β_1 antibody (Anti- β_1 ; 30 μ g/ml), integrin α_1 antibody (Anti- α_1 ; 15 μ g/ml), integrin α_2 antibody (Anti- α_2 ; 15 μ g/ml), or both antibodies (Anti- α_1 + Anti- α_2 ; 30 μ g/ml). The samples were assayed in triplicate, and the experiments were repeated at least twice with similar results. *, statistically significant differences in means of treated versus control samples using the *t* test ($p < 0.05$). BSA, bovine serum albumin.

To determine whether integrins were involved in CHL1-mediated migration, haptotactic migration of HEK293 cells toward collagen I was further evaluated in the presence of function-blocking integrin antibodies. Collagen I was chosen as a substrate, because collagen I, in addition to collagens III and IV, increases at the site of nerve injury and promotes Schwann cell migration (41), and CHL1 is up-regulated in Schwann cells during peripheral nerve regeneration (20, 23). Treatment of cells with function-blocking β_1 integrin antibodies (44) strongly inhibited CHL1-mediated HEK293 cell migration toward collagen I, as well as CHL1-independent migration of HEK293 cells (Fig. 2). Residual migration of CHL1-HEK293 cells in the presence of anti- β_1 integrin antibodies was low but significantly elevated over that of HEK293 cells ($p < 0.05$), suggesting that CHL1 might function to a small extent through a non- β_1 containing collagen receptor. Because the principal collagen I receptors are known to be $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (45), function-blocking antibodies against α_1 and α_2 integrins were evaluated next for effects on migration. Antibodies against either α_1 or α_2 integrin strongly inhibited CHL1-mediated migration toward collagen I, whereas α_1 and α_2 integrin antibodies added together were as effective as β_1 integrin antibodies (Fig. 2). The α_1 and α_2 integrin antibodies were not as effective as β_1 integrin antibodies in inhibiting the basal migration of HEK293 cells, thus it is likely that these cells use $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins in addition to an unidentified β_1 integrin heterodimer for migration to collagen. In summary, these results indicated that CHL1 potentiated the migration of HEK293 cells toward collagen I primarily through $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins.

The DGEA Motif in the CHL1 Ig6 Domain Is Required for Potentiating Migration to Collagen I—The mouse CHL1 and human CALL proteins contain a conserved Asp-Gly-Glu-Ala (DGEA) motif in the Ig6 domain of their extracellular regions (22, 24). Interestingly, the DGEA motif is also present in collagen I where it serves as a recognition site for $\alpha_2\beta_1$ integrin in platelets (31). Mutation of the DGEA sequence in the CHL1 Ig6 domain to Ala-Gly-Glu-Val (AGEV) effectively inhibited CHL1-potentiated migration of HEK293 cells toward collagen I, reducing migration to nearly the level of CHL1-nonexpressing HEK293 cells (Fig. 3). In the Ig6 domain of L1, an Arg-Gly-Asp (RGD) sequence is required for potentiating migration to fi-

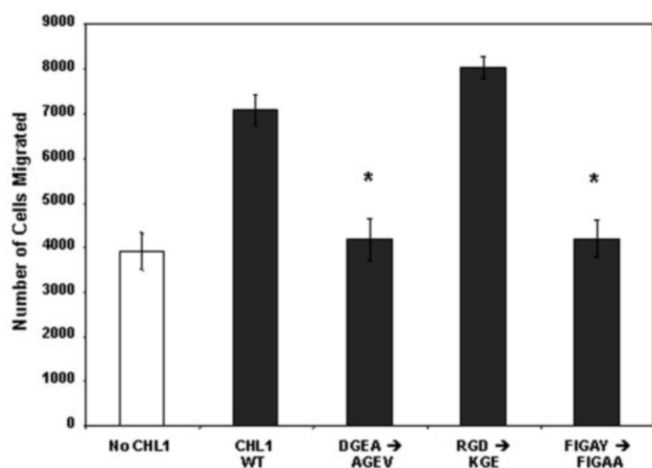


FIG. 3. **CHL1 mutations in extracellular Ig6 (DGEA) and cytoplasmic (FIGAY) domains suppress CHL1-potentiated migration to collagen.** HEK293 cells transiently expressing wild type CHL1, CHL1 with mutations DGEA → AGEV, RGD → KGE, FIGAY → FIGAA, or no CHL1 were assayed for haptotactic migration to collagen I for 3 h. Each sample was assayed in triplicate, and the experiments were repeated at least twice with similar results. *, statistically significant differences in means compared with CHL1 wild type (WT) using the *t* test ($p < 0.05$).

bronectin (37). The Ig2 domain of CHL1 contains an Arg-Gly-Asp (RGD) motif (22), and a corresponding sequence (Lys-Gly-Asp) in the human CALL protein is closely conserved though nonidentical (24). This motif might interact with integrins, because RGD is a known adhesion motif that is present in collagen I, fibronectin, and vitronectin, and it is recognized by at least eight integrins (46). However, mutation of the RGD in the CHL1 Ig2 domain to Lys-Gly-Glu (KGE) did not perturb CHL1-potentiated migration to collagen (Fig. 3). These results illustrated the importance of the DGEA sequence in the CHL1 Ig6 domain in mediating haptotactic migration toward collagen I through $\alpha_1/\alpha_2 \beta_1$ integrins.

CHL1 Enhances Migration through *c*-Src, PI 3-Kinase, and MAP Kinase—The nonreceptor tyrosine kinase *c*-Src, PI 3-kinase, and MAP kinase are key signaling intermediates downstream of cell adhesion molecules such as L1 (47, 48). There is evidence that PI 3-kinase also has a role in integrin receptor recycling (49), whereas MAP kinase has well characterized functions in regulating cell migration through regulating new protein synthesis that may be required for synthesis of receptors (50, 51). It is not known whether CHL1 stimulates signaling pathways such as those utilized by L1 in potentiating haptotactic migration through integrins. To determine whether Src was required for haptotactic cell migration to collagen I, HEK293 cells and CHL1-HEK293 cells were treated with PP2, a pyrazolopyrimidine inhibitor of Src family tyrosine kinases. PP2, but not its inactive analog, PP3, is a selective inhibitor of Src family catalytic activity (52). PP2 was a potent inhibitor of CHL1-dependent migration of HEK293 cells to collagen I, whereas PP3 had no effect (Fig. 4). PP2 also reduced basal migration of nonexpressing HEK293 cells, whereas PP3 did not. To assess the involvement of PI 3-kinase, HEK293 cells and CHL1-HEK293 cells were treated with the PI 3-kinase inhibitor LY294002. LY294002 partially inhibited CHL1-dependent migration of HEK293 cells to collagen, indicating that PI 3-kinase also participated in the CHL1 response (Fig. 4), but it had no effect on basal migration. To assess the role of MAP kinase in haptotactic migration, the cells were treated with an inhibitor (U0126) of the dual specificity kinase MEK, which normally phosphorylates and activates MAP kinase. The MEK inhibitor selectively suppressed migration of CHL1-expressing

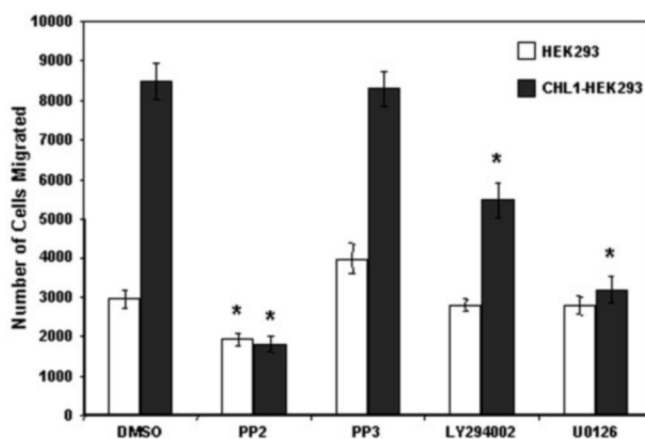


FIG. 4. ***c*-Src, PI 3-kinase, and MAP kinase are required for CHL1-potentiated migration of HEK293 cells.** Migration of CHL1-expressing or nonexpressing HEK293 cells was measured toward collagen I for 3 h. Cells migration was conducted in the presence of 0.05% dimethyl sulfoxide (DMSO, control), Src family inhibitor PP2 (5 μ M), PP3 (5 μ M), an inactive analog of PP2, PI 3-kinase inhibitor LY294002 (25 μ M), or MEK inhibitor U0126 (15 μ M). Each sample was assayed in triplicate, and the experiments were repeated at least twice with similar results. *, statistically significant differences in means of treated and untreated (control) cells using the *t* test ($p < 0.05$).

cells, indicating that MAP kinase was required for the ability of CHL1 to potentiate haptotactic migration through $\alpha_1/\alpha_2 \beta_1$ integrins to collagen I (Fig. 4). Thus, CHL1 appeared to promote integrin-mediated haptotactic migration to collagen I through the signaling intermediates Src and MAP kinase and to a lesser extent through PI 3-kinase.

CHL1 Co-caps with β_1 Integrins on the Cell Surface—Because these results showed that CHL1 promoted cell migration toward collagen mediated by integrins, we investigated whether CHL1 and β_1 integrins were laterally associated within the plasma membrane on the same cell by co-capping experiments (53). Monoclonal antibody MAB2000 specific for β_1 integrins was used to cross-link β_1 integrins on the surface of CHL1-expressing HEK293 cells. Formation of integrin caps was observed as a cluster of fluorescent spots on the cell surface (Fig. 5A). CHL1 expression was detected on the same cells by indirect immunofluorescence staining with CHL1 polyclonal antibodies (Fig. 5B). The overlay image showed CHL1 co-localization within the integrin cap (Fig. 5C), suggesting that these proteins were associated either directly or indirectly in *cis* within the plasma membrane. CHL1 immunofluorescence staining did not co-localize with all of the β_1 integrin staining, consistent with the interpretation that CHL1 interacted with a subset of β_1 integrins, most likely only the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ subclasses. Integrin caps were formed under these conditions on the majority of cells, and CHL1 co-localized with the integrin caps on all CHL1-expressing cells. In control assays, non-immune mouse IgG did not induce integrin capping or CHL1 clustering. Interestingly, mutation of the DGEA sequence in the Ig6 domain of CHL1 to AGEV did not perturb its ability to co-cap with β_1 integrins (data not shown), thus indicating that the DGEA motif was neither necessary nor sufficient for the association between CHL1 and β_1 integrins on the cell surface.

The FIGAY Motif in the CHL1 Cytoplasmic Domain Is Required for Ankyrin Recruitment and Potentiating Migration—L1 family members are coupled to the actin cytoskeleton in part through interaction of the intracellular cytoskeletal adapter protein ankyrin with the sequence Phe-Ile-Gly-Gln-Tyr (FIGQY), which is conserved in the cytoplasmic domains of L1, NrCAM, NgCAM, and neurofascin (16, 54) as well as in the single L1 homolog LAD-1 in *Caenorhabditis elegans* (55) and

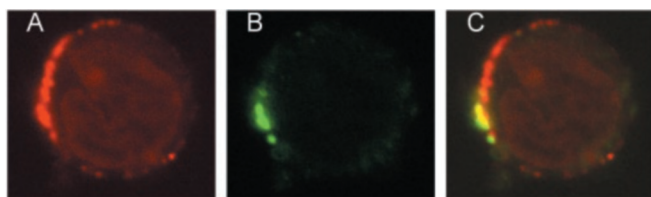


FIG. 5. β_1 integrin and CHL1 co-cap on the surface of CHL1-expressing HEK293 cells. A, β_1 integrin capping (red) was induced by cross-linking with β_1 integrin monoclonal antibody MAB2000 and was detected with a TRITC-labeled secondary antibody. B, CHL1 protein (green) labeled with CHL1 polyclonal antibodies and fluorescein isothiocyanate-labeled secondary antibody was recruited to the integrin cap. C, partial co-localization of β_1 integrin and CHL1 was indicated by the yellow color in the overlay image.

Drosophila neuroglian (56, 57). Although the structure of CHL1 reveals conservation of most of the functional domains found in other L1 family members (22), the cytoplasmic domain of CHL1 bears a nonconservative amino acid substitution in the putative ankyrin interaction motif, resulting in the sequence Phe-Ile-Gly-Ala-Tyr (FIGAY). To examine whether CHL1 was able to bind ankyrin, a cellular ankyrin recruitment assay was carried out as described (38, 58) using transfected HEK293 cells transiently expressing CHL1 and an ankyrin G fusion protein tagged with green fluorescent protein (ankyrin-GFP). In this assay, the interaction of ankyrin-GFP with CHL1 in the plasmalemma was detected indirectly through the recruitment of the ankyrin-GFP fluorescent protein from the cytosol to the cell membrane visualized by confocal microscopy. When HEK293 cells were transfected with the ankyrin-GFP plasmid in the absence of CHL1, the fluorescent ankyrin-GFP fusion protein remained cytosolic and was not recruited to the plasma membrane (Fig. 6, A and B) possibly as a result of saturation of binding sites by endogenous ankyrin (58). In contrast, when HEK293 cells were co-transfected with plasmids expressing ankyrin-GFP and CHL1, ankyrin-GFP was redistributed to the plasma membrane, where CHL1 was localized (Fig. 6, C–E). The cells shown in panels C–H of Fig. 6 were more sparsely plated than those in panels A and B and were not in contact with other cells throughout most of their cell surfaces. The recruitment of ankyrin to the plasma membrane of HEK293 cells by CHL1 appeared to be independent of cell-cell adhesion, thus differing from L1, which binds ankyrin in a cell contact-dependent manner in *Drosophila* S2 cells (56). This may be due to differences in the levels of CHL1 expression or phosphorylation state or to the unique structural features of CHL1. These results showed that CHL1, like the other members of the L1 family, was able to bind ankyrin. No proteins cross-reacting with the anti-mouse CHL1 antibody were detected in this human cell line by Western blot or indirect immunofluorescence analysis (data not shown).

Mutation of the tyrosine residue within the FIGYQ motif of the cytoplasmic domain of L1 family members disrupts their ability to recruit ankyrin (38, 54). Mutation of tyrosine 1229 in L1 to histidine occurs in some patients with the L1 mental retardation syndrome (59) and may contribute to the pathogenic mechanism of the disease because of its inability to bind ankyrin (38). The tyrosine residue contained within the FIGYQ motif of L1 and other family members appears to have a regulatory capability because it undergoes reversible phosphorylation by an unidentified kinase that modulates binding to ankyrin and the microtubule-associated protein doublecortin (60). To investigate whether the corresponding residue Tyr¹¹⁸⁶ in the FIGAY sequence of the CHL1 cytoplasmic domain was essential for ankyrin binding, this residue was mutated to alanine, and the resulting CHL1 mutant was evaluated for the

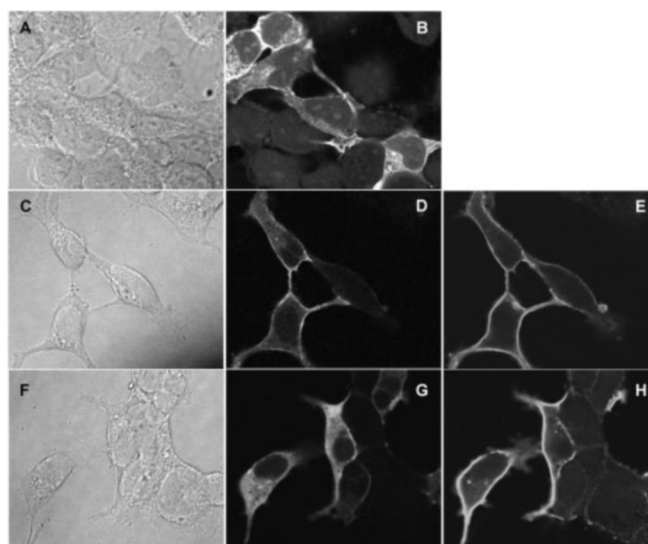


FIG. 6. CHL1 is able to recruit ankyrin to the cell membrane through the FIGAY sequence. HEK293 cells were transfected with the ankyrin-GFP expression plasmid alone (A and B) or together with a second plasmid expressing wild type CHL1 (C–E) or FIGAA mutant CHL1 (F–H). A, differential interference contrast image of HEK293 cells transfected with the ankyrin-GFP construct alone. B, HEK293 cells shown in A expressed ankyrin-GFP with a diffuse cytoplasmic localization. C, differential interference contrast image of cells co-transfected with the wild type CHL1 and ankyrin-GFP constructs. D, ankyrin-GFP was recruited to the membrane of cells expressing wild type CHL1, shown in C. E, CHL1 was localized on the cell membranes, as shown by immunofluorescence of cells in C using CHL1 antibodies and TRITC-conjugated secondary antibody. F, differential interference contrast image of cells co-transfected with the CHL1 FIGAA mutant and ankyrin-GFP constructs. G, ankyrin-GFP was not recruited to the membrane of cells expressing the mutant CHL1, shown in F, and assumes the same diffuse cytoplasmic localization as in cells not expressing CHL1 (B). H, the FIGAA mutant was normally inserted in the plasma membrane.

ability to recruit ankyrin in the cytofluorescence assay. When co-expressed with ankyrin-GFP, the mutant FIGAA failed to recruit fluorescent ankyrin-GFP to the plasma membrane (Fig. 6, F–H). The CHL1 FIGAA mutant was expressed at a level similar to that of the wild type CHL1 protein and was normally inserted in the plasmalemma of HEK293 cells (Fig. 6H).

To determine whether ankyrin binding by CHL1 and, in particular, the Tyr¹¹⁸⁶ residue within the FIGAY sequence were important for haptotactic migration to extracellular matrix substrates, wild type CHL1 and the FIGAA mutant were compared for their ability to promote migration of HEK293 cells to collagen I. When the CHL1 FIGAY sequence was mutated to FIGAA, CHL1-potentiated migration was reduced to the level of HEK293 cells not expressing CHL1 (Fig. 3). Mutation of the CHL1 FIGAY motif to FIGAA did not affect co-capping with β_1 integrins (not shown). These results suggested that CHL1-potentiated cell migration to extracellular matrix proteins may be mediated by physical linkage of its cytoplasmic domain to the actin cytoskeleton through its interaction with ankyrin and that the Tyr¹¹⁸⁶ residue was crucial for this interaction.

DISCUSSION

This study describes a novel function for CHL1 in potentiating integrin-dependent cell migration toward extracellular matrix proteins. CHL1-potentiated migration of HEK293 cells to collagen I was dependent on $\alpha_1\beta_1$ or $\alpha_2\beta_1$ integrins and was mediated by early integrin signaling intermediates c-Src, PI 3-kinase, and MAP kinase. Co-capping studies demonstrated that CHL1 and β_1 integrins were capable of associating on the

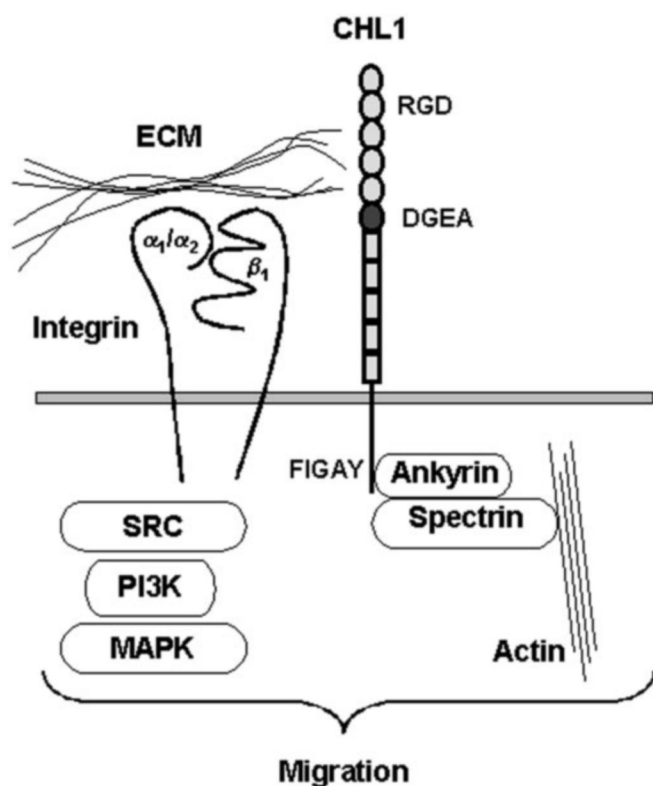


FIG. 7. Hypothetical model of the molecular interactions involved in CHL1-potentiated migration through β_1 integrins. CHL1 interacts with $\alpha_1/\alpha_2\beta_1$ integrins in *cis* on the cell surface and promotes intracellular signaling through c-Src, PI 3-kinase, and MAP kinase, which stimulates cell migration on extracellular matrix (ECM) proteins such as collagen I. The CHL1 Ig6 domain, including the DGEA motif, may transiently interact with a binding site on β_1 integrins to stimulate cell signaling, but it is not sufficient for the interaction. Linkage of CHL1 to the actin cytoskeleton through ankyrin binding to the FIGAY sequence is necessary for enhanced cell motility and may stabilize adhesive contacts with the substrate.

cell surface, and a DGEA motif in the Ig6 domain was required for enhancing migration. Through a key tyrosine residue in the FIGAY sequence within the CHL1 cytoplasmic domain, CHL1 was able to recruit ankyrin, an adapter for the spectrin-actin cortical skeleton, suggesting that this capacity was necessary for the promotion of integrin-dependent migration.

Our results describing the functional interaction of CHL1 with β_1 integrins are consistent with a model in which CHL1 associates with β_1 integrins in *cis* on the plasma membrane to promote cell migration on extracellular matrix substrates (Fig. 7). A direct or indirect association between CHL1 and β_1 integrins is supported by their ability to co-cap on the cell surface upon antibody-induced cross-linking. A potential integrin interaction motif, the DGEA sequence in the Ig6 domain of CHL1, was necessary for potentiating migration to collagen I, but it was not essential for co-capping of CHL1 and β_1 integrin. Other determinants within the CHL1 protein thus may be more important in the association with β_1 integrin, although DGEA or conformational elements of the Ig6 domain may contribute to the interaction. L1 (36, 37, 61–64) and NrCAM (65) also cooperate functionally with β_1 integrins, although a physical association has only been reported for L1 (64). Like CHL1, the Ig6 domain in L1 is important for enhancing migration, but in the case of L1, an intrinsic RGD sequence in this domain is the critical motif in promoting migration on fibronectin (37). Although CHL1 contains an RGD domain located in the second Ig domain (22), our experiments showed that the mutation of

this sequence did not affect the ability of CHL1 to potentiate migration through $\alpha_1\beta_1$ or $\alpha_2\beta_1$ integrins.

CHL1 and L1 are closely related structurally, but they have different effects in regulating haptotactic migration toward extracellular matrix proteins. CHL1 promoted migration of HEK293 cells toward collagen I through the collagen receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, but L1 did not promote migration to collagen I. Although both cell adhesion molecules stimulated migration to fibronectin, L1-potentiated migration is strongly inhibited by function blocking antibodies against the fibronectin receptor, $\alpha_5\beta_1$ integrin (37), whereas CHL1-potentiated migration to fibronectin was not affected substantially by these antibodies.² It is not known whether CHL1 promotes migration toward other matrix substrates such as fibronectin, laminin, or vitronectin through interactions with other integrin subtypes or alternatively through integrin cross-talk (43, 66, 67). An interesting possibility is that different L1 family members may interact with distinct or overlapping subclasses of integrins to potentiate haptotactic migration on diverse extracellular matrix substrates.

CHL1-potentiated migration of HEK293 cells to extracellular matrix proteins depended on c-Src, PI 3-kinase, and MAP kinase. These kinases are components of an early integrin signaling pathway that elicits membrane ruffling and lamellipodia formation and enhances cell migration (68–70). Because L1-potentiated migration also relies on these intermediates (37), they may be sites for integration of signaling by L1 cell adhesion molecules and integrins. Although we do not know whether CHL1 can directly activate these kinases, their catalytic function was necessary for enhancing migration. c-Src may act in this capacity through its ability to down-regulate RhoA GTPase, which has been shown to be necessary for integrin-mediated cell migration (71). Targets of MAP kinase involved in CHL1-stimulated cell migration have not been identified, but a possible candidate is myosin light chain kinase, which is required for FG carcinoma cell motility on collagen (72). CHL1-stimulated migration also relied in part on the activity of PI 3-kinase, an enzyme that could contribute to cell motility through its ability to influence integrin endocytosis and recycling (49, 73).

The process of cell migration requires dynamic regulation of cell adhesion through coupling of adhesion receptors with the cytoskeleton (74, 75). The motif FIGQY in the cytoplasmic domain motif of all L1 family members except CHL1 is part of the binding site for ankyrin, a multivalent adapter of the spectrin/actin cortical skeleton (18). Although CHL1 possesses a cytoplasmic motif, FIGAY, with a nonconservative substitution, our experiments show that CHL1 recruited ankyrin to the cell membrane and that this motif was essential for potentiated cell migration. This result suggested that ankyrin binding may play a role in the mechanism of cell migration, although other functions of the CHL1 cytoplasmic domain might be perturbed by this mutation, accounting for the abrogation of migration. In any case, ankyrin-binding domains of L1 family members do not have an absolute requirement in regard to sequence specificity. Several studies have shown that the association of L1 family members with ankyrin is regulated by tyrosine phosphorylation of the FIGQY sequence (54, 76) and that mutations of the conserved tyrosine perturb the ankyrin-binding function (38, 58). It is not known whether the tyrosine within the FIGAY sequence of CHL1 is phosphorylated, but its importance is evident from the perturbation of both ankyrin recruitment and enhancement of haptotactic cell migration toward extracellular

² M. Buhusi, B. R. Midkiff, A. M. Gates, M. Richter, M. Schachner, and P. F. Maness, unpublished data.

matrix proteins when it is mutated to an alanine residue. CHL1 binding to the spectrin-actin skeleton may increase the adhesion between the leading edge of the cell and substrate, which could promote cell migration (74). Finally, it did not appear that CHL1 required cell-cell contact for ankyrin binding. Although CHL1 is not known to engage in homophilic binding, interaction with the extracellular matrix substrate through *cis* integrin association could provide necessary contact that may regulate ankyrin recruitment.

Taken together these results are consistent with a model (Fig. 7) in which CHL1 interacts in *cis* with $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins on the cell surface to promote intracellular signaling through c-Src, PI 3-kinase, and MAP kinase, which stimulate cell migration on extracellular matrix proteins such as collagen I. The CHL1 Ig6 domain may transiently interact with a binding site on β_1 integrins to stimulate cell signaling, but it is not sufficient for the interaction. Linkage of CHL1 to the actin cytoskeleton through ankyrin binding to the FIGAY sequence is also an important determinant of cell motility and may help stabilize adhesive contacts with the extracellular matrix substrate.

The capacity of CHL1 to potentiate haptotactic migration toward extracellular matrix proteins through integrins in these *in vitro* studies may reflect a physiological role for CHL1 in integrin-dependent cell migration during nerve regeneration. CHL1 may regulate the migration of Schwann cells at sites of nerve injury, because it is strongly up-regulated in Schwann cells during regeneration (23). Schwann cell migration on regenerating nerves is supported by collagens I, III, IV, and fibronectin, which, along with $\alpha_1\beta_1$ integrin, increase at the injury site (41, 77, 78). CHL1 expression is also up-regulated in dorsal root ganglion and thalamic neurons upon injury (23, 79) and is a strong promoter of neurite growth (20), suggesting that it may function analogously to promote axon growth on extracellular matrix substrates. The ability of CHL1 to promote cell migration toward extracellular matrix proteins may also play a role in radial glia-guided migration of cortical neurons in the developing neocortex, where integrin receptors and their ligands are distributed (80) and CHL1 is prominently expressed (21).

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