L1-dependent neuritogenesis involves ankyrinB that mediates L1-CAM coupling with retrograde actin flow

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The cell adhesion molecule L1 (L1-CAM) plays critical roles in neurite growth. Its cytoplasmic domain (L1CD) binds to ankyrins that associate with the spectrin–actin network. This paper demonstrates that L1-CAM interactions with ankyrinB (but not with ankyrinG) are involved in the initial formation of neurites. In the membranous protrusions surrounding the soma before neuritogenesis, filamentous actin (F-actin) and ankyrinB continuously move toward the soma (retrograde flow). Bead-tracking experiments show that ankyrinB mediates L1-CAM coupling with retrograde F-actin flow in these perisomatic structures. Ligation of the L1-CAM ectodomain by an immobile substrate induces L1CD–ankyrinB binding and the formation of stationary ankyrinB clusters. Neurite initiation preferentially occurs at the site of these clusters. In contrast, ankyrinB is involved neither in L1-CAM coupling with F-actin flow in growth cones nor in L1-based neurite elongation. Our results indicate that ankyrinB promotes neurite initiation by acting as a component of the clutch module that transmits traction force generated by F-actin flow to the extracellular substrate via L1-CAM.

Introduction

Complex neural networks are formed by nerve processes that have emerged and elongated from nascent neurons. Various functional molecules, including cell adhesion molecules (CAMs) and cytoskeletal elements, are cooperatively involved in process outgrowth. Developmental defects in major axon tracts, such as the corpus callosum and the corticospinal tract, are found in humans with X-linked hydrocephalus that is caused by mutations in the gene of cell adhesion molecule L1 (L1-CAM), a member of the Ig superfamily of CAMs (Kamiguchi et al., 1998). L1-CAM is a single-pass transmembrane protein that is predominantly expressed by developing neurons, and promotes neuronal migration and neurite growth. Human pathogenic mutations in the L1-CAM cytoplasmic domain (L1CD) produce less severe phenotypes than those in the L1-CAM extracellular domain (L1ED). However, mutations in the L1CD almost always cause abnormal axon tract development, suggesting that the L1CD is critical for axon growth (Kamiguchi et al., 1998). The majority of mutations in the L1CD reported so far are either nonsense or frame shift mutations that produce a premature stop codon (Van Camp et al., 1996), eliminating its COOH-terminal tail that contains a binding site for ankyrins. The amino acid residues SFIGQY1229 in the human L1CD are conserved among the majority of L1 family CAMs including NrCAM and neurofascin (Hortsch, 2000), and are critical for their ankyrin-binding activity (Garver et al., 1997; Hortsch et al., 1998a).

The ankyrin family currently includes three genes encoding 480/270-kD ankyrinG and 440/220-kD ankyrinB, as well as multiple alternatively spliced variants (Bennett and Chen, 2001). The ankyrinG and ankyrinB are the major isoforms expressed in the developing nervous system. Ankyrins interact with structurally diverse membrane proteins,
including ion channels and L1 family CAMs, through ANK repeats in their membrane-binding domains. Ankyrins also associate with the spectrin–actin network via their spectrin-binding domains, thereby coupling the membrane proteins to the actin cytoskeleton. L1-CAM and ankyrin are colocalized in premylinated axon tracts during development. Ankyrin<sup>[1–7]</sup> mice exhibit axon tract hypoplasia similarly to L1-CAM<sup>[c–h]</sup> mice and X-linked hydrocephalus patients (Scotland et al., 1998). Furthermore, missense point mutations in the ankyrin-binding region of the L1CD, Y1229H and S1224L, are sufficient to produce the phenotype in humans (Van Camp et al., 1996), suggesting that the interaction of L1-CAM with ankyrins plays an important role in axon growth. However, it remains to be determined whether and how ankyrins are involved in axon growth stimulated via L1-CAM.

Generally, it is thought that cell migration and process outgrowth are driven by traction force generated by F-actin that flows in a backward direction (Lin and Forscher, 1995; Mitchison and Cramer, 1996). CAMs transmit this force by linking the retrograde F-actin flow with immobile ligands present on neighboring cells or in the ECM (Sheetz et al., 1998). For example, several Ig superfamily CAMs, such as apCAM, NrCAM, and L1-CAM, have been shown to couple with retrograde F-actin flow in nerve growth cones (Suter et al., 1998; Faivre-Sarrailh et al., 1999; Kamiguchi and Yoshihara, 2001), thereby promoting neurite elongation. It has also been hypothesized that the initial formation of neurites is induced by coupling between extracellular substrates and retrograde F-actin flow in membranous protrusions surrounding the soma (Smith, 1994). However, poorly understood is the molecular identity of the clutch module that mediates CAM–actin linkages during neurite formation and elongation. The present work shows that L1-dependent neuritogenesis involves an interaction between ankyrin<sub>B</sub> and the SFIGQY-containing region in the L1CD, and that this interaction mediates L1-CAM coupling with retrograde F-actin flow. We propose that ankyrin<sub>B</sub> constitutes the clutch module that regulates neurite initiation stimulated by L1 family CAMs.

**Results**

**L1CD–ankyrin<sub>B</sub> interactions are induced by L1ED ligation**

The interaction of ankyrins (270-kD ankyrin<sub>C</sub> and 220-kD ankyrin<sub>B</sub>) with the L1CD was tested indirectly by the ankyrin recruitment assay as described previously (Zhang et al., 1998). When HEK 293 cells were transfected with an expression plasmid coding for either GFP-tagged ankyrin<sub>C</sub> (Zhang et al., 1998) or GFP-tagged ankyrin<sub>B</sub> (Mohler et al., 2002), the fluorescent signals were diffusely distributed in the cytoplasm with slight enrichment along the plasma membrane (Fig. 1, A and B). This indicates that endogenous spectrin and other ankyrin-binding proteins such as Na/K ATPase in 293 cells were insufficient to recruit the majority of exogenous ankyrins to the plasma membrane. Cotransfection of a neuronal form of L1-CAM caused GFP-ankyrin<sub>C</sub> (but not GFP-ankyrin<sub>B</sub>) to become recruited to the plasma membrane (Fig. 1, C and D). In contrast, cotransfection of L1-CAM<sub>ΔC77</sub>, a COOH-terminal truncation mutant that completely lacks the ankyrin-binding region (Kamiguchi and Lemmon, 1998), did not influence the cytoplasmic distribution of GFP-ankyrin<sub>C</sub> (Fig. 1 E). These results are consistent with a previous report (Needham et al., 2001) stating that the L1CD expression is necessary and sufficient for recruitment of exogenous ankyrin<sub>C</sub> to the plasma membrane. However, our results indicate that the L1CD is not sufficient to recruit ankyrin<sub>B</sub> to the plasma membrane.

Next, we examined the distribution pattern of ankyrin<sub>B</sub> in contacting cells, because the recruitment of *Drosophila* ankyrin to the plasma membrane was dependent on L1-mediated cell–cell adhesion (Hortsch et al., 1998b). While ankyrin<sub>B</sub> remained cytoplasmic in L1-CAM–negative cells in contact (Fig. 2, A and B), L1-CAM coexpression recruited ankyrin<sub>B</sub> to the plasma membrane that attaches to the membrane of neighboring L1-CAM–positive cells, but not of L1-CAM–negative cells (Fig. 2, C–E). As controls, human pathogenic mutations in the L1CD, such as ΔC77, Y1229H, and S1224L, impaired L1-CAM’s ability to recruit ankyrin<sub>B</sub> to cell contact sites (Fig. 2, F–K). These results support the idea that homophilic trans-adhesion via L1-CAM induces ankyrin<sub>B</sub> binding to the SFIGQY<sup>1229</sup>-containing region in the L1CD. Although the L1CD expression was sufficient to recruit ankyrin<sub>C</sub> to the plasma membrane (Fig. 1), L1-mediated cell–cell adhesion induced an even greater recruitment of ankyrin<sub>C</sub> to the contacting membrane (Fig. 2, L–O).

To further investigate L1-CAM interactions with ankyrin<sub>B</sub>, we used fluorescent resonance energy transfer (FRET) microscopy using CFP tagged to the COOH terminus of

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**Figure 1.** The L1CD is sufficient for recruitment of exogenous ankyrin<sub>C</sub>, but not ankyrin<sub>B</sub>, to the plasma membrane. Confocal fluorescent images of GFP-ankyrin<sub>C</sub> (A, C, and E) and GFP-ankyrin<sub>B</sub> (B, D, and F) expressed by 293 cells. The cells did not express L1-CAM (A and B) or did coexpress either wild-type L1-CAM (C and D) or L1-CAM<sub>ΔC77</sub> (E and F). L1-CAM expression was visualized by immunofluorescence (not depicted). Bar, 10 μm.
the L1CD (L1-CFP) as a donor and Venus tagged to the NH₂ terminus of ankyrinB (Venus-ankyrinB) as an acceptor. Venus is a variant of YFP with efficient maturation and increased resistance to environmental changes (Nagai et al., 2002). Because FRET from CFP to YFP occurs only if the two proteins are in very close proximity (<50Å), L1-CAM–ankyrinB interactions should be assessed by measuring FRET efficiency (FRET<sup>E</sup>) that is defined as the percentage of donor signal loss due to FRET (Miyawaki and Tsien, 2000). FRET<sup>E</sup> can be mathematically deduced from three fluorescent measurements (a donor excitation/donor emission image; a donor excitation/acceptor emission image; and an acceptor excitation/acceptor emission image) after correcting uncertain stoichiometries of CFP to YFP expression and their spectral cross talk (Gordon et al., 1998). Using their method (see Materials and methods), we calculated FRET<sup>E</sup> from L1-CFP to Venus-ankyrinB. In 293 cells expressing L1-CFP and Venus-ankyrinB, increased FRET<sup>E</sup> was detected at cell contact sites (Fig. 3, A and D). This distribution pattern of increased FRET<sup>E</sup> was confirmed by independent measurement using the acceptor photobleaching method (Miyawaki and Tsien, 2000; Fig. 3, I–K). As controls, such an increase in FRET<sup>E</sup> at cell contact sites was not observed when a single amino acid mutation, Y1229H (Fig. 3, B and E) or S1224L (Fig. 3, C and F), was introduced to the ankyrin-binding region of L1-CFP. Furthermore, the cross-linking of L1-CAM on the cell surface by anti-L1-CAM antibody increased FRET<sup>E</sup> from wild-type L1-CFP to Venus-ankyrinB in a punctate pattern (Fig. 3 G), whereas the treatment with control antibody had no effect (Fig. 3 H). These results indicate that L1CD–ankyrinB binding, as assessed by FRET<sup>E</sup>, is induced by L1ED ligation.

**L1CD–ankyrinB interactions are involved in L1-stimulated neurite initiation**

We tested whether ankyrins are required for neurite growth on an L1-Fc substrate and two other substrates, N-cadherin–Fc and laminin, using ankyrin<sub>B</sub>(<sup>−/−</sup>) and ankyrin<sub>G</sub>(<sup>−/−</sup>) mouse strains described previously (Scotland et al., 1998; Zhou et al., 1998). It has been reported that L1-CAM and N-cadherin presented as a culture substrate stimulate neurite growth in vitro by binding homophilically to L1-CAM and N-cadherin expressed on the neuronal surface, respectively (Lemmon et al., 1989; Bixby and Zhang, 1990). Although heterophilic trans-interactions of L1-CAM with integrins have also been reported (Yip et al., 1998), L1-CAM knockout neurons completely lose their ability to extend neurites on an L1 substrate (Dahme et al., 1997; Fransen et al., 1998), indicating that L1-stimulated neurite growth from mouse neurons depends, for the most part, on homophilic L1-CAM interactions. In contrast, laminin promotes neurite growth upon binding to integrins (Bozyczko and Horwitz, 1986; Tomaselli et al., 1986). As shown in Fig. 4, ankyrin<sub>B</sub>(<sup>−/−</sup>) neurons derived from both the cerebellum and dorsal root ganglion (DRG) had an impaired ability to ini-
Figure 3.  **L1CD–ankyrin$_B$ interactions as assessed by quantitative FRET microscopy.** (A–H) Three fluorescent images of 293 cells expressing L1-CFP and Venus-ankyrin$_B$ were obtained, and FRET$^E$ was calculated and displayed using quantitative pseudocolor (see Materials and methods). (A–F) 293 cells were cotransfected with Venus-ankyrin$_B$ and wild-type L1-CFP (A and D), Venus-ankyrin$_B$ and L1$^{Y1229H}$-CFP (B and E), or Venus-ankyrin$_B$ and L1$^{S1224L}$-CFP (C and F). Shown are FRET$^E$ (A–C) and CFP images (D–F) of the cells. (G and H) Time-lapse images of FRET$^E$ in living cells expressing wild-type L1-CFP and Venus-ankyrin$_B$. Anti-L1-CAM antibody (G) or anti-L1CD antibody (H) was added to the culture medium at 0 min. (I–K) 293 cells were transfected with L1-CFP and Venus-ankyrin$_B$, and FRET$^E$ was measured using the acceptor photobleaching method.

Figure 4.  **Ankyrin$_B$ is involved in L1-stimulated neurite initiation.** (A and B) DIC images of cerebellar granule cells derived from wild-type mice (A) or ankyrin$_B^{-/-}$ mice (B). The cells have been cultured on an L1-Fc substrate for 20 h. Bar, 50 $\mu$m. (C–F) Neurite initiation from cerebellar granule cells plated on L1-Fc (C), N-cadherin–Fc (D), laminin (E), or a control substrate coated with anti-Fc antibody but no CAM-Fc (F). The percentage of neurons bearing neurites was plotted against hours in culture. Neurons from ankyrin$_B^{-/-}$ mice (open circles) and their wild-type littermates (closed circles) were analyzed. (G) L1-stimulated neurite initiation from wild-type DRG neurons (closed circles) and ankyrin$_B^{-/-}$ DRG neurons (open circles). (H) DRG neurons were cultured for 4 h on the indicated substrates, and the percentage of neurons bearing neurites was quantified. Each value is from four determinations involving $>$200 neurons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; compared with wild-type neurons under the same culture conditions.

Shown are Venus images before (I) and after (J) bleaching, and a FRET$^E$ image (K) given as $1 - I_d/I_a$ where $I_a$ and $I_d$ are CFP image intensities before and after the acceptor bleaching, respectively. Bars, 20 $\mu$m.
tiate neurites on an L1 substrate, but not on the other two substrates. Considering the level of background neurite initiation on a control substrate coated with anti-Fc antibody but no CAM-Fc (Fig. 4, F and H), the loss of ankyrin expression resulted in 30–50% decrease in L1-dependent neurite initiation. In contrast, the length of neurites did not depend on ankyrin expression on any of the substrates tested (Table III), indicating that ankyrin B is not involved in neurite elongation. The loss of ankyrin expression did not affect viability of the neurons, and the populations of the surviving neurons did not depend on the substrates tested (Table I and Table II). We also compared ankyrinB(−/−) cerebellar granule cells with wild-type neurons, but there was no significant difference in neurite initiation and elongation on any of the substrates tested (Table III).

Next, we tested whether neurite initiation is affected by overexpression of L1-CAM mutants (L1-CAMΔC77, L1-CAMΔ512291, and L1-CAMΔ1224L) in a dominant-negative manner. To ensure a high level expression of transfected L1-CAM and endogenous mouse L1-CAM, Fluo-3 fluorescent images of DRG neurons were acquired with a 12-bit CCD camera followed by background subtraction. The L1CD immunoreactivity was quantified by measuring the average fluorescent intensities (0–4095) of pixels within a 203 by 200 region of interest. This result indicated that the level of transfected L1-CAM was >10 times the amount of endogenous L1-CAM, which should be sufficient to exert a dominant-negative effect. As shown in Fig. 5 (C and D), the neurons overexpressing any of the L1-CAM mutants had an impaired ability to initiate neurites in response to L1-Fc as compared with those transfected with wild-type L1-CAM. These data, together with our results on ankyrinB(−/−) neurons, demonstrate that L1CD–ankyrinB interactions are involved in L1-mediated neurite initiation. The dominant-negative effect of these L1-CAM mutants ap-

### Table I. Characteristics of ankyrinB(−/−) cerebellar granule cells

| Genotype       | Substrate | Surviving cellsa (n = 4) | Zic-positive cellsb (n = 4) | Neurite length at 37 h after plating (n = 200) |
|----------------|-----------|--------------------------|----------------------------|-----------------------------------------------|
|                |           | %                        | %                          | µm                                            |
| Wild type      | L1-Fc     | 95.5 ± 1.9               | 89.5 ± 1.4                 | 86.9 ± 3.8                                   |
|                | N-cadherin-Fc | 94.5 ± 0.6             | 90.9 ± 1.7                 | 81.6 ± 4.0                                   |
|                | Laminin   | 92.0 ± 1.3               | 90.8 ± 0.9                 | 67.9 ± 3.4                                   |
|                | Control   | 84.0 ± 2.9               | ND                         | ND                                            |
| AnkyrinB(−/−)  | L1-Fc     | 95.7 ± 1.7               | 91.0 ± 1.9                 | 85.0 ± 3.6                                   |
|                | N-cadherin-Fc | 97.0 ± 0.7            | 91.7 ± 1.0                 | 78.8 ± 3.7                                   |
|                | Laminin   | 90.9 ± 1.5               | 92.5 ± 2.1                 | 65.4 ± 2.8                                   |
|                | Control   | 89.7 ± 3.8               | ND                         | ND                                            |

ND, not determined.

aCell viability was determined by trypan blue staining.
bZic is a marker for granule cells in the cerebellum (Aruga et al., 1994).

### Table II. Characteristics of ankyrinB(−/−) DRG neurons

| Genotype       | Substrate | Surviving cellsa (n = 4) | TrkA-positive cellsb (n = 4) | TrkC-positive cellsb (n = 4) | Neurite length at 12 h after plating (n = 91–100) |
|----------------|-----------|--------------------------|----------------------------|-----------------------------|--------------------------------------------------|
|                |           | %                        | %                          | %                           | µm                                               |
| Wild type      | L1-Fc     | 93.9 ± 0.9               | 48.7 ± 3.8                 | 28.8 ± 2.2                  | 129.4 ± 7.5                                     |
|                | N-cadherin-Fc | 94.1 ± 1.1            | 49.7 ± 3.5                 | 31.2 ± 5.3                  | 175.9 ± 8.9                                     |
|                | Laminin   | 90.7 ± 2.4               | 49.5 ± 2.6                 | 32.4 ± 5.2                  | 183.1 ± 12.8                                    |
|                | Control   | 85.8 ± 0.9               | 51.1 ± 4.4                 | 34.1 ± 3.6                  | 28.8 ± 1.5                                     |
| AnkyrinB(−/−)  | L1-Fc     | 92.3 ± 1.2               | 47.3 ± 3.9                 | 29.7 ± 1.7                  | 120.7 ± 6.9                                     |
|                | N-cadherin-Fc | 89.6 ± 1.7            | 49.4 ± 4.8                 | 34.4 ± 1.4                  | 183.0 ± 8.7                                     |
|                | Laminin   | 94.4 ± 1.5               | 45.6 ± 1.9                 | 29.7 ± 2.6                  | 191.1 ± 10.5                                    |
|                | Control   | 90.6 ± 1.7               | 48.6 ± 4.8                 | 34.2 ± 1.9                  | 25.2 ± 1.4                                     |

aCell viability was determined by trypan blue staining.
AnkyrinG neurite initiation was not affected by L1-CAM. As another control, L1-stimulated neurite initiation from untransfected neurons was quantified (D). N-cadherin–stimulated L1-CAM. As another control, L1-stimulated neurite initiation from overexpression of wild-type L1-CAM.

In the process of neuritogenesis, DRG neurons always formed membranous protrusions surrounding the soma (Fig. 6 A), which were termed the perisomatic lamellae and filopodia in this paper. Dynamic properties of F-actin in these perisomatic structures were investigated by fluorescent speckle microscopy, in which low concentrations of fluorescently labeled phalloidin were used to generate fiducial marks (speckles) on F-actin (Schaefer et al., 2002a). As shown in Fig. 6 (B and C), F-actin moved toward the soma. The speed of retrograde F-actin flow did not depend on ankyrinB expression: 6.3 ± 0.2 μm/min (n = 48) in 12 wild-type neurons and 6.1 ± 0.2 μm/min (n = 56) in 14 ankyrinB−/− neurons.

To test whether ankyrinB associates with retrograde F-actin flow, the movement of Venus-ankyrinB expressed in DRG neurons was monitored by time-lapse fluorescent microscopy. As shown in Fig. 6 (D and E), ankyrinB formed clusters of various sizes in the perisomatic lamellae and filopodia. Smaller clusters of ankyrinB often moved toward the soma, whereas larger clusters tended to be stationary. The representative movement of a small ankyrinB cluster in the perisomatic filopodia is shown in Fig. 6 F. We simultaneously acquired differential interference contrast (DIC) images to exclude the possibility that the observed ankyrinB movement was due to filopodial retraction. An ankyrinB cluster was often found in a bulbous structure in the filopodia (Fig. 6 F, DIC images). We showed that ankyrinB associates with retrograde F-actin flow.

AnkyrinB mediates L1-CAM coupling with retrograde F-actin flow in membranous protrusions surrounding the soma

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also tested whether spectrin mediates ankyrinB coupling with F-actin flow, using an ankyrinB mutant that had a single amino acid substitution, A1000P, in the spectrin-binding domain and did not interact with spectrin (unpublished data). When expressed in DRG neurons, this ankyrinB mutant was less likely to form clusters than wild-type ankyrinB, and the mutant clusters did not show the retrograde directional movement in the perisomatic filopodia. This suggests that ankyrinB associates with F-actin flow via spectrin. Interestingly, neurons cultured on an L1-Fc substrate for 2 h were more likely to have large stationary ankyrinB clusters than those cultured on an N-cadherin–Fc substrate: 43.6 ± 4.8% (n = 4) out of ≥200 neurons on L1-Fc had such clusters, compared with 25.1 ± 3.1% (n = 4) on N-cadherin–Fc. Endogenous ankyrinB also formed large clusters colocalizing with endogenous L1-CAM in the perisomatic lamellae of DRG neurons on an L1-Fc substrate (Fig. 7, A–C). These results, together with the FRET data (Fig. 3), suggested to us that L1ED ligation induced the formation of a molecular complex involving F-actin, ankyrinB, L1-CAM, and an immobile extracellular substrate (L1-Fc in this case). Such a complex would be able to transmit traction force generated by F-actin to the substrate, leading to the formation of neurites.

This hypothesis was supported by longer term time-lapse imaging of Venus-ankyrinB–transfected neurons (Fig. 6, J and K) and by FRET imaging of neurons (Fig. 8). L1-stimulated neurite initiation preferentially occurred at the site of large stationary ankyrinB clusters (Fig. 6, J and K; neurites at the bottom), although some neurites were formed independent of ankyrinB clustering (Fig. 6, J and K; neurites at the top). Analyses of 11 neurons stimulated with L1-Fc revealed that 14 of 17 neurites were formed at the site of stationary ankyrinB clusters, whereas only 4 of 20 neurites from 11 neurons stimulated with N-cadherin–Fc were formed at the site of such clusters. FRET microscopy of fixed neurons indicated that the L1CD interacted with ankyrinB at the site of large ankyrinB clusters in the perisomatic lamellae if neurons had been stimulated with L1-Fc, but not with N-cadherin–Fc (Fig. 8, A–G). Quantitative analyses gave the following results: 20 of 68 (29.4%) ankyrinB clusters in the perisomatic lamellae of L1-treated neurons showed FRET E > 8%, whereas only 1 of 46 (2.1%) ankyrinB clusters of N-cadherin–treated neurons showed FRET E > 8%. We also found that growth cones rarely (4.5%; 2 of 44 growth cones with strong Venus-ankyrinB expression) showed FRET E > 8%
even if neurite initiation and elongation had been stimulated by L1-Fc (Fig. 8, H–J). Although the FRET analysis does not exclude the possibility of L1-CAM interaction with ankyrinB in growth cones, the interaction is unlikely to occur in the peripheral domain because expression of endogenous ankyrinB was restricted to the central domain in growth cones as assessed by immunocytochemistry (Fig. 7, D–F). Collectively, our results so far strongly suggest that ankyrinB, in response to L1ED ligation, links the L1CD with retrograde F-actin flow in the perisomatic membranous structures, but not in the growth cone peripheral domain.

Direct evidence that endogenous ankyrinB is involved in L1-CAM coupling with retrograde F-actin flow has been obtained by bead-tracking experiments. As we reported previously (Kamiguchi and Yoshihara, 2001), microbeads coated with either L1-Fc or anti-L1-CAM antibody bound to L1-CAM expressed on the growth cone lamellae and showed retrograde directional movement when coupled with F-actin flow. In the present work, we monitored the movement of beads coated with L1-Fc, anti-L1-CAM antibody, or laminin on the perisomatic lamellae of wild-type or ankyrinB(−/−) DRG neurons. On either group of neurons, 88–92% of the beads showed retrograde directional movement regardless of the coating proteins, whereas the remaining 8–12% showed Brownian motion on the cell surface. On wild-type neurons pretreated with 0.5 μg/ml cytochalasin D, none of 25 L1-
Fc–coated beads that had bound to the cell surface showed retrograde directional movement, indicating that this type of bead movement is driven by F-actin flow. To assess the coupling efficiency of L1-CAM with F-actin flow, we measured the speed of retrograde bead movement. Note that the speed of F-actin flow does not depend on ankyrinB expression as described in the previous paragraph. The movement of beads coated with either L1-Fc or anti-L1-CAM antibody on ankyrinB−/− neurons was significantly retarded compared with that on wild-type neurons (Fig. 9, A–F), indicating that ankyrinB is required for the full engagement of L1-CAM with retrograde F-actin flow in the perisomatic lamellae. As a control, the loss of ankyrinB expression did not affect the movement of laminin-coated beads (Fig. 9 I). The partial slippage between L1-CAM and F-actin flow in ankyrinB−/− neurons indicated the existence of other clutch molecules. One candidate is a member of the ERM (ezrin-radixin-moesin) family, an actin-binding protein that has recently been demonstrated to interact with the Arg-Ser-Leu-Glu (RSLE)–containing region in the L1CD (Dickson et al., 2002). To explore this possibility, we generated cDNA constructs of wild-type and RSLE-minus human L1-CAM tagged to GFP, and transfected mouse DRG neurons with each construct. Neurons expressing the transgene were identified by GFP fluorescence, and a microbead coated with 200 µg/ml 5G3 was placed on the tip of a perisomatic filopodium. Out of the 26 beads tested on the perisomatic filopodia expressing wild-type human L1-CAM, 12 beads moved retrogradely at 2.4 ± 0.2 µm/min. In contrast, the beads (n = 27) never showed retrograde directional movement on the perisomatic filopodia expressing RSLE-minus human L1-CAM. Therefore, a member of the ERM family might be another component of the clutch module that regulates L1-based neurite initiation.

Our result that ankyrinB was not required for neurite elongation led us to test for the involvement of ankyrinB in L1-CAM coupling with F-actin flow in growth cone lamellae. As expected, the loss of ankyrinB expression did not affect retrograde movement of beads coated with either L1-Fc or anti-L1-CAM antibody on growth cone lamellae (Fig. 9, G and H). This was also consistent with our result that ankyrinB did not interact with the L1CD in the growth cone peripheral domain. Collectively, the data indicate that neurons change components of the clutch module during neurite growth and that ankyrinB acts as a clutch component only before neurite formation.

Discussion

The clutch hypothesis states that cell motility is controlled by regulated engagement between retrograde F-actin flow and CAMs bound to an immobile substrate (Mitchison and Kirschner, 1988). Although many pieces of evidence for this hypothesis have been reported, the question has not been answered as to what molecules constitute the clutch module (Jay, 2000). With respect to CAM–cytoskeletal linkages, integrins are by far the best-studied family of CAMs. Focal adhesion proteins are obvious candidates for the clutch module that connects integrins with F-actin flow. The cytoskeletal linkage via focal adhesion proteins, such as talin and vinculin, has been implicated in cell motility and neurite growth (Nuckolls et al., 1992; Varnum-Finney and Reichardt, 1994; Sydor et al., 1996). Furthermore, it has been reported that focal adhesions, as labeled with GFP-integrin chimera, exhibit nonmotile and motile states coordinated with cell migration, suggesting the existence of a molecular clutch that alternates between these states (Smilenov et al., 1999). However, it remains to be determined what molecules connect CAMs with F-actin flow in such a regulated manner. The most important aspect of our research is the direct demonstration that ankyrinB mediates L1-CAM coupling with F-actin flow. In addition, we provide the following results that support the idea that ankyrinB constitutes the clutch module regulating L1-mediated neurite initiation (Fig. 10): (1) ankyrinB showed retrograde movement that was associated with F-actin flow; (2) in response to L1ED ligation by an immobile substrate, ankyrinB interacted with the L1CD and formed stationary clusters; (3) neurite initiation preferentially occurred at the site of stationary ankyrinB clusters; and (4) neurite initiation was impaired by loss of L1CD–ankyrinB interactions.
Another important topic in this paper is the differences between ankyrinG and ankyrinB: (1) coexpression of the L1CD with ankyrinG is sufficient for their interaction, whereas the L1CD binds to ankyrinB in response to L1ED ligation; and (2) L1-CAM binding to ankyrinB (but not to ankyrinG) is involved in neurite initiation. This suggests that ankyrinB collaborates with L1-CAM in dynamic cell functions, whereas ankyrinG may be involved in rather static adhesion. The idea that different forms of ankyrins have distinct functional significance could be supported by a recent report (Gil et al., 2003). They showed that the ankyrin-binding activity of the L1CD is required for its interactions with static components of the cytoskeleton in ND-7 neuroblastoma hybrid cells, and also that the binding activity inhibits retrograde L1-CAM movement on the cell surface. Although not identified in their report, some form of ankyrins expressed in ND-7 cells should mediate such static interactions, which is in striking contrast to the role of ankyrin demonstrated in our paper. It is likely that a large number of ankyrin isoforms play diverse roles in different cell types and at different developmental stages.

As has been shown in neurofascin–ankyrin binding (Garver et al., 1997), phosphorylation of Y1229 in the L1CD might be responsible for the lack of ankyrin interactions with nonligated L1-CAM. However, the ankyrin recruitment assay using Drosophila S2 cells showed that ankyrin recruitment was dramatically reduced, but still limited to cell contact sites when the corresponding tyrosine residue of neuroglian, a Drosophila homologue of L1-CAM, was mutated to a phenylalanine (Hortsch et al., 1998a). We also obtained a similar result with L1-CAMY1229F-expressing 293 cells (unpublished data). Therefore, another mechanism must be responsible for the outside-in regulation of L1CD–ankyrin binding. There are at least five other phosphorylation sites in the L1CD (Kamiguchi and Lemmon, 1997; Schaefer et al., 1999, 2002b), but none of the amino acid substitutions of these phosphorylation sites affected the selective recruitment of ankyrin to cell contact sites (unpublished data). Alternatively, a conformational change in the L1CD induced by oligomerization of the L1ED (Silletti et al., 2000) could be the mechanism, as proposed by Jefford and Dubreuil (2000), but further research will be required to solve this problem.

Materials and methods

Antibodies

Rabbit antisera against human L1-CAM, rat L1-CAM, and L1CD were provided by Dr. Vance Lemmon (Case Western Reserve University, Cleveland, OH). These antisera have been described previously (Hävin and Lemmon, 1991; Schaefer et al., 1999; Long et al., 2001). Rabbit anti-Zic antibody was a gift of Dr. Jun Aruga (RIKEN Brain Science Institute, Saitama, Japan; Aruga et al., 1994). Mouse anti-human L1-CAM antibody (5G3) was purchased from BD Biosciences, mouse anti-ankyrinB antibody from Zymed Laboratories, rabbit antibodies against TrkA and TrkC from CHEMICON International, and Alexa-conjugated secondary antibodies from Molecular Probes, Inc.

cDNA constructs

The generation of cDNA constructs is explained in detail in the supplemental Materials and methods section (available at http://www.jcb.org/cgi/content/full/jcb.200303060/DC1). In brief, the pcDNA3-based expression plasmids (Invitrogen), which contain a cDNA encoding for the neuronal form or its mutant forms of human L1, were generated using the site-directed mutagenesis kit (CLONTECH Laboratories, Inc.). The plasmids containing L1-CAM(C7), cDNA have been described previously (Kamiguchi and Lemmon, 1998). The pECFP-N1 vector (CLONTECH Laboratories, Inc.) encoding for L1-CFP, in which CFP is tagged to the COOH terminus of human L1-CAM via a multiple (GGSGG)-GGSGG-α-amino acid linker, was generated by PCR. Similarly, the vector encoding for Venus-ankyrinB, in which Venus is tagged to the NH2 terminus of human ankyrinB, was generated by PCR. The linker sequences in both L1-CFP and Venus-ankyrinB were optimized based on FRET measurements in transfected 293 cells: L1-(GGSGGTTGGGGSG)-CFP and Venus-(GGSGGGS)-ankyrinB. Human L1-CAM cDNA was provided by Dr. Vance Lemmon, and Venus cDNA by Dr. Atsushi Miyawaki (RIKEN Brain Science Institute).

Cell culture

The cerebelli and DRGs dissected from P0 mice were dissociated as described previously (Nakai and Kamiguchi, 2002), and were plated on a dish coated with 9 μg/cm2 laminin (Life Technologies) or CAM-Fc. Production of CAM-Fc, which consists of the whole extracellular domain of a CAM (chick L1-CAM or chick N-cadherin) and the Fc region of human IgG, was performed as described previously (Kamiguchi and Yoshihara, 2001). CAM-Fc–coated dishes were prepared by sequential coating with 0.1 mg/ml poly-ơ-lysine (70–150 kD; Sigma-Aldrich), 40 μg/ml anti-Fc antibody (Sigma-Aldrich), and CAM-Fc. The N-cadherin-Fc cDNA construct was a gift of Dr. Patrick Doherty (Guy’s Hospital, London, UK). Neurons were cultured in serum-free media with essential supplements as described previously (Nakai and Kamiguchi, 2002).

HEK 293 cells (American Type Culture Collection) were seeded on a dish coated with 6 μg/cm2 fibronectin (Life Technologies) and cultured in RPMI 1640 medium supplemented with 10% FBS.

The cultures were maintained in a humid atmosphere of 95% air, 5% CO2 at 37°C. For live-cell imaging, 293 cells were cultured in Leibovitz’s L-15 medium (Life Technologies), and DRG neurons in L-15 supplemented with N-2 (Life Technologies) and 50 ng/ml NGF (Promega), in a humid atmosphere of 100% air at 37°C on a microscope stage.

Transfection

HEK 293 cells were transfected with expression plasmids using FuGENE™ 6 transfection reagent (Roche) according to the manufacturer’s protocol. DRG neurons were transfected with LipofectAMINE™ 2000 (Life Technologies). To allow for high level expression of transgene products before neurite initiation, we used the following protocol: (1) DRG neurons were plated on a dish coated sequentially with poly-ơ-lysine and anti-Fc antibody and incubated for 3 h. (2) The cells were incubated in the presence of DNA–LipoFectAMINE™ 2000 complexes for 2 h according to the manufacturer’s protocol. (3) After an additional 12-h incubation, CAM-Fc was added to the culture medium to stimulate neurite growth.

Immunocytochemistry

HEK 293 cells were fixed with 4% formaldehyde in PBS for 20 min, blocked with 10% horse serum in PBS for 1 h, and incubated with rabbit anti-human L1-CAM antiserum (1:5,000 dilution) for 1 h at 37°C. L1-CAM was labeled by incubating the cells with 10 μg/ml Alexa 594–conjugated anti-rabbit IgG for 1 h at RT.

Neurons were fixed with 4% formaldehyde and blocked with 10% horse serum. In some cases, the cells were permeabilized with 0.1% Triton X-100. The following primary antibodies were used: 10 μg/ml 5G3, anti-L1CD antiserum (1:10,000 dilution), anti-Zic antibody, 2 μg/ml anti-TrkA antibody, 1 μg/ml anti-TrkC antibody, and 1 μg/ml anti-ankyrinB antibody. Expression of the proteins was visualized with 10 μg/ml Alexa-conjugated secondary antibodies.

Neurite growth assay

Neurite-bearing neurons were defined as those that possess cellular processes longer than the diameter of the soma. Included in this paper was a neuron in isolation whose neurites did not contact other cells or neurites. Neurite initiation was assessed by calculating the percentage of neurons bearing neurites. The length of neurites was measured as described previously (Kamiguchi and Yoshihara, 2001).

Confocal microscopy

Images of 293 cells were taken with a confocal imaging system (Radiance 2000; Bio-Rad Laboratories) attached to a microscope (Eclipse TE300; Nikon), using an argon/krypton laser (488 and 566 nm) and a 100X Plan
Apochromat (NA 1.4) objective lens. Pinhole settings were chosen to give single optical sections of 0.6 μm in thickness.

**FRET microscopy**

To measure FRET, three fluorescent images of a cell expressing CFP and YFP (Venus) were acquired in the same order in all experiments through (1) a FRET filter set (excitation 440/21 nm, emission 480/30 nm); and (3) a YFP filter set (excitation 500/25 nm, emission 545/35 nm). A single dichroic mirror was used with all three filter sets. The images were acquired with a 12-bit digital CCD camera (CoolSNAP HQ2; Roper Scientific) and a microscope (Axiovert S100; Carl Zeiss MicroImaging, Inc.) using a 100× Plan Apochromat (NA 1.4) objective lens. Exposure time and binning (4 × 4 or 8 × 8) were adjusted so that pixel intensity values were 20–80% saturation in the three channels. A background image was subtracted from each raw image before carrying out FRET calculations.

Quantitative FRET measurements were performed as described previously (Gordon et al., 1998). In brief, corrected FRET (FRET) was calculated on a pixel-by-pixel basis for the entire image by subtracting the cross-talks as follows: $FRET = \frac{IFRET}{IYFP} - 0.33 (0.05 ICFP)$, where $IFRET$, $IYFP$, and $ICFP$ are image intensities under the FRET, CFP, and YFP filter sets, respectively. 0.33 and 0.05 are the fractions of CFP bleed-through and YFP cross-excitation, respectively, through the FRET filter channel. These coefficients were rounded up from average cross-bled values determined in cells expressing only CFP- or YFP-tagged constructs alone. Then, FRET was calculated by using the following equation: $FRET = FRET_{ Coppa, J., N. Yokota, M. Hashimoto, T. Fusushi, M. Fukuda, and K. Mikoshiba. 1994. A novel zinc finger protein, zic, is involved in neurogenesis, especially in the cell lineage of cerebellar granule cells. J. Neurochem. 63:1880–1890.

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