Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration

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Introduction

Ror2 is a member of the Ror family of receptor tyrosine kinases, which possess characteristic structural domains, i.e., extracellular Frizzled-like cysteine-rich domain (CRD), cytoplasmic tyrosine kinase domain, and proline-rich domain (PRD; Yoda et al., 2003). Ror2 is expressed primarily in neural crest–derived cells and mesenchymal cells during mouse embryogenesis (Matsuda et al., 2001), and plays crucial roles in developmental morphogenesis (DeChiara et al., 2000; Takeuchi et al., 2000). Ror2-deficient mice exhibit skeletal, genital, and cardiovascular abnormalities (Takeuchi et al., 2000; Oishi et al., 2003), presumably caused by partially disrupted convergent extension (CE) movements during gastrulation. In addition, CAM-1, which is the Caenorhabditis elegans orthologue of Ror2, has been implicated in cell migration, asymmetric cell division, and axonal extension during embryogenesis (Forrester et al., 1999). However, the molecular mechanisms by which Ror2 and/or CAM-1 regulate cell migration during embryogenesis remain poorly defined. Importantly, it has recently been shown that Ror2 acts as an alternative receptor or coreceptor for Wnt5a (Oishi et al., 2003; Mikels and Nusse, 2006). In fact, Ror2 mediates Wnt5a signaling by activating the Wnt–JNK pathway and inhibiting the β-catenin–TCF pathway. Wnt proteins constitute a large family of cysteine-rich secreted glycoproteins that play crucial roles in various developmental processes and tissue homeostasis in the adult (Logan and Nusse, 2004). Binding of Wnt proteins to their cognate Frizzled receptors elicits several distinct signaling pathways, including the canonical β-catenin–TCF and noncanonical planar cell polarity (PCP)–CE pathways (Kuhl et al., 2000; Mlodzik, 2002; Logan and Nusse, 2004). According to conventional classification, Wnt5a is a representative noncanonical Wnt signaling protein. Indeed, previous studies have shown that loss or gain of Wnt5a function results in dysregulated CE movements in vertebrates (Moon et al., 1993; Kilian et al., 2003).
We show that Ror2 is required for filopodia formation and Wnt5a-induced cell migration. Irrespective of Wnt5a stimulation, ectopic expression of Ror2 can induce filopodia formation by actin reorganization via coupling with the actin-binding protein filamin A (FLNa), and this requires the PRD of Ror2. Intriguingly, disruption of filopodia formation by disrupting the expression of either Ror2 or FLNa inhibits Wnt5a-induced cell migration, indicating the critical role of Ror2-mediated filopodia formation in Wnt5a-induced cell migration. However, Ror2-mediated filopodia formation is not sufficient for Wnt5a-induced cell migration. In fact, Dishevelled proteins (Dvls), which are regulatory cytoplasmic proteins mediating both canonical and noncanonical Wnt signaling (Wallingford and Habas, 2005), are also required for Wnt5a-induced cell migration, but not Ror2-mediated filopodia formation.

Results and discussion

Both the CRD and PRD of Ror2 are required for Wnt5a-induced cell migration

We first examined the role of Ror2 in Wnt5a-induced cell migration using mouse embryonic fibroblasts (MEFs) from wild-type (Ror2+/+) and Ror2-deficient (Ror2−/−) mice. Treatment of Ror2+/+ MEFs with conditioned medium (CM) containing Wnt5a (Wnt5a CM), but not control CM (prepared from culture of mock-transfected L cells) or DME, resulted in a drastic increase in cell motility (Fig. 1 A). Interestingly, Wnt5a-induced cell migration was impaired in Ror2−/− MEFs compared with Ror2+/+ MEFs. In contrast, Wnt3a could induce considerable cell migration of both Ror2+/+ and Ror2−/− MEFs at comparable levels (Fig. 1 A), demonstrating that Ror2 is indeed required for Wnt5a-induced cell migration. We next addressed the important question of which domains or functions of Ror2 are required for Wnt5a-induced cell migration. Because MEFs are a somewhat heterogeneous population, we used L cells stably expressing similar amounts of wild-type (WT) or various Ror2 mutants (Fig. 1 B). Ror2DK lacks intrinsic tyrosine kinase activity, and Ror2∆CRD, Ror2∆C, or Ror2Tc lack the extracellular CRD, the cytoplasmic C-terminal region containing PRD, or most of the cytoplasmic region of Ror2, respectively. As shown in Fig. 1 C, L cells expressing Ror2WT or Ror2DK, but not mock-transfected L cells, exhibited drastically enhanced cell motility after Wnt5a stimulation, indicating that tyrosine kinase activity of Ror2 is dispensable for Wnt5a-induced cell migration. Importantly, no obvious cell migration was observed after Wnt5a stimulation of L cells expressing Ror2∆CRD, Ror2∆C, or Ror2Tc (Fig. 1 C). The results indicate that both the CRD (binding site for Wnt5a) and PRD of Ror2 are required for Wnt5a-induced cell migration.

The PRD of Ror2 is required for Ror2-mediated filopodia formation

Surprisingly, we observed that ectopic expression of Ror2 in various cell types (human embryonic kidney [HEK] 293T, MCF7, B16BL6, and L cells) induced remarkable filopodia formation in the absence of Wnt5a stimulation (Fig. 2 A, Fig. 4 A, Fig. 5 B, and not depicted). Ror2 was colocalized with actin at the filopodia (Fig. 2 A). We next examined which domains within Ror2 are responsible for Ror2-mediated filopodia formation. Ror2WT and a series of Ror2 mutants (Fig. 1 B) were

Figure 1. Both the CRD and PRD of Ror2 are required for Wnt5a-induced cell migration. (A) Migratory responses of Ror2+/+ and Ror2−/− MEFs in the absence or presence of control (neo CM), Wnt5a CM, or Wnt3a CM were determined in the Transwell chamber. The data are expressed as the mean ± the SD of three independent experiments. (B) Schematic representations of the wild-type (WT) Ror2 and its derived mutants. (C) L cells stably transfected with expression plasmid for Ror2-Flag (WT, DK, ∆CRD, ∆C, or Tc) or the control vector (pcDNA3), respectively, were analyzed in a Transwell migration assay in the presence of neo CM or Wnt5a CM. The data are expressed as the mean ± the SD of three independent experiments. Expression of the respective Ror2 proteins was analyzed by anti-Flag immunoblotting (inset).
each fused to GFP, and expressed in HEK293T cells, followed by rhodamine-phalloidin staining. HEK293T cells expressing Ror2WT, Ror2DK, or Ror2ΔCRD exhibited drastic formation of the filopodia at the site where Ror2 and actin were colocalized (Fig. 2 A), indicating that neither tyrosine kinase activity nor the CRD of Ror2 is required for Ror2-mediated filopodia formation. In contrast, cells expressing Ror2Tc or Ror2ΔC, both of which lack the cytoplasmic PRD, failed to form filopodia (Fig. 2 A). Cell surface expression levels of Ror2WT and the respective Ror2 mutants on transient or stable transfectants are generally comparable as assessed by cell surface biotin-labeling experiment (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200607127/DC1; and not depicted). Similar results were obtained when Ror2 and its mutants were expressed in MCF7, B16BL6, or L cells (unpublished data). These results indicate that the cytoplasmic region of Ror2, particularly the PRD, is responsible for Ror2-mediated filopodia formation. Importantly, Ror2-mediated filopodia formation by itself failed to induce cell migration in the absence of Wnt5a (Videos 1 and 2).
Although ectopic expression of Ror2WT, Ror2DK, or Ror2ΔCRD in L cells, lacking the expression of endogenous Ror2, could induce filopodia formation irrespective of Wnt5a (Fig. 2 A), Wnt5a stimulation of cultured fibroblasts (e.g., NIH3T3 cells) expressing Ror2 endogenously also induced apparent filopodia formation (not depicted). Importantly, Ror2+/+/MEFs, but not Ror2−/− MEFs, clearly exhibited Wnt5a-induced filopodia formation (Fig. 2 B), indicating that Ror2, indeed, plays a crucial role in Wnt5a-induced filopodia formation. On the other hand, Wnt3a stimulation of Ror2+/+ and Ror2−/− MEFs resulted in the formation of lamellipodia (or pseudopod)-like structures, but not filopodia (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200607127/DC1).

The actin-binding protein FLNa is associated with the cytoplasmic PRD of Ror2 and is colocalized with Ror2

We next examined the role of the cytoplasmic region of Ror2 in filopodia formation. We performed yeast two-hybrid screening using the cytoplasmic region of Ror2 as bait to identify potential associating molecules. We identified FLNa, an actin-binding protein that plays important roles in actin cytoskeletal reorganization, cell migration, and various aspects of signal transmission (Stossel et al., 2001; Feng and Walsh, 2004). We thus examined the association of Ror2 with FLNa in HeLa and MCF7 cells that express Ror2 endogenously. Anti-Ror2 immunoprecipitates prepared from either HeLa or MCF7 cells also contained FLNa, indicating that endogenous Ror2 and FLNa, indeed, associate in these cells (Fig. 3 A). Consistent with the result, Ror2-GFP, but not Ror2ΔC-GFP, expressed ectopically in MCF7 cells was found essentially colocalized with FLNa at the filopodia (Fig. 3 B), although expression of FLNa was rather concentrated at the roots of filopodia (Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200607127/DC1).

FLNa consists of an N-terminal actin-binding domain and 24 tandem repeats (FLN repeats) of ~96 amino acids each, and dimerizes via the FLN repeat 24 (Stossel et al., 2001; Feng and Walsh, 2004). Thus, we performed pull-down assays...
using either GST-FLNa/15-19 or GST-FLNa/20-24 bound to glutathione–Sepharose, and found that GST-FLNa/20-24, but not GST-FLNa/15-19, could associate with Ror2WT expressed in HEK293T cells (unpublished data). To determine the cytoplasmic domains within Ror2 that are required for its association with FLNa, we performed a pull-down assay using GST-FLNa/20-24 bound to glutathione–Sepharose. Ror2WT expressed in HEK293T cells was found coprecipitated with GST-FLNa/20-24, whereas Ror2ΔC was not (Fig. 3 C). This indicates that the cytoplasmic C-terminal region of Ror2,

Figure 4. Association of FLNa with Ror2 is indispensable for Ror2-mediated filopodia formation. (A) Ror2-GFP (green; top) or Ror2ΔC-GFP (green; bottom) was expressed in FLNa-deficient M2 or FLNa-expressing A7 cells, and F-actin (red) was stained with rhodamine-phalloidin. (right) Merged images. Magnified images of boxed regions are shown (magnified). (B) M2 cells were transfected with Ror2-CFP (red) along with YFP-FLNa (green; top) or YFP-FLNaΔ20 (green; bottom). (right) Merged images. Magnified images of boxed regions are shown (magnified). (C) Quantitative analysis of Ror2-induced filopodia formation. M2 and A7 cells expressing Ror2-GFP or M2 cells expressing Ror2-CFP together with YFP-FLNa or FLNaΔ20 were stained with rhodamine-phalloidin for F-actin. The percentages of cells with the indicated number of filopodia per cell, identified by F-actin staining, on a GFP- or CFP/YFP-positive cell, were measured (n > 60). (D) M2 and A7 cells were assayed in a Transwell migration assay in the presence of neo CM or Wnt5a CM. The data are expressed as the mean ± the SD of four independent experiments. *, P < 0.005, t test. Expression levels of FLNa, Ror2, and α-tubulin were analyzed by immunoblotting (inset). Bars, 10 μm.
containing the PRD, is required for its association with FLNa in vitro. We also investigated which domains within FLNa are required for its association with Ror2. GST-FLNa/20-21, but not GST-FLNa/21-22, GST-FLNa/22-23, or GST-FLNa/23-24, could associate with Ror2 in a manner similar to GST-FLNa/20-24 (Fig. 3 D), showing that FLN repeat 20-21, in particular repeat 20, is responsible for its association with Ror2. Conversely, FLNaΔ20 lacking FLN repeat 20, expressed in HEK293T cells, was incapable of associating with GST-Ror2 (Fig. 3 E). These results indicate that the PRD of Ror2 and FLN repeat 20 of FLNa are required for the association of Ror2 and FLNa, respectively.

Association of Ror2 with FLNa is indispensable for Ror2-mediated filopodia formation

Next, we used two human melanoma cell lines, M2 and A7, to examine the role of FLNa in Ror2-mediated filopodia formation. M2 lacks FLNa expression, whereas A7 is a derivative of M2 stably transfected with the FLNa cDNA. Expression of Ror2-GFP in A7, but not in M2 cells, resulted in considerable formation of the filopodia at the site where Ror2 and actin were colocalized (Fig. 4, A and C). Furthermore, filopodia formation in A7 cells was not induced by expression of Ror2ΔC, which fails to associate with FLNa (Fig. 4 A). In M2 cells, coexpression of FLNaWT and Ror2 resulted in the considerable formation of filopodia at the site where FLNa and Ror2 colocalize, whereas coexpression of FLNaΔ20 and Ror2 failed to induce filopodia (Fig. 4, B and C). The results indicate that Ror2-mediated filopodia formation requires the presence of Ror2-associated FLNa.

Because Ror2 protein was expressed endogenously in M2 and A7 cells (Fig. 4 D, insert), we evaluated the abilities of M2 or A7 cells to induce cell migration after Wnt5a stimulation. We observed that Wnt5a could induce significant cell migration of A7, but not M2, cells (Fig. 4 D). The result indicates the critical role of Ror2/FLNa-mediated filopodia formation in Wnt5a-induced cell migration. At present, it remains unclear about the mechanism underlying Ror2/FLNa-mediated filopodia formation. Further study will be required to clarify this issue.

Dvl3s are required for Wnt5a-induced cell migration, but not Ror2-mediated filopodia formation

Because previous studies have shown that Dvl proteins are required for noncanonical PCP–CE movements (Wallingford and Habas, 2005), we next examined, using siRNA-mediated gene knockdown, whether the Dvl proteins (Dvl2 and Dvl3) might also be required for Ror2-mediated filopodia formation and/or Wnt5a-induced cell migration. To this end, we used L cells where expression of Dvl2 and Dvl3, but not Dvl1, were detectable (Fig. 5 A and not depicted). Intriguingly, in L cells expressing Ror2WT, suppression of Dvl2 and Dvl3 expression resulted in the drastic inhibition of Wnt5a-induced cell migration, but not of Ror2-mediated filopodia formation (Fig. 5, B and C). This indicates that the Dvl proteins are required for Wnt5a-induced cell migration, but not for Ror2-mediated filopodia formation. It remains unclear whether or not Dvl3s are involved...
in Wnt5α–Ror2-mediated signaling pathways, leading to cell migration. It will be important to elucidate how Dvl3 regulates Wnt5α-induced cell migration in vitro.

Materials and methods

Plasmids and antibodies

Plasmids for Ror2 and its derivative mutants were constructed by subcloning the respective Ror2 cDNAs (Ror2 WT, DK, ∆CRD, ∆C, or Tc cDNAs) into pcDNA, pEGFP, pEYFP, and pMYC vectors, respectively (Matsuda et al., 2003; Oishi et al., 2003; Kani et al., 2004). Plasmid for YFP-actin was provided by K. Mizuno. Human FNa and its ∆20 mutant lacking aa 2,151–2,257 were subcloned into the pEYFP- and pMYC-C1 vectors. Plasmids expressing the series of GST-FNα vectors were constructed by subcloning cDNAs encoding the following FNα fragments into the pGEX vector, respectively: 15–19 (aa 1,640–2,130), 20–24 (aa 2,151–2,647), 20–21 (aa 2,151–2,330), 21–22 (aa 2,326–2,424), 22–23 (aa 2,331–2,539), and 23–24 (aa 2,425–2,647). The siRNA plasmids, pSUPER-Dvl2 and -Dvl3, were constructed as previously described (Lu et al., 2004). The control siRNA plasmid containing a nonspecific siRNA sequence (GTAGCGCAAGTACTTGC) was used. An anti-Ror2 antibody was prepared as previously described (Kani et al., 2004); Antibodies against Flag (M2; Sigma-Aldrich), Myc (9E10; Santa Cruz Biotechnologies, Inc.), α-tubulin (Ab-1; Calbiochem) were purchased commercially.

Cells, transfection, and conditioned media

L cells were cultured in DME containing 5% FCS. HEK293T, MCF7, and Hela cells, and Ror2 WT, DK, and ∆CRD were transfected from mouse embryos (E13.5) were cultured in DME containing 10% FCS. Human melanoma cell lines, M2 and A7, were maintained as previously described (Cunningham et al., 1992). To establish L cells stably expressing Ror2 or its mutants, transfected cells were selected with G418 (1 mg/ml) and single cell clones were isolated and screened for Ror2 expression by immunoblot analysis. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. For electroporation, cells were suspended in 400 μl of OPTI-MEM medium containing the respective plasmids and electroporated at 250 V/975 μF, using Gene Pulser II (Bio-Rad Laboratories, Inc.). Wnt5α, Wnt3a, and FLNa were cultured for 72 h in serum-free DME, respectively.

Immunoprecipitation, immunoblotting, and GST-pull down

Cells were lysed in lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 5% glycerol, 1 mM MgCl2, 1 mM MnCl2, 20 mM NaF, 1 mM Na3VO4, 1 mM diithiothreitol, 0.25 mM PMSF, and 10 μg/ml leupeptin). Whole-cell lysates were subjected to analyses by immunoprecipitation and immunoblotting as previously described (Kani et al., 2004). Whole-cell lysates prepared from HEK293T cells expressing Ror2-Flag (WT or ∆C) or Myc-FNα (WT or ∆20) were incubated with purified-GST or -GST fusion proteins bound to glutathione–Sepharose beads. Pelleted beads were subjected to SDS-PAGE, followed by immunoblot analyses.

Cell staining and image analysis

Cells cultured on coverslips were fixed and stained with the respective antibodies, as previously described (Matsuda et al., 2003). To visualize Factin, fixed cells were stained with rhodamine-phalloidin (Invitrogen). Fluorescent images were obtained at room temperature using an inverted microscope (Axiovert 200M) equipped with a laser scanning confocal imaging system (LSM510) and a 63×/NA 1.4 oil immersion objective lens (Plan Apochromat; all from Carl Zeiss Microlmaging, Inc.). Images were processed using Photoshop CS (Adobe).

Cell migration assays

MEFs (2 × 10^4 cells) or L cells (6 × 10^5 cells) suspended in 100 μl DME were loaded into the upper well of the Transwell chamber (8-μm pore size; Costar) that was precoated on both sides with 0.1% gelatin for 2 h at 37°C. The lower well was filled with 600 μl of CM or DME. For the migration assay using M2 and A7 cells, the underside of a Transwell chamber was precoated with 0.1% gelatin, and 3 × 10^5 cells in 100 μl DME containing 0.1% BSA were loaded into the upper well. The lower well was filled with 600 μl of CM containing 0.1% BSA. After incubation for 7 h for MEFs and L cells or 3 h for M2 and A7 cells, the membrane was fixed in 3.7% formaldehyde. Nonmigrating cells on the top of the membrane were removed by wiping and rinsing, and migrating cells on the lower face of the membrane were stained with DAPI and counted at room temperature under an Axiovert 200M inverted microscope equipped with a LSM510 laser scanning confocal imaging system and a Plan-Neofluar NA 0.3 10× objective lens [Carl Zeiss Microlmaging, Inc.].

Online supplemental material

Fig. S1 shows surface expression of WT and mutant Ror2 proteins on HEK293T cells. Fig. S2 shows the effect of Wnt3a stimulation on morphol- ogy of Ror2−/− and Ror2 −/+ MEFs. Videos 1 and 2 show the time-lapse fluorescence of B16F1 cells expressing YFP-actin alone (video 1) or YFP-actin and Ror2-CFP (Video 2). Video 3 shows the time-lapse fluorescence of M2 cells coexpressing Ror2-CFP and YFP-FNa. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200607127/DC1.

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