The Receptor Tyrosine Kinase Ror2 Associates with the Melanoma-associated Antigen (MAGE) Family Protein Dlxin-1 and Regulates Its Intracellular Distribution*

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Takeru Matsuda‡§, Hiroaki Suzuki‡§, Isao Oishi‡, Shuichi Kani‡, Yoshikazu Kuroda‡, Takahide Komori‖, Aya Sasaki**, Ken Watanabe**, and Yasuhiro Minami‡ ‡‡
From the ‡Department of Genome Sciences, the §Department of Clinical Molecular Medicine, and the ‖Department of Oral and Maxillofacial Surgery, Faculty of Medical Sciences, Graduate School of Medicine, Kobe University, Kobe 650-0017 and the **Department of Geriatric Research, National Institute for Longevity Sciences, Aichi 474-8522, Japan

The mammalian Ror family receptor tyrosine kinases, Ror1 and Ror2, play crucial roles in developmental morphogenesis. Although the functions of Ror1 and Ror2 are redundant, Ror2 exhibits more specific functions during development. We show that when expressed in mammalian cells, Ror2, but not Ror1, associates with the melanoma-associated antigen (MAGE) family protein, Dlxin-1, which is known to bind to the homeodomain proteins Msx2 and Dlx5 and regulate their transcriptional functions. This association requires the cytoplasmic C-terminal region of Ror2, containing proline-rich and serine/threonine-rich domains, and the C-terminal neadin homology domain of Dlxin-1. Interestingly, the cytoplasmic C-terminal region of Ror2 is missing in patients with brachydactyly type B. Interestingly, transient expression and immunohistochemical analyses reveal that both Dlxin-1 and Msx2 are co-localized in the nuclei in the absence of Ror2. In the presence of Ror2, Dlxin-1 is colocalized with Ror2 at the membranous compartments and Msx2 is retained in the nuclei. It was also found that the majority of cellular Dlxin-1 is retained in the membranous fractions of wild-type but not Ror2−/− mouse embryonic fibroblasts. Furthermore, we show that transcriptional activity of Msx2, irrespective of Ror2 kinase activity, is regulated by ectopic expression of Ror2 using a reporter plasmid containing the WIP element. Thus, Ror2 sequesters Dlxin-1 in membranous compartments, thereby affecting the transcriptional function of Msx2.

Receptor tyrosine kinases (RTKs) play crucial roles in developmental morphogenesis by regulating cellular proliferation, differentiation, migration, and death (1). The Ror family of RTKs are orphan RTKs, characterized by the presence of extracellular Frizzled-like cysteine-rich domains, membrane proximal Kringle domains, and intracellular distal proline-rich domains that are assumed to mediate protein-protein interactions (2–9). Pairs of structurally related Ror family RTKs are found in Drosophila (Dror and Dnrk) and mammals (Ror1 and Ror2). Because the spatiotemporal expression patterns of Ror1 and Ror2 overlap considerably, and both transcripts are detected in the face, limbs, heart, and lungs during mouse embryogenesis (10, 11), it has been assumed that the developmental functions of Ror1 and Ror2 may be at least partially redundant.

Previous genetic studies have demonstrated that both mouse Ror2 and Ror2 play important roles in developmental morphogenesis, in particular in skeletal and cardiac development (12–14). Mice lacking Ror2 exhibit dwarfism, short limbs (with dysomorphic dysplasia) and tails, facial abnormalities, ventricular septal defects, and respiratory dysfunction resulting in neonatal lethality (12, 14). Ror1-deficient mice also die soon after birth due to respiratory dysfunction, yet they do not show any apparent skeletal or cardiac phenotypes (13). Furthermore, Ror1/Ror2 double mutant mice exhibit markedly enhanced skeletal and cardiac abnormalities compared with Ror2 mutant mice, indicating that Ror1 and Ror2 interact genetically and functionally during the development of these organs (13). These findings demonstrate the pleiotropic and specific, yet partially redundant, functions of Ror2 in mouse development. Interestingly, it has recently been reported that mutations within Ror2 are responsible for brachydactyly type B (BDB), a dominant skeletal disorder characterized by hypoplasia/aplasia of distal phalanges (15, 16), and Robinow syndrome, a recessive condition characterized by short stature, limb bone shortening, segmental defects of the spine, and a dysmorphic facial appearance (17, 18) in humans. This finding further indicates the crucial function of Ror2 in developmental morphogenesis. However, little is known about the signaling mechanisms mediated by Ror1 and Ror2.

To gain insights into Ror1- and/or Ror2-mediated signaling, yeast two-hybrid screening was employed to identify proteins that interact with Ror1 and/or Ror2, and a cDNA encoding Dlxin-1, a member of the melanoma-associated antigen (MAGE) family (19–21), was obtained. Dlxin-1 has been shown to associate with the homeodomain proteins Msx2 and Dlx5 and to regulate the transcriptional function of Dlx5 (22). The Rat ortholog of Dlxin-1, designated NRAGE or MAGE-D, has
also been characterized (23, 24). NRAGE has been shown to bind to the p75 neurotrophin receptor (p75NTR) and to confer nerve growth factor-dependent apoptosis on developing neuroblastoma cells (4). In addition, it has been reported that NRAGE interacts with an inhibitor of apoptosis protein (IAP) and augments interleukin-3 withdrawal-induced apoptosis of the pro-myeloid leukemic cell line 32D (25). Furthermore, it has recently been reported that NRAGE and its downstream protein kinase cascade is activated by a mitochondrial apoptotic caspase pathway through a C-Jun NH₂-terminal kinase- and c-Jun-dependent pathway (26). Here we show that Ror2, but not Ror1, associates with Dlxin-1 when expressed in mammalian cells. The association of Ror2 with Dlxin-1 requires the cytoplasmic C-terminal region of Ror2, which containing proline-rich and serine/threonine-rich domains. This region is deleted in Ror2 from BDB patients, suggesting a possible role of Dlxin-1 in the pathogenesis of BDB. Interestingly, although Dlxin-1 and Mxs2 are co-localized in the nuclei in the absence of Ror2, the subcellular distribution of Dlxin-1, but not Mxs2, is altered by the presence of Ror2. These data indicate that Dlxin-1 is an important mediator of Ror2 function and that its subcellular distribution is regulated by Ror2.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Mouse Ror1 and Ror2 cDNAs were isolated as described previously (7). For the construction of bait plasmids, the cDNAs encoding the cytoplasmic domains of Ror1 and Ror2 were each subcloned into pAS2-1. Expression vectors encoding the FLAG- or HA-tagged Ror proteins (pcDNA-Ror1-FLAG, pcDNA-Ror2-FLAG, pcDNA-Ror2-HA) were constructed as follows. The cDNA fragments corresponding to Ror1 and Ror2 were obtained by PCR using a combination of specific primers that create HindIII/NotI sites for Ror1 and EcoRI/XhoI site for Ror2, respectively, at the ends of each cDNA region. Subsequently, these cDNA fragments were ligated into pcDNA3 (Invitrogen). To generate pcDNA-Ror2ΔC-FLAG and pcDNA-Ror2ΔC-HA, the C-terminal region of Ror2, i.e. amino acids 788–944 containing the proline-rich (Pro-rich) and serine/threonine-rich (Ser/Thr-rich 2) domains, were deleted. To generate pcDNA-Ror2(ΔRob)-FLAG, amino acids 502–944, containing the Pro-rich and serine/threonine-rich (Ser/Thr-rich 1, Ser/Thr-rich 2) domains and the major part of the kinase domain, were deleted. The expression vector pcDNA-Ror2ΔDK-FLAG, encoding a kinase-dead mutant of Ror2, was constructed by replacing lysine 507, crucial for ATP binding, with arginine. In some experiments, the expression vectors pEAK12 (Edge Biosystems) encoding Ror1, Ror2, and Dlxin1 were utilized, and essentially identical results were obtained compared with those obtained by utilizing cDNA expression vectors (data not shown). The expression vectors pcDNA-HA-Dlxin1, pcDNA-HA-Dlxin1ΔN, pcDNA-HA-Dlxin1ΔKBD, pcDNA-HA-Dlxin1ΔC, pcDNA-FLAG-Msx2, and pcDNA-HA-Msx2 were constructed as described previously (22). The pG2L-WIP reporter gene plasmid was constructed as described previously (27).

Antibodies, Cells, and Transfection—Mouse monoclonal antibody M2 (Eastman Kodak) recognizes the FLAG peptide sequence (DYKD DDDK). Mouse monoclonal antibody 12CA5 (Roche Applied Science), rat monoclonal antibody 3F10 (Roche Applied Science), and rabbit polyclonal antibody 12CA5-NHD antibody was used to detect endogenous mouse Dlxin-1 (28). Mouse monoclonal anti-phosphotyrosine antibodies, PY20 and 4G10, were purchased from Transduction Laboratories and Upstate Biotechnology, respectively. Alexa Fluor 546 (goat anti-mouse IgG, red) and Alexa Fluor 488 (goat anti-rabbit IgG, green) were purchased from Molecular Probes. Mouse embryonic fibroblasts (MEFs) were obtained from Charles C. Thomas (29) and were cultured in DMEM/HAM’s F12 (50:50) supplemented with 10% fetal bovine serum. HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DME, Nissui) supplemented with 10% (v/v) fetal calf serum (FCS). Transient cDNA transfection into cells was performed using the calcium phosphate method (30), or LipofectAMINE reagent (Invitrogen) according to the manufacturer’s instructions.

Yeast Two-Hybrid Screening—Yeast two-hybrid screening was performed following the manufacturer’s instructions (Matchmaker, Clontech). Briefly, the bait plasmids, pAS2-1-Ror1DK and pAS2-1-Ror2DK, were constructed by inserting the cDNA fragments encoding the cytoplasmic regions of kinase-dead version of mouse Ror1 and Ror2, respectively, in-frame at the NotI site of pAS2-1 (Clontech). A yeast strain, Y190, was co-transformed with the bait plasmids pAS2-1-Ror1DK or pAS2-1-Ror2DK along with the mouse embryo (17 days post-coital (d.p.c.)) cDNA library carried in the prey plasmid pGAD10 (Clontech). Approximately 5 × 10⁶ transformants were selected for their ability to grow on SD plates lacking histidine (containing 50 μg ml⁻¹ 3-amino-1,2,4-triazole). The colonies grown on SD plates were subsequently analyzed for β-galactosidase activity by filter assays, and –20 and 30 β-galactosidase-positive clones were obtained when pAS2-1-Ror1DK and pAS2-1-Ror2DK were co-transformed, respectively. Among them, three and six independent clones, respectively, were found to possess the prey plasmids containing the cDNA fragments that encode Dlxin-1 protein, as assessed by sequence analysis with an ABI PRISM 310 Genetic Analyzer (PerkinElmer Life Sciences). prey plasmid cloneSize positive yeast colonies were re-examined by co-transformation into Y190 with the original bait plasmid followed by a β-galactosidase assay.

Preparation of Cell Lysates and Subcellular Fractions—Cells were solubilized with lysis buffer (50 mM Tris-HCl (pH 7.4), 0.5% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml apro tinin), and cell lysates were prepared by centrifugation at 12,000 × g for 15 min. Preparation of subcellular fractions from MEFs (wild type and Ror2⁻/⁻) was carried out as described previously (31).

Immunoprecipitation and Immunoblotting—Cell lysates were pre-cleared for 1 h at 4 °C with protein A-Sepharose (Amersham Biosciences). The precleared supernatants were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and blocked in 5% nonfat dry milk in 1× Tris-buffered saline (TBS) at room temperature and then permeabilized with PBS containing 0.1% Triton X-100 for 15 min at room temperature. After blocking in PBS with 10% FCS for 30 min, cells were incubated with primary antibodies, anti-FLAG monoclonal antibody (M2, 1:500), and/or anti-HA monoclonal antibody (3F10, 1:200), in PBS, 10% FCS for 30 min at room temperature. Cells were washed twice with PBS and then incubated with secondary antibodies, Alexa Fluor 546 (anti-mouse IgG antibody, 1:500), and/or Alexa Fluor 488 (anti-rat IgG antibody, 1:200) in PBS, 10% FCS at room temperature for 30 min. After two washes in PBS, the cells were mounted with Pristine Mount (Research Genetics) and analyzed with an upright fluorescence microscope (Zeiss).
Fig. 1. Dlxin-1 interacts with Ror2 in vivo. A, Dlxin-1 interacts with Ror2 but not Ror1 in transfected 293T cells. Cell lysates were prepared from 293T cells singly or doubly transfected with the expression vectors shown in the panel. Whole cell lysates (WCL) or anti-FLAG immunoprecipitates from the respective cell lysates were subjected to SDS-PAGE and analyzed by anti-HA or anti-FLAG immunoblotting. Expression of Ror1, Ror2 and Dlxin-1 were confirmed by anti-FLAG immunoblotting of anti-FLAG immunoprecipitates and by anti-HA immunoblotting of the whole cell lysates, respectively. B, schematic representation of the WT and mutant forms of Ror2. Lysine 507 was replaced with arginine (K507R) in the kinase-dead mutant of Ror2 (Ror2 DK). The C-terminal regions containing the Pro-rich, Ser/Thr-rich 1, and Ser/Thr-rich 2 domains, whereas Ror2(Rob) lacks amino acids 502–944, containing the Pro-rich and Ser/Thr-rich domain. We evaluated the Proline-rich Domain of Ror2 and the C-terminal Necdin Homology Domain (NHD) of Dlxin-1 Are Required for the Association of Ror2 with Dlxin-1. To identify the regions within Ror2 that are required for its association with Dlxin-1, we generated two truncated mutants of Ror2: Ror2(WT) and Ror2(Rob) (see Fig. 1C). Ror2(WT), Dlxin-1 along with either FLAG-tagged wild-type (WT) or a kinase-dead mutant of Ror2 (see B) and/or Dlxin-1 as shown. Tyrosine phosphorylation of Ror2 WT and Ror2 DK was monitored by anti-phosphotyrosine immunoblotting of anti-FLAG immunoprecipitates (left panel). The association of Ror2 WT or Ror2 DK with Dlxin-1 were examined as described in A (right panel).

We next examined whether or not association of Ror2 with Dlxin-1 requires Ror2 kinase activity. To this end, HA-tagged Dlxin-1 specifically co-immunoprecipitated with FLAG-tagged Ror2, but not with FLAG-tagged Ror1, indicating that Ror2, but not Ror1, associates with Dlxin-1 in vivo.

Expression Patterns of Ror2 and Dlxin-1 Overlap during Mouse Embryogenesis—To verify the physiological significance of the Ror2-Dlxin-1 association observed in HEK293T cells, we compared the embryonic expression patterns of Ror1, Ror2, and Dlxin-1 by whole-mount in situ hybridization analyses on mouse embryos at E10.5 and E12.5 (Fig. 2, data not shown). At E10.5, Ror2 and Dlxin-1 exhibited remarkably similar expression patterns, especially in the pharyngeal arches and limb buds, whereas Ror1 transcripts exhibited a somewhat different pattern of localization (Fig. 2). Both Ror2 and Dlxin-1 were expressed throughout the limbs, whereas Ror1 expression was more restricted to the proximal regions of the limb buds. However, the expression patterns of these three transcripts were similar in the pharyngeal arches. At a later stage (E12.5), Dlxin-1, like Ror2, was expressed in the perichondrium of the digits and the marginal regions of the limbs; this overlapping expression pattern was maintained at E13.5 (Ref. 11; data not shown). On the other hand, Ror1 transcripts were detected in the anterior and posterior portions of the limbs (Ref. 11; data not shown). The results reveal that the expression patterns of Ror2 and Dlxin-1 overlap significantly, especially in the developing face and limbs. This suggests that Ror2 does indeed associate with Dlxin-1 during mouse embryogenesis.

The C-terminal Proline-rich Domain of Ror2 and the C-terminal Necdin Homology Domain (NHD) of Dlxin-1 Are Required for the Association of Ror2 with Dlxin-1—To identify a region(s) within Ror2 that is required for its association with Dlxin-1, we generated two truncated mutants of Ror2: Ror2 WT and Ror2(Rob) (see Fig. 1B). Ror2 WT lacks the C-terminal amino acids 788–944, containing the Pro-rich and Ser/Thr-rich 2 domains, whereas Ror2(Rob) lacks amino acids 502–944, containing the Pro-rich, Ser/Thr-rich 1, and Ser/Thr-rich 2 domains and most of the kinase domain. We evaluated the
Dlxin-1 were expressed similarly in the pharyngeal arches.

Extended throughout the limbs. It should also be noted that Ror2 and scripts were detected in the proximal region of the limbs as shown in.

On the other hand, as shown in.

performed as described under “Experimental Procedures.” Ror1 transcripts were detected in the proximal region of the limbs as shown in A. On the other hand, as shown in B and C, expression of Ror2 and Dlxin-1 extended throughout the limbs. It should also be noted that Ror2 and Dlxin-1 were expressed similarly in the pharyngeal arches.

abilities of these mutants to associate with Dlxin-1 in HEK293T cells. As shown in Fig. 3A, neither Ror2 C nor Ror2(Rob) could associate with Dlxin-1, indicating that the C-terminal region of Ror2, containing the Pro-rich and Ser/Thr-rich 2 domains, is required for association. Although the cytoplasmic tyrosine kinase domains of Ror1 and Ror2 exhibit an amino acid identity of $>70\%$, their C-terminal regions exhibit a lower degree of similarity (an amino acid identity of $<30\%$).

It should also be noted that Ror2 isolated from BDB patients exhibits frameshift and non-sense mutations that generate a truncated version of Ror2 lacking either the C-terminal proline-rich and serine/threonine-rich domains or the entire cytoplasmic region (15, 16).

It has been reported that p75NTR associates with the rat ortholog of Dlxin-1 via the cytoplasmic juxtamembrane domain (amino acids 276–328) of p75NTR (24). However, no apparent amino acid sequence similarity was found between the cytoplasmic C-terminal region of Ror2 and the cytoplasmic juxtamembrane domain of p75NTR. It will be of interest to test whether Ror2 and p75NTR can compete for association with Dlxin-1 (NRAGE) in cells.

Dlxin-1 belongs to the MAGE family of proteins, characterized by the presence of an NHD in their C-terminal portions (Refs. 22 and 24; see Fig. 3B). Necdin is one of the best characterized proteins in this family, and has been shown to associate with E2 promoter binding factor 1 (E2F1) and p53 and mimic the function of retinoblastoma (pRb) during cell cycle progression (35, 36). It therefore seems likely that the NHD is involved in protein-protein interaction. In the central region of Dlxin-1 protein, there is a repeated motif, consisting of the sequence WQXPXX, which is hypothesized to serve as site of interaction with Dlx5 (22, see Fig. 3B). The N-terminal domain of Dlxin-1 does not show significant homology to any protein in the public data bases. We thus examined several deletion mutants of Dlxin-1 for their association with Ror2, as shown in Fig. 3C. We found that HA-tagged Dlxin-1 (ΔN), but not Dlxin-1 (DlxBD) or Dlxin-1 (ΔC), co-immunoprecipitated with FLAG-tagged Ror2. This indicates that the NHD of Dlxin-1 is responsible for its association with Ror2.

Both the WQXPXX Repeat (25 WQXPXX) and the NHD of Dlxin-1 Are Required for Its Association with Msx2—Although Dlxin-1 was originally identified as a Dlx5-interacting protein, it was subsequently shown that Dlxin-1 associates with Dlx5, Dlx7, and Msx2 (22). Dlxin-1 associates with Msx2 more efficiently than with Dlx5 or other Dlx family proteins, including Dlx7 (22, data not shown). We examined which domain(s) of Dlxin-1 is responsible for its interaction with Msx2. FLAG-tagged Msx2 and HA-tagged Dlxin-1 (ΔC), Dlxin-1 (DlxBD), or Dlxin-1 (ΔN) were expressed in HEK293T cells, and their association was examined by anti-FLAG immunoprecipitation followed by anti-HA immunoblotting. As shown in Fig. 3D, HA-tagged Dlxin-1 (DlxBD) and Dlxin-1 (ΔN), but not Dlxin-1 (ΔC), co-immunoprecipitated with FLAG-tagged Msx2, indicating that both the WQXPXX repeat domain (25 WQXPXX) and the NHD of Dlxin-1 are required for association with Msx2. Considering the fact that the NHD of Dlxin-1 is required for association with both Ror2 and Msx2, it was assumed that the association of Ror2 and Msx2 with Dlxin-1 is competitive. Indeed, we found that Msx2 was not co-immunoprecipitated with Ror2 even in the presence of Dlxin-1 (Fig. 3E). On the other hand, the association between Ror2 and Dlxin-1 was almost completely unaffected by the absence or presence of Msx2 (Fig. 3E). This is consistent with competitive binding between Msx2 and Ror2 for Dlxin-1, if one assumes that the interaction between Ror2 and Dlxin-1 predominates to the extent that it prevents any interaction between Msx2 and Dlxin-1.

Subcellular Localization of Dlxin-1 Is Regulated by Msx2 and Ror2—To understand the biological relevance of the observed molecular associations of Ror2 and Msx2 with Dlxin-1, we examined the subcellular distribution of Ror2, Dlxin-1, and of Msx2 proteins in 293 cells by immunofluorescence (see “Experimental Procedures”). When FLAG-tagged Ror2, HA-tagged Dlxin-1, and FLAG-tagged Msx2 were individually expressed in 293 cells, Ror2 was found to be localized in the membranous compartments (mainly the plasma membrane and endoplasmic reticulum), whereas Dlxin-1 and Msx2 were localized exclusively in the cytoplasm and nuclei, respectively (Fig. 4A).

We found that HA-tagged Dlxin-1 was co-localized with either FLAG-tagged Ror2 or Msx2 when expressed in 293 cells (Fig. 4, B and C). This is consistent with our previous biochemical analyses (see Figs. 1A and 3D). Dlxin-1 is co-localized with Msx2 in the nuclei (Fig. 4B), whereas Dlxin-1 is co-localized with Ror2 at the plasma membrane and endoplasmic reticulum (Fig. 4C). On the other hand, when HA-tagged Ror2 and FLAG-tagged Msx2 were coexpressed in 293 cells, Ror2 and Msx2 proteins were detected at the membranous compartments and in the nuclei, respectively, and their intracellular distribution did not overlap at all (Fig. 4D). Importantly, when all three molecules were expressed in 293 cells, HA-Dlxin-1 was detected exclusively at the plasma membrane and endoplasmic reticulum (Fig. 4E, green tag), whereas, as expected, HA-Ror2 plus Flag-Msx2 were detected in the membranous compartments and in the nuclei (Fig. 4E, red tag). These results indicate that Dlxin-1 is co-localized with Ror2, but not with Msx2 in the membranous compartments, whereas Msx2 alone is retained in the nuclei. This is consistent with our biochemical analyses showing that Ror2 and Msx2 associate competitively with Dlxin-1, with Ror2 binding predominating. Co-localization of Dlxin-1 with Ror2, but not with Msx2, was further confirmed by a similar immunostaining analysis using HA-tagged Dlxin-1, HA-tagged Ror2, and FLAG-tagged Msx2 expressed simultaneously in 293 cells (data not shown).

The Ror2 Mutant, Ror2 ΔC, Fails to Sequestrate Dlxin-1 in Intracellular Membranous Compartments—The Ror2 mutant Ror2 ΔC fails to associate with Dlxin-1 in a co-immunoprecipitation assay (see Fig. 3A). We therefore next examined whether Ror2 ΔC is co-localized with Dlxin-1. When FLAG-Ror2 ΔC was expressed by itself in 293 cells, it localized mainly at the plasma membrane and endoplasmic reticulum (Fig. 5A), similar to wild-type Ror2 (Ror2 WT). When FLAG-tagged Ror2 ΔC and HA-tagged Dlxin-1 were co-expressed, Ror2 ΔC was detected in the membranous compartments, whereas Dlxin-1 stained diffusely throughout the cytoplasm (Fig. 5B). As expected, when HA-tagged Ror2 ΔC and FLAG-tagged Msx2 were co-expressed in 293 cells, both proteins were detected in the...
membranous compartments and in the nuclei, respectively, and their intracellular distribution showed no overlap at all (Fig. 5C). When HA-tagged Dlxin-1, FLAG-tagged Ror2 ΔC, and FLAG-tagged Max2 were co-expressed in 293 cells, HA-Dlxin-1 was detected exclusively in the nuclei (Fig. 5D, green tag), whereas Ror2 ΔC plus Max2 exhibited cell surface, reticular, and nuclear staining (Fig. 5D, red tag). This result suggests that Dlxin-1 co-localizes with Max2 in the nuclei, whereas Ror2 ΔC localizes in the membranous compartments. Co-localization of Dlxin-1 with Max2, but not Ror2 ΔC, was also verified by a similar immunostaining analysis using HA-tagged Dlxin-1, HA-tagged Ror2 ΔC, and FLAG-tagged Max2 co-expressed in 293 cells (data not shown). Taken together, these results indicate that the Ror2 mutant, Ror2 ΔC, fails to sequester Dlxin-1 in the membranous compartments. As expected, another Ror2 mutant (Ror2(Rob)) bearing more extensive cytoplasmic deletion than Ror2 ΔC (see Fig. 1B) also failed to sequester Dlxin-1 in the membranous compartments (data not shown).

Subcellular Distribution of Dlxin-1 Regulated by Ror2—To further confirm that Ror2 sequesters Dlxin-1 in the membranous compartments, we performed subcellular fractionation of...
FIG. 4. Subcellular localization of Ror2, Dlxin-1, and Msx2. 293 cells transfected singly or in combination with the expression vectors encoding FLAG-tagged or HA-tagged Ror2, HA-tagged Dlxin-1, and/or FLAG-tagged Msx2 were fixed, permeabilized, and incubated with anti-FLAG monoclonal antibody (M2, red) and anti-HA monoclonal antibody (3F10, green) followed by treatment with Alexa Fluor 546 (anti-mouse IgG antibody, red) and Alexa Fluor 488 (anti-rat IgG antibody, green). The proteins expressed are indicated in each panel. The results shown are representative of three independent experiments. A, FLAG-tagged Ror2, HA-tagged Dlxin-1, and FLAG-tagged Msx2 were expressed singly in 293 cells, and their intracellular distributions were visualized as described under “Experimental Procedures.” Ror2 proteins exhibit cell surface and reticular staining, characteristic of transmembrane cell surface proteins. Dlxin-1 proteins are localized diffusely in the cytoplasm, whereas Msx2 proteins are localized predominantly in the nuclei of the transfected cells. B, Dlxin-1 and Msx2 proteins are co-localized in the nuclei in the absence of Ror2 protein expression. C, Ror2 and Dlxin-1 proteins are co-localized at the cell surface and intracellular membranous compartments in 293 cells co-expressing FLAG-tagged Ror2 and HA-tagged Dlxin-1. D, The intracellular distribution of Ror2 and Msx2 proteins do not overlap in 293 cells co-expressing HA-tagged Ror2 and FLAG-tagged Msx2. Their distribution was essentially identical with that observed when the respective proteins were expressed singly in 293 cells (see A). E, Dlxin-1 co-localizes with Ror2 but not Msx2 when all three molecules are expressed concomitantly in 293 cells. FLAG-tagged Ror2, HA-tagged Dlxin-1, and FLAG-tagged Msx2 were expressed in 293 cells, and the intracellular distribution of Ror2 plus Dlxin-1 and of Msx2 was examined (data not shown).

FIG. 5. Ror2 ∆C fails to sequester Dlxin-1 in intracellular membranous compartments. 293 cells were transfected singly or in combination with the respective expression vectors, i.e. FLAG- or HA-tagged Ror2 WT, Ror2 ∆C, Dlxin-1, and/or Msx2 (see below). Subsequently, cells were fixed, permeabilized, and incubated with anti-FLAG monoclonal antibody (M2, red) and anti-HA monoclonal antibody (3F10, green), followed by treatment with Alexa Fluor 546 (anti-mouse IgG antibody, red) and Alexa Fluor 488 (anti-rat IgG antibody, green). The proteins expressed are indicated in each panel. The results shown are representative of three independent experiments. A, FLAG-tagged Ror2 WT, FLAG-tagged Ror2 ∆C, and HA-tagged Dlxin-1 were expressed singly in 293 cells, and their intracellular distribution was visualized as described under “Experimental Procedures.” Both Ror2 WT and Ror2 ∆C proteins exhibit cell surface and reticular staining, characteristic of transmembrane cell surface proteins. Dlxin-1 proteins are localized diffusely in the cytoplasm of the transfected cells. B, FLAG-tagged Ror2 ∆C and HA-tagged Dlxin-1 were co-expressed in 293 cells. The intracellular distribution of Ror2 ∆C and Dlxin-1 was essentially identical to that observed when the respective proteins were expressed singly in 293 cells (see A). C, the intracellular distribution of Ror2 ∆C and Msx2 proteins do not overlap in 293 cells co-expressing HA-tagged Ror2 ∆C and FLAG-tagged Msx2. Their distribution was essentially identical with that observed when the respective proteins were expressed singly in 293 cells (see A). D, Dlxin-1 and Msx2 proteins are co-localized in the nuclei in the absence of Ror2 ∆C proteins. FLAG-tagged Ror2 ∆C, HA-tagged Dlxin-1, and FLAG-tagged Msx2 were expressed in 293 cells, and the intracellular distribution of Ror2 ∆C plus Msx2 and of Dlxin-1 was examined as described under “Experimental Procedures.” In some experiments, HA-tagged Ror2 ∆C, HA-tagged Dlxin-1, and FLAG-tagged Msx2 were expressed in 293 cells, and the intracellular distribution of Ror2 ∆C plus Dlxin-1 and of Msx2 was examined (data not shown).

MEFs from the wild-type and Ror2−/− mice (see “Experimental Procedures”). Because antibodies against endogenous mouse Ror2 are currently unavailable, the presence or absence of Ror2 expression in the wild-type and Ror2−/− MEFs was confirmed by RT-PCR and Northern blot analyses (data not shown). Ex-
expression of Dlxin-1 did not differ significantly between the wild-type and Ror2−/− MEFs (data not shown), suggesting that expression of Dlxin-1 is unaffected by Ror2. As shown in Fig. 6, the vast majority of cellular Dlxin-1 was localized in the membrane fractions in wild-type MEFs, with somewhat lower levels present in the cytosol. On the other hand, in Ror2−/− MEFs, only a small percentage of cellular Dlxin-1 was detected in the membrane fraction (Fig. 6), whereas the amount of cytosolic Dlxin-1 was comparable between Ror2−/− and wild-type MEFs.

The amount of total cellular Dlxin-1 was also comparable between Ror2 WT and wild-type MEFs. As shown in Fig. 6, the vast majority of cellular Dlxin-1 in Ror2−/− MEFs may be localized in the nucleocytoskeletal fractions. This result suggests that Ror2 is responsible for the subcellular localization of Dlxin-1 and that it normally sequesters Dlxin-1 in the membrane fractions.

Transcriptional Activity of Mx2 Is Modulated by Ectopic Expression of Ror2—To pursue the functional significance of observed sequestration of Dlxin-1 by Ror2 in the membranous compartments, we examined whether expression of Ror1 WT, Ror2 WT, or Ror2 DK affects the transcriptional activity of Mx2. To this end, luciferase assays were performed with 293 cells transfected with the respective expression vectors along with a reporter plasmid containing a WIP element upstream of the simian virus 40 minimal promoter and a luciferase reporter gene (pGL2-WIP) (27). Experiments were carried out without ectopic co-expression of Dlxin-1, because it was found that Dlxin-1 is expressed endogenously in 293 cells at a relatively high level (data not shown). Consistent with a previous report (27), transfection of the Mx2 expression vector alone resulted in drastic transcriptional repression of the WIP reporter plasmid (Fig. 7). As shown in Fig. 7, this transcriptional repression by Mx2 was cancelled at least partially by ectopic expression of Ror2 WT but not Ror1 WT. Interestingly, it was also cancelled at least partially by ectopic expression of Ror2 DK (Fig. 7), suggesting that Ror2, irrespective of its kinase activity, regulates the transcriptional activity of Mx2 in the nuclei by sequestrating Dlxin-1, a transcriptional co-factor for Mx2, in the membranous compartments.

Dlxin-1 Is a Possible Mediator of Ror2 Function—in mammals, the Ror family RTKs consist of two structurally related proteins, Ror1 and Ror2 (3, 7). Although both Ror1 and Ror2 play crucial roles during development, Ror2 exhibits more specific functions compared with Ror1 (13). We have shown that Dlxin-1, a member of the MAGE family of proteins, associates with Ror2, but not with Ror1 (Fig. 1). This suggests that Dlxin-1 may be involved in mediating some if not all of the developmental functions of Ror2. Interestingly, Ror2 and Dlxin-1 exhibit remarkably similar expression patterns in the developing face and limbs, in contrast to Ror1, which shows a different pattern (Fig. 2). This suggests that the molecular association between Ror2 and Dlxin-1 may be biologically significant during development. Furthermore, it has been shown that the cytoplasmic C-terminal region of Ror2, containing proline-rich and serine/threonine-rich domains, and the C-terminal necdin homology domain of Dlxin-1 are required for the association between Ror2 and Dlxin-1 (Fig. 3, A and C). It should also be noted that the cytoplasmic C-terminal region of Ror2 is missing in BDB patients, suggesting a possible involvement of Dlxin-1 in the pathogenesis of BDB. Intriguingly, Dlxin-1 is co-localized with the wild-type Ror2 in the membranous compartments as the result of sequestration by Ror2 (Figs. 4 and 6), whereas the mutant Ror2 fails to sequesterate Dlxin-1 in the membranous compartments (Fig. 5).

Until recently, the functions of the MAGE family of proteins were largely unknown, except for the protein necdin. Necdin was shown to mediate growth arrest of postmitotic neurons, presumably by interacting with E2F1 and p53 (20, 35, 36). Interestingly, it has recently been shown that Dlxin-1 (and its rat ortholog, NRAGE) is a multifunctional adaptor protein: (i) Dlxin-1 associates with the homeodomain proteins Mx2 and Dlx5 and regulates the transcriptional function of the latter (22); (ii) NRAGE associates with p75NTR and is required for nerve growth factor-dependent apoptosis of developing neurons (24); and (iii) NRAGE associates with the IAPs ITA and XIAP and augments the apoptosis of 32D cells upon growth factor withdrawal (25). Similar to our observation that Ror2 and Mx2 bind to an overlapping region within Dlxin-1 (Fig. 3, C

Fig. 6. Distribution of Dlxin-1 in subcellular fractions from MEFs (wild-type and Ror2−/−). Cytosolic and membrane fractions as well as total cell extracts were prepared from MEFs (wild-type and Ror2−/−) as described under “Experimental Procedures.” The amounts of Dlxin-1 in the cytosolic and membrane fractions as well as total cell extracts from MEFs (wild-type and Ror2−/−) were determined by immunoblotting with anti-Dlxin-1 antibody (anti-DXN-NHD Ab). The amount of proteins recovered in the cytosolic and membrane fractions as well as in total cell extracts is almost comparable between the wild-type and Ror2−/− MEFs. The results shown are representative of three independent experiments.

Fig. 7. Transcriptional suppression activity of Mx2 is cancelled partially by co-expression of Ror2 WT and Ror2 DK, respectively. 293 cells were transfected with the respective expression vectors along with pGL2-WIP reporter plasmid as indicated. Cell extracts were prepared and luciferase assays performed as described under “Experimental Procedures.” Luciferase activities are expressed as fold increase relative to cells transfected with the empty vector alone. Expression levels of Ror1 WT, Ror2 WT, and Ror2 DK in the respective transfectants were almost comparable as assessed by anti-FLAG immunoblotting (data not shown). Data are expressed as the mean ± S.D. of relative luciferase activity in three replicate wells from one representative experiment of three.
Subcellular Distribution of Dlxin-1 Regulated by Ror2

and D), it has been reported that NRAGE and TrkA association with p75NTR are also physically exclusive (24).

Collectively, our findings help elucidate the pathophysiological roles of Ror2 during development. Dlxin-1 has previously been shown to associate with and regulate the transcriptional activity of Dlx5 (22), a member of the Dlx family of homeodomain proteins involved in skeletal development. Therefore, it is likely that the transcriptional activity of Mx2, another homeodomain protein involved in skeletal development (37, 38), would be affected similarly by its interaction with Dlxin-1. Hence, the expression levels of Ror2, Dlxin-1, and of the main proteins involved in skeletal development. Therefore, it is been shown to associate with and regulate the transcriptional function of Ror2 at the membranous compartments, irrespective of its kinase activity, indirectly affects the transcriptional function of Dlx5 (22), a member of the Dlx family of homeodo-

Dlxin-1. Hence, the expression levels of Ror2, Dlxin-1, and of Meox2 in a particular cell may be the critical determinants that regulate the behavior and function of the cell under unstimu-

ated or ligand-stimulated conditions. Although the cognate ligand of Ror2 is currently unknown, its identification may help elucidate the mechanisms regulating the subcellular distribution and function of Dlxin-1 and/or Meox2.

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The Receptor Tyrosine Kinase Ror2 Associates with the Melanoma-associated Antigen (MAGE) Family Protein Dlxin-1 and Regulates Its Intracellular Distribution

Takeru Matsuda, Hiroaki Suzuki, Isao Oishi, Shuichi Kani, Yoshikazu Kuroda, Takahide Komori, Aya Sasaki, Ken Watanabe and Yasuhiro Minami

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