The Receptor Tyrosine Kinase Ror2 Associates with and Is Activated by Casein Kinase Iε

Shuichi Kani‡§§, Isao Oishi‡‡§§, Hiroyuki Yamamoto‡, Akinori Yoda‡, Hiroaki Suzuki‡, Akira Nomachi‡, Kengo Iozumi‡, Michiru Nishita‡, Akira Kikuchi‡, Toru Takumi**, and Yasuhiro Minami‡‡

From the ‡Department of Genome Sciences, Faculty of Medical Sciences, Graduate School of Medicine, Kobe University, Kobe 650-0017, Japan, the ‡Department of Biochemistry, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 734-8551, Japan, and the **Osaka Bioscience Institute, Osaka 565-0874, Japan

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Ror2, a member of the mammalian Ror family of receptor tyrosine kinases, plays important roles in developmental morphogenesis, although the mechanism underlying activation of Ror2 remains largely elusive. We show that when expressed in mammalian cells, Ror2 associates with casein kinase Iε (CKIε), a crucial regulator of Wnt signaling. This association occurs primarily via the cytoplasmic C-terminal proline-rich domain of Ror2. We also show that Ror2 is phosphorylated by CKIε on serine/threonine residues, in its C-terminal serine/threonine-rich 2 domain, resulting in autophosphorylation of Ror2 on tyrosine residues. Furthermore, it was found that association of Ror2 with CKIε is required for its serine/threonine phosphorylation by CKIε. Site-directed mutagenesis of tyrosine residues in Ror2 reveals that the sites of phosphorylation are contained among the five tyrosine residues in the proline-rich domain but not among the four tyrosine residues in the tyrosine kinase domain. Moreover, we show that in mammalian cells, CKIε-mediated phosphorylation of Ror2 on serine/threonine and tyrosine residues is followed by the tyrosine phosphorylation of G protein-coupled receptor kinase 2, a kinase with a developmental expression pattern that is remarkably similar to that of Ror2. Intriguingly, a mutant of Ror2 lacking five tyrosine residues, including the autophosphorylation sites, fails to tyrosine phosphorylate G protein-coupled receptor kinase 2. This indicates that autophosphorylation of Ror2 is required for full activation of its tyrosine kinase activity. These findings demonstrate a novel role for CKIε in the regulation of Ror2 tyrosine kinase.

Receptor tyrosine kinases (RTKs) play important roles in developmental morphogenesis by regulating growth, differentiation, motility, adhesion, and death of many types of cells (1). It has been well documented that the interactions of RTKs with their cognate ligands trigger their dimerization or oligomerization, resulting in tyrosine autophosphorylation and tyrosine kinase activation of RTKs. This induces various intracellular signaling events. In contrast, it has been reported that tyrosine autophosphorylation and the tyrosine kinase activities of several RTKs, including the insulin and epidermal growth factor receptors, can be negatively regulated by ligand-independent transphosphorylation of these RTKs by cytoplasmic serine/threonine kinases (2–8). However, little is known about the positive regulation of RTK tyrosine autophosphorylation and tyrosine kinase activation caused by cytoplasmic serine/threonine kinases.

The mammalian Ror family of RTKs, consisting of two structurally related proteins, Ror1 and Ror2, are orphan RTKs, characterized by several conserved domain structures, the extracellular Frizzled-like cysteine-rich domains, and the membrane-proximal Kringle domains that are assumed to mediate protein-protein interactions (9–13). It has been reported that in nematodes and mammals, Ror family RTKs play crucial roles in various developmental processes. CAM-1, the Caenorhabditis elegans ortholog of Ror2, is implicated in cell migration, asymmetric cell division, and axon outgrowth during embryogenesis, and these processes may be either tyrosine kinase-dependent or -independent (14). Previous studies with Ror2-deficient mice have further revealed that Ror2 plays crucial roles in the development of the skeletal, genital, and cardiovascular systems (15–17). In humans, Ror2 is responsible for two heritable skeletal disorders; recessive Robinow syndrome and dominant brachydactyly type B (BDB) (18–23). Interestingly, it has recently been reported that the developmental pathology of Ror2−/− mice can explain many of the developmental malformations found in patients with Robinow syndrome (24).

We have recently shown that Ror2 associates with the melanoma-associated antigen family protein, Dlxin-1, which exhibits a similar developmental expression pattern with Ror2 and is known to bind to the homeodomain proteins Msx2 and Dlx5. Ror2 appears to affect transcriptional functions of Msx2 and Dlx5 by regulating intracellular distribution of Dlxin-1 in a tyrosine kinase-independent manner (25). Furthermore, our

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§ These two authors contributed equally to this work.

‡‡ To whom correspondence may be addressed: Dept. of Genome Sciences, Faculty of Medical Sciences, Graduate School of Medicine, Kobe University, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Tel.: 81-78-382-5560; Fax: 81-78-382-5579; E-mail: kani@med.kobe-u.ac.jp.

§§ To whom correspondence may be addressed: Dept. of Genome Sciences, Faculty of Medical Sciences, Graduate School of Medicine, Kobe University, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Tel.: 81-78-382-5560; Fax: 81-78-382-5579; E-mail: minami@kobe-u.ac.jp.

1 The abbreviations used are: RTK, receptor tyrosine kinase; CKIε, casein kinase Iε; GRK2, G protein-coupled receptor kinase 2; BDB, brachydactyly type B; GST, glutathione S-transferase; HA, hemagglutinin; WT, wild-type; GMCSF, granulocyte macrophage colony-stimulating factor; WCL, whole cell lysate.
recent genetic and biochemical analyses have indicated that Ror2 interacts with Wnt5a both physically and functionally to activate the noncanonical Wnt5a/JNK pathway in a tyrosine kinase-independent manner (16). In Xenopus, Xror2, a putative Xenopus ortholog of Ror2, has also been shown to interact with Xenopus Wnts and to modulate convergent extension movements of axial mesoderm and neuroectoderm by modulating the planar cell polarity pathway of Wnt signaling in a tyrosine kinase-independent manner (26). However, nothing is known about the molecular mechanisms underlying Ror2 tyrosine kinase activation and the consequent tyrosine kinase-dependent functions of Ror2.

To gain insights into new functions of Ror2, we performed yeast two-hybrid screening using Ror2 as bait to identify a candidate molecule(s) that interacts with Ror2. From this screen, we identified casein kinase Iε (CKIε), a member of the CKI family of protein serine/threonine kinases, as a molecule that interacts with Ror2. Recently, much attention has been paid to CKIε as a crucial regulator of the canonical Wnt signaling, although its exact role(s) in this regulation remains controversial (27). It has been demonstrated that CKIε can phosphorylate various Wnt signaling mediators, including Dvl (Dishevelled), adenomatous polyposis coli, axin, and β-catenin, thereby contributing to the regulation of the canonical Wnt pathway (28–33). Here we show that Ror2 associates with and is phosphorylated on serine/threonine residues by CKIε when expressed in mammalian cells. Interestingly, serine/threonine phosphorylation of Ror2 by CKIε is followed by the autophosphorylation of Ror2 tyrosine residue(s) within its cytoplasmic Pro-rich domain. Moreover, Ror2 associates with G protein-coupled receptor kinase 2 (GRK2) and tyrosine phosphorylates it following activation of Ror2 by CKIε. These results indicate that the tyrosine kinase activity and tyrosine autophosphorylation of Ror2 can be positively regulated by CKIε. We further provide evidence indicating that tyrosine autophosphorylation of Ror2 is required for activation of Ror2 tyrosine kinase.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—Wild-type and mutant cDNAs were constructed in the mammalian expression vector pcDNA3 (Invitrogen). Expression vectors encoding the FLAG-tagged Ror proteins were constructed as described previously (25). The Ror2 mutant constructs (ΔC, ΔDBB, Rs, TC, S860A, S861A, S864A, S866A, S868A, S870A, S878A, and S882A, T871A, T875A, T876A, and T881A) were constructed by site-directed mutagenesis. Ror2 mutants bearing substitutions of serine and threonine with alanines, Ror2 1S/TA (S860A, S861A, and S864A), Ror2 2S/T2 (S871A and S876A), and Ror2 S/T1,2 were generated by deleting amino acids 788–944, 749–944, 502–944, 434–944, 883–944, 783–859, 744–782, 860–882, 744–782 and 860–882, respectively, in the C-terminal region of Ror2. An expression vector encoding a kinase-dead mutant of Ror2 was constructed by replacing lysine 507, crucial for ATP binding, with arginine. Ror2 mutants bearing substitutions of serine with alanines, Ror2 1S/T1 (S860A, S861A, S864A, S866A, S868A, S870A, S878A, and S882A, T871A, T875A, T876A, and T881A) were constructed by site-directed mutagenesis. Ror2 mutants bearing substitutions of tyrosines with phenylalanines, Ror2 4YF (Y641F, Y645F, Y646F, and Y722F) and Ror2 5YF (Y641F, Y645F, Y646F, and Y722F) were constructed by site-directed mutagenesis by replacing lysine 38, crucial for ATP binding, with arginine.

Antibodies, Cells, and Transfection—Rabbit polyclonal anti-mouse Ror2 antibody was raised against GST mouse Ror2 (amino acids 726–945). The mouse monoclonal antibodies M2 (Sigma) and 12CA5 (Roche Applied Science) recognize the FLAG peptide and human influenza HA protein peptide sequence.

Cell Signaling and Upstate Biotechnology, Inc., respectively. Rabbit polyclonal anti-phosphoserine and anti-phosphothreonine antibodies were from Zymed Laboratories and Cell Signaling, respectively. HEK293T (293T) and NIH3T3 (3T3) cells were maintained in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% (v/v) fetal calf serum. Transient cDNA transfection was performed using the calcium phosphate method (12). Immuno precipitation and Immunoblotting—The 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 aprotinin), and the cell lysates were prepared by centrifugation at 12,000 × g for 15 min. The cell lysates were preclarified for 1 h at 4 °C with protein A-Sepharose (Amersham Biosciences). The preclarified supernatants were then immunoprecipitated with anti-FLAG or anti-HA antibody conjugated to protein A-Sepharose beads for 2 h at 4 °C. The immunoprecipitates were washed five times with 1 ml of the above lysis buffer and eluted with Laemmli sample buffer. Immunoprecipitates or whole cell lysates were separated by SDS-PAGE (9% PAGE) and transferred to polyvinylidene difluoride membrane filters (Immobilon, Millipore). The membranes were immunoblotted with the respective antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated anti-mouse IgG antibodies using chemiluminescence reagent (Western Lightning; PerkinElmer Life Sciences) as described previously (12).

Expression and Purification of GST Fusion Proteins—The GST fusion proteins, GST-CKIε WT and GST-CKIε DK, expressed in Escherichia coli DH5α were extracted with phosphate-buffered saline containing 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin and were isolated using glutathione-Sepharose beads (Amersham Biosciences). Fusion proteins were then eluted from beads by 25 mM glutathione (reduced), followed by dialysis prior to use in kinase assays.

In Vitro Kinase Assay—For in vitro kinase assay, 293T cells were solubilized 60 h after transfection, and Ror2 WT-FLAG or Ror2 DK-FLAG proteins were immunoprecipitated as described above. Precipitates were washed five times with lysis buffer and resuspended in 50 μl kinase buffer containing 50 mM Tris-HCl, pH 7.5, 1 μM MgCl2, and 40 μM ATP. The in vitro kinase reaction was initiated by the addition of purified GST-CKIε WT or GST-CKIε DK and allowed to incubate for 30 min at 30 °C. The reaction was terminated by the addition of Laemmli sample buffer, and the samples were separated by SDS-PAGE (10% PAGE) and transferred to polyvinylidene difluoride membrane filters, followed by immunoblot analysis with anti-phospho-serine/threonine antibodies.

In Situ Hybridization—In situ hybridization analyses were performed essentially as described previously (34). The 0.86-kb HindII/ EcoRI fragment of Ror2 or the 0.87-kb HindII/apaf fragment of GRK2 were utilized as templates to synthesize single strand RNA probes.

**RESULTS**

**Ror2 Associates with CKIε**—To identify a Ror2-interacting protein(s), we performed a yeast two-hybrid screening using the cytoplasmic region of Ror2 as bait (25). From this screen we identified a protein serine/threonine kinase, CKIε (data not shown). To determine whether Ror2 associates with CKIε in mammalian cells, FLAG-tagged wild-type Ror2 (Ror2 WT) and HA-tagged CKIε were coexpressed in 293T cells. As shown in Fig. 1A, HA-tagged CKIε was coimmunoprecipitated with FLAG-tagged Ror2, indicating that Ror2 associates with CKIε in vitro. We also found that endogenous CKIε was detected in anti-Ror2 immunoprecipitates from 293T cells, confirming association between endogenous Ror2 and CKIε (Fig. 1B).

**Antibodies, Cells, and Transfection**—Rabbit polyclonal anti-mouse Ror2 antibody was raised against GST mouse Ror2 (amino acids 726–945). The mouse monoclonal antibodies M2 (Sigma) and 12CA5 (Roche Applied Science) recognize the FLAG peptide and human influenza HA protein peptide sequence. Mouse monoclonal anti-phosphotyrosine antibodies PY20 and 4G10 were purchased from Transduction Laboratories. The mouse monoclonal anti-phosphorysine antibodies PY20 and 4G10 were purchased from Transduction Laboratories.
Serines (860, 861, 864, 866, 868, 870, 879, and 882) and threonines (869, 871, 875, 876, and 881) were replaced with alanines in the 13S/TA mutant.

To test whether CKIε could phosphorylate Ror2 directly, GST-CKIε WT and GST-CKIε DK proteins purified from E. coli (see “Experimental Procedures”) were subjected to in vitro kinase assay using FLAG-tagged WT or kinase-inactive Ror2 mutant (DK) as substrates. As shown in Fig. 2B, GST-CKIε WT, but not GST-CKIε DK, could phosphorylate serine/threonine residues within both Ror2 WT and Ror2 DK in vitro, supporting the idea that CKIε phosphorylates Ror2 directly.

We also examined the phosphorylation status of the Ror2 ΔS/T1, ΔS/T2, ΔS/T1,2, and Δpro in mammalian cells coexpressing CKIε. The Ror2 ΔS/T2, ΔS/T1,2, and Δpro exhibited a complete loss of serine/threonine phosphorylation of Ror2, whereas the Ror2 ΔS/T1 showed somewhat weak, yet apparent serine/threonine phosphorylation of Ror2, compared with the Ror2 WT (Fig. 2C).

Next, we attempted to identify serine/threonine phosphorylation sites within Ror2 by CKIε. Because serine/threonine phosphorylation of Ror2 was found in the Ror2 ΔS/T1 but not Ror2 ΔS/T2 mutants, we generated the Ror2 13S/TA mutant in which all of the serines and threonines in the S/T2 domain of Ror2 were replaced with alamines (Fig. 1C). As shown in Fig. 2C, the Ror2 13S/TA mutant could associate with CKIε but failed to be phosphorylated by CKIε, indicating that CKIε phosphorylates primarily serine/threonine residues in the S/T2 domain of Ror2.

Tyrosine Autophosphorylation of Ror2 Following Its Serine/Threonine Phosphorylation by CKIε—To better understand the biological consequence of Ror2 phosphorylation by CKIε, we transiently coexpressed FLAG-tagged Ror2 WT with either CKIε WT or CKIε DK in 293T cells. Tyrosine phosphorylation of Ror2 was detected when Ror2 and CKIε WT were coexpressed, but not when Ror2 was expressed alone or coexpressed with CKIε DK (Fig. 3A). The results indicate that CKIε kinase...
activity is required for tyrosine phosphorylation of Ror2. Next, FLAG-tagged Ror2 WT or Ror2 DK was expressed along with CKIWT, and Ror2 tyrosine phosphorylation was evaluated. Tyrosine phosphorylation of Ror2 WT, but not Ror2 DK, was observed under the same experimental setting, although Ror2 DK was also associated with and serine/threonine-phosphorylated by CKIWT to similar extents when compared with Ror2 WT (Fig. 3B). Thus, CKI-mediated tyrosine phosphorylation of Ror2 requires the intrinsic tyrosine kinase activity of Ror2.

We further attempted to identify tyrosine phosphorylation sites within Ror2 induced by coexpression of CKI. We have previously shown that the tyrosine kinase domains of the Ror family RTKs are most similar to those of the neurotrophin receptor Trk family of RTKs and that the four autophosphorylated tyrosine residues found in the activation loops within the tyrosine kinase domains of the Trk family RTKs are also conserved in Ror2 (Tyr641, Tyr645, Tyr646, and Tyr722) (12). We also found that coexpression of CKI failed to induce tyrosine phosphorylation of Ror2 ΔC (data not shown). The Ror2 ΔC lacks the C-terminal portion of Ror2, which contains six tyrosine residues, including five (Tyr818, Tyr824, Tyr830, Tyr833, and Tyr838) that are found in the Pro-rich domain. Thus, we generated the two Ror2 mutants, 4YF and 5YF (Fig. 3C and see “Experimental Procedures”), in which the tyrosines were replaced with phenylalanines. When FLAG-tagged Ror2 4YF and 5YF were expressed in 293T cells along with CKIWT, it was found that both the Ror2 4YF and 5YF could associate with CKIWT and were phosphorylated on serine/threonine residues to a similar extent as Ror2 WT (Fig. 3D). Interestingly, when coexpressed with CKI, Ror2 4YF but not Ror2 5YF was found to be phosphorylated on tyrosine residues (Fig. 3D). This indicates that the sites of tyrosine autophosphorylation are among the five tyrosine residues contained within the Pro-rich domain but not among the four tyrosine residues contained within the tyrosine kinase domain. We then examined whether serine/threonine phosphorylation of Ror2 by CKI is required for subsequent tyrosine autophosphorylation of Ror2.

Tyrosine Phosphorylation of GRK2 by Ror2 Following Coexpression of CKI—The Ror2-interacting proteins, CKI and

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**Fig. 2.** Phosphorylation of serine/threonine residues within the serine/threonine rich 2 domain of Ror2 following expression of CKI. A, serine/threonine phosphorylation of Ror2 following expression of CKI WT but not CKI DK. FLAG-tagged Ror2 protein was expressed transiently in 293T cells along with either the WT or kinase inactive mutant (DK) of CKI proteins. WCLs or anti-FLAG immunoprecipitates (IP) from the respective WCLs were analyzed by anti-phosphoserine/threonine (top panel), anti-FLAG (second panel), or anti-CKI (third and bottom panels) immunoblotting. B, Ror2 serine/threonine residues are phosphorylated in vitro by purified CKI WT, but not CKI DK. FLAG-tagged Ror2 WT and Ror2 DK were prepared by overexpressing the respective proteins in 293T cells followed by anti-FLAG immunoprecipitation. In vitro kinase assay was performed as described under “Experimental Procedures.” Top, middle, and bottom panels indicate anti-phosphoserine/threonine, anti-FLAG, and anti-CKI immunoblot analyses of kinase reactions, respectively. C, serine/threonine phosphorylation within the serine/threonine-rich 2 domain of Ror2 by CKI. WCLs were prepared from 293T cells expressing FLAG-tagged WT or a series of Ror2 mutant proteins along with HA-tagged CKI protein, as shown in the panel. Serine/threonine phosphorylation of Ror2 WT and the respective Ror2 mutants were examined as described for A.
Dlxin-1 (see Fig. 5A), were not tyrosine-phosphorylated by Ror2 when coexpressed with CKIe (data not shown). We therefore searched for other candidate molecule(s) that could be tyrosine-phosphorylated by Ror2 under the same experimental conditions. The gene encoding the GRK2 was reported to exhibit a developmental expression pattern very similar to those of Ror2 and Dlxin-1 (see Fig. 5A). We therefore examined whether or not tyrosine phosphorylation of GRK2 correlates with CKIe-induced tyrosine autophosphorylation of Ror2. Consistent with the result shown in Fig. 5A, coexpression of Ror2 WT and CKIe WT resulted in the tyrosine phosphorylation of GRK2, whereas Ror2 WT or CKIe WT alone did not (Fig. 5B). Furthermore, coexpression of Ror2 WT plus CKIe DK or Ror2 DK plus CKIe WT failed to induce tyrosine phosphorylation of GRK2. These results indicate that tyrosine phosphorylation of Ror2, following coexpression of Ror2 and CKIe, leads to Ror2-mediated tyrosine phosphorylation of GRK2.

Next, we examined whether or not tyrosine phosphorylation of GRK2 was observed when Ror2 WT or Ror2 4YF, but not Ror2 5YF, was coexpressed with CKIe. This indicates that autophosphorylation of one or more of the five tyrosine residues within the Pro-rich domain of Ror2 is required for tyrosine phosphorylation of GRK2 by Ror2. Finally, we also examined whether serine/threonine phosphorylation of Ror2 by CKIe is indeed required for Ror2-mediated tyrosine phosphorylation of GRK2. As expected, tyrosine phosphorylation of GRK2 was detected in cells expressing Ror2 WT, but not 13S/TA, along with CKIe (Fig. 5D). The result indicates that serine/threonine phosphorylation of Ror2 by CKIe is also required for tyrosine phosphorylation of GRK2 by activated Ror2.
The receptor tyrosine kinase Ror2 plays important roles in developmental morphogenesis, including the development of skeletal, genital, and cardiorespiratory systems (15, 17). Genetic analyses in nematodes have revealed that CAM-1, the C. elegans ortholog of Ror2, possesses both tyrosine kinase-dependent and -independent functions in development (9, 13, 14). However, little is known about the tyrosine kinase-dependent functions of Ror2, in particular, the mechanisms underlying tyrosine kinase activation of Ror2. In fact, Wnt5a stimulation of Ror2, antibody-mediated cross-linking of Ror2, and granulocyte macrophage colony-stimulating factor (GMCSF)-induced dimerization of a chimeric GMCSF/Ror2 receptor (GMCSF receptor extracellular region/Ror2 transmembrane and intracellular regions) fail to induce Ror2 tyrosine autophosphorylation or tyrosine kinase activity (data not shown). Here we show that tyrosine autophosphorylation and tyrosine kinase activation of Ror2 are induced by the cytoplasmic protein serine/threonine kinase CKIε-mediated phosphorylation of Ror2 (Fig. 3).

Our structure-function analyses of Ror2 indicate that Ror2 associates with CKIε primarily via its C-terminal proline-rich domain (Fig. 1, D and E) and that Ror2 is phosphorylated by CKIε in its C-terminal S/T2 domain (Fig. 2C). Because all of the deletional mutants of Ror2 lacking the proline-rich domain, Ror2 AC, BDB, RS, Te (data not shown), and Δpro (Fig. 2C), exhibited both drastically decreased levels or complete loss of CKIε binding and of serine/threonine phosphorylation by CKIε (Fig. 2C; data not shown), it is likely that association of Ror2 with CKIε is required for its phosphorylation by CKIε. Furthermore, we present evidence indicating that phosphorylation of Ror2 by CKIε is prerequisite to induce autotyrosine phosphorylation and activation of Ror2 (Figs. 3 and 5). In fact, the Ror2 13S/T2A mutant, which is assumed to lack serine/threonine phosphorylation sites by CKIε, can associate with CKIε but fail to be autotyrosine-phosphorylated or activated by CKIε (Figs. 2C, 3E, and 5D). It is of importance to determine the pivotal serine/threonine residues within the S/T2 domain of Ror2 that are phosphorylated by CKIε. Ror2 mutants lacking the C-terminal portion (e.g. Ror2 AC and BDB) are also neither autotyrosine-phosphorylated nor activated by CKIε (data not shown). Interestingly, mutant Ror2 proteins from BDB patients lack this C-terminal portion of Ror2, suggesting that the pathogenesis of BDB may be attributable to a defect in tyrosine autophosphorylation and activation of Ror2 tyrosine kinase activity.

To clarify the mechanism of tyrosine kinase activation of Ror2 following phosphorylation by CKIε, we attempted to identify the sites of tyrosine autophosphorylation within the cytoplasmic region of Ror2. It had been reported that autophosphorylation of the tyrosine residues in the activation loop of the tyrosine kinase domains of the Trk family RTKs is required for activating the Trk family RTKs (38), the closest relatives of Ror family RTKs. However, we mapped the Ror2 tyrosine autophosphorylation sites to the proline-rich domain, not the activation loop (Fig. 3D). Further study will be required to identify more precisely the autophosphorylated tyrosine residue(s). In this study, we also identified the cytoplasmic protein serine/threonine kinase GRK2, as a substrate for activated Ror2 (Fig. 5). Interestingly, the Ror2 5YF, but not 4YF, failed to tyrosine phosphorylate GRK2 (Fig. 5C), indicating that autophosphorylation of tyrosine residue(s) within the proline-rich domain of Ror2 is required for (full) activation of Ror2 tyrosine kinase. We have previously shown that Ror1, in addition to Ror2, plays an important role in developmental morphogenesis and that most functions of Ror1 during mouse development can be compensated for by Ror2 (37). We found that Ror1 could also associate with CKIε in vivo and is phosphorylated on serine/threonine residues by CKIε (data not shown). However, at present tyrosine autophosphorylation and tyrosine kinase activation of Ror1 have not been detected, probably because of a very low level of Ror1 expression compared with Ror2 in our transfection experiments. The results also suggest that the mechanism underlying activation of Ror2 tyrosine kinase may be distinct from those of other Trk family RTKs.

Our results demonstrate that the tyrosine kinase activity of Ror2 in vivo is regulated by CKIε. It has been reported that several RTKs can be transphosphorylated by cytoplasmic protein kinases. For example, the insulin receptor is phosphorylated by cAMP-dependent protein kinase (7), protein kinase C (8), and casein kinase 2 (6), and the epidermal growth factor receptor is phosphorylated by cAMP-dependent protein kinase (2), protein kinase C (4, 5), and calmodulin-dependent protein kinase II (3). In these cases, serine/threonine phosphorylation of these RTKs results in the drastic down-regulation of their auto-tyrosine phosphorylation and tyrosine kinase activities. Compared with these RTKs, Ror2 RTK is rather unique in that serine/threonine phosphorylation of Ror2 by CKIε results in the stimulation of its tyrosine autophosphorylation and tyrosine kinase activity.
activity. Importantly, it has been reported that CKI\(\alpha\)/H9280 regulates the canonical Wnt pathway by interacting both physically and functionally with various Wnt signal mediators (28–30, 32, 33). Taken together with our findings, we envisage that there may be significant cross-talk between the Ror2 and canonical Wnt signal pathways. Although we have previously shown that Ror2 is also involved in the noncanonical Wnt pathway (16), it is currently unclear whether or not CKI\(\alpha\)/H9280 is likewise involved in noncanonical Wnt signaling. The rat Frizzled-2 (rFz2) is a putative receptor for Wnt5a and has been shown to activate several protein serine/threonine kinases, including protein kinase C, CaMKII, TAK1, and NLK, which mediate the noncanonical Wnt signaling pathway following engagement of upstream receptors (39–42). It would therefore be of interest to examine whether or not these protein kinases may also be able to phosphorylate and regulate Ror2 tyrosine kinase.

GRK2 is known as a key modulator in the internalization of seven transmembrane-spanning G protein-coupled receptors. Following agonist stimulation, GRK2 phosphorylates the most C-terminal cytoplasmic region of G protein-coupled receptors, resulting in the recruitment of \(\beta\)-arrestin and eventual agonist-induced internalization of the G protein-coupled receptors (43). It has previously been shown that the kinase activity and/or stability of GRK2 can also be modulated by tyrosine phosphorylation by the Src protein tyrosine kinase (44–47). Therefore, it is important to determine whether tyrosine phosphorylation of GRK2 by Ror2 may affect GRK2 kinase activity and consequent endocytosis of G protein-coupled receptors. We have shown that Ror2 forms a complex with rFz2 or human Frizzled 5 (hFz5), putative seven transmembrane-spanning type receptors for Wnt5a (16). Furthermore, it has recently been reported that mouse Frizzled 4 (mFz4), another putative seven transmembrane-spanning type receptor for Wnt5a, can be internalized following stimulation with Wnt5a and phorbol myristoyl...
acetate, a potent activator of protein kinase C, and that phosphorylated Dvl 2 recruits β-arrestin 2 to mediate internalization of mFz4 (48). The Ror2-associated CKI may be located in proximity to the putative seven transmembrane-spanning type receptors for Wnt5α (mFz4, fR2, and fH5α) (16). In addition, CKI has been shown to phosphorylate the Dvl proteins (30, 32, 49). Therefore, it is conceivable that the Dvl proteins may be phosphorylated by Ror2-associated CKI, recruit β-arrestin to these Wnt5α receptors, and thereby mediate internalization of these receptors. Alternatively, activated Ror2 tyrosine kinase may phosphorylate and regulate the function of GRK2, which may be located in proximity to the putative seven transmembrane-spanning type receptors for Wnt5α (mFz4, fR2, and fH5α) (48). The Ror2-associated CKI may phosphorylate and regulate the function of GRK2, which in turn phosphorylates these receptors, resulting in their internalization. Further study will be required to clarify the biological significance of Ror2 tyrosine kinase activation.

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