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Biochemical and functional characterization of the Ror2/BRIb receptor complex

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ABSTRACT

Ror2 belongs to the Ror family of receptor tyrosine kinases. Two distinct human disorders result from mutations in Ror2 suggesting a role in cartilage formation, chondrocyte differentiation, and joint formation. We have previously demonstrated functional and physical association of Ror2 with the BMP receptor type Ib (BRIb). The interaction site was mapped to the extracellular CRD domain of Ror2. Here we show specific association with and transphosphorylation by BRIb, but not BMP receptors Ia or II. This association is independent of *N*-glycosylation, excluding the possibility that the interaction is mediated by carbohydrate moieties present in the CRD region of Ror2. The Ror2/BRIb complex proved very stable under high ionic and reducing conditions, yet it appeared sensitive to SDS-treatment. Besides we provide evidence that the Ror2/BRIb complex forms in distinct microdomains at the plasma membrane (DRMs), indicating that Ror2 may interfere with BMP signaling complexes within these membrane domains.

Ror2 is a receptor tyrosine kinase related to muscle specific kinase [1,2] and Trk neurotrophin receptors [3]. Ror2 orthologs were identified in several species including human [1] and mouse [4]. Ror2 is expressed in a wide spectrum of tissues during embryogenesis including the heart, lung, and bones of the head and limbs [5]. Homozygous knock out mice die shortly after birth and display dwarfism, facial abnormalities, shortened snouts, limbs and tail besides abnormalities in axial skeleton and respiratory dysfunctions [4].

Two different human genetic disorders, recessive Robinow syndrome characterized by short-limbed dwarfism [6] and Brachydactyly type B (BDB) characterized by shortened fingers and toes, were found to result from mutations in human Ror2 [7]. We have shown that mutations within the human bone morphogenetic protein receptor type Ib (BRIb) result in Brachydactyly type A2 (BDA2), suggesting that Ror2 and BRIb are both components which might act in concert to regulate skeletogenesis during embryonic development [8].

Bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs) are multifunctional growth factors, belonging to the TGFb superfamily [9]. Many of them play a central role in skeletal development. BMP2 induces bone and cartilage in vivo and in vitro [10]. GDF5 plays a central role in chondrogenesis, joint formation, and establishment of tendons [11]. These factors signal through two types of transmembrane serine/threonine kinase receptors classified as type I and type II. GDF5 binds BRIb with high affinity, whereas BMP2 preferentially binds BRIa [12]. The ligand-bound type I receptor is subsequently activated through transphosphorylation by the low affinity type II receptor (BRII) [13]. We have previously demonstrated that the BMP receptors BRII, BRIa, and BRIb reside in cholesterol-rich, detergent-resistant microdomains (DRMs) of the plasma membrane. More precisely, BRIa and BRIb possess a strong affinity to DRMs, while BRII exhibits a much less confined distribution within membrane fractions. Our studies revealed that the localization of BMP receptors in distinct microdomains and subsequent internalization is required for downstream signaling [14]. Most recently, it was shown that Caveolin-1 and its interaction with BRII are required for BRII membrane localization and subsequent Smad-dependent signaling [15]. Smads are intracellular substrates that are recruited to and phosphorylated by activated type I receptors at the plasma membrane. The ligand–receptor–Smad complex then undergoes endocytosis via clathrin-coated pits (CCPs). Finally, phosphorylated Smads can dissociate from the receptor complex and translocate to the nucleus, where they participate in the transcriptional regulation of genes involved in cartilage and bone formation [13,14,16].

Previously, we provided evidence for crosstalk between the Ror2 tyrosine and the BRIb serine/threonine kinase receptors. Ror2 was found to form a complex with BRIb in a ligand-indepen-

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dent manner and to subsequently modulate the GDF5/BRIb induced Smad 1/5 signaling cascade [17]. Here we show this being specific for BRIb, since no crosstalk is observed with BRIa or BRII. Moreover, crosstalk between Ror2 and BRIb is mediated through direct complex formation of these receptors in a ligand-independent fashion at the extracellular domains not requiring *N*-glycosylation of Ror2. The heteromeric complex of Ror2 and BRIb is strong enough to resist high ionic strength. It also forms independent of disulfide bonds as it is not affected by DTT. Furthermore, we describe that BRIb and Ror2 are co-localized in DRM sites, where BMP-mediated Smad-dependent signaling is initiated.

Materials and methods

Constructs, cells, and reagents. Flag-tagged Ror2 [17], HA-tagged BRIa, BRIb, and BRII receptors [18,19], and stably transfected C2C12-BRIb cells [20] were described previously. Cells, antibodies, and reagents were purchased from the following companies: COS7 and C2C12 cells from ATCC, anti-Flag M2 monoclonal antibody and Protein-A-Sepharose (PAS) from Sigma, anti-HA antibody and anti-Caveolin-1α antibodies from Santa Cruz Biotechnology, secondary antibodies from Dianova, the protease inhibitor cocktail (Complete) and tunicamycin from Roche Diagnostics, EndoH from Calbiochem, and Opti-Prep from Axis-Shield.

Production of antibodies. Polyclonal anti-Ror2 antibodies were produced in cooperation with Eurogentec, Brussels. Two KLH-coupled peptides were selected for rabbit immunization: peptide #1 DTLGQPDGPDSPLPT, corresponding to amino acids 42–56 and peptide #2 RLGPTHSPNHNFQ, corresponding to amino acids 153–165 of Ror2. All experiments were performed using a 1:1 mixture of both antibodies. The antibodies were successfully tested in IP and Western blot for both overexpressed Ror2 in COS7 cells and endogenous Ror2 in C2C12 cells (data not shown).

Immunoprecipitation, in vitro kinase assay, Western blotting. COS7 cells were transfected with the indicated receptor constructs using the DEAE-dextran method [21].

In vitro kinase assay and co-immunoprecipitation (IP) were described earlier [17]. Briefly, transfected COS7 cells were lysed in TNE lysis buffer supplemented with 1% NP-40, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM NaF, 10 mM Na_3VO_4, and 1× protease inhibitor cocktail. Lysates were precleared with PAS and proteins were captured using the indicated antibodies. The receptor–antibody complexes were precipitated with PAS.

Soluble proteins were separated by SDS–PAGE, transferred to a nitrocellulose membrane and analyzed by immunoblotting using the indicated antibodies. Secondary goat anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase were used and signals were visualized by chemiluminescence reagents [18].

Deglycosylation studies using tunicamycin and endoglycosidase. To inhibit *N*-glycosylation in vivo, we treated transfected COS7 cells with 2 mg/ml tunicamycin for 36 h. Equivalent amount of DMSO was added to control cells.

Alternatively, to remove the Ror2 N-linked carbohydrates, immunoprecipitated Ror2-Flag was incubated in the presence of EndoH endoglycosidase according to the manufacturer's instructions.

Separation of detergent-insoluble membrane domains. Separation of detergent-insoluble membrane domains was performed as described before [14]. Briefly, C2C12-BRIb cells were lysed with TNE-CHAPS buffer and homogenized. The lysate was adjusted to an OptiPrep concentration of 40%, and a discontinuous OptiPrep gradient from 30% to 5% was formed above the lysate. Following ultracentrifugation, the gradient was fractionated on ice from top to bottom by removing 12 fractions of 1 ml each. Fractions were analyzed using SDS–PAGE and Western blot as described.

Results

Ror2 is selectively associated with and transphosphorylated by BRIb

As we have demonstrated earlier, Ror2 and BRIb interact in a ligand-independent manner, and Ror2 does not associate with either BRIa or BRII [17]. To further investigate the specificity of this interaction, we performed an in vitro kinase assay with the related BMP receptors BRIa and BRII. COS7 cells were co-transfected with Ror2 and BRIa, BRIb, or BRII in both its alternative splice variants (SF or LF). Ror2 was immunoprecipitated with anti-Flag antibody, while the BMP receptors were precipitated with anti-HA antibody. The precipitates were subjected to an in vitro kinase assay as depicted in Fig. 1A. Specific autophosphorylation of Ror2 with an expected molecular mass of approximately 120 kDa was observed in the absence of exogenous stimuli (lane 2). We could confirm earlier observations that Ror2 is not only associated with BRIb, but also serves as a phosphorylation substrate for BRIb (lane 3). In contrast to BRIb, BRIa did not undergo autophosphorylation (lane 6). In addition, there is no difference in the Ror2 phosphorylation state upon co-expression and co-immunoprecipitation with BRIa (lane 5). BRII LF possesses a strong autophosphorylation capacity (lane 8). In comparison, BRII SF which lacks the tail domain shows a reduction in autophosphorylation capacity (lane 10). This suggests that the BRII tail has a regulatory effect on the kinase activity of BRII or includes additional phosphorylation sites. Co-expression of Ror2 with either BRII LF (lane 7) or SF (lane 9) did not alter the phosphorylation state of Ror2. These data support previous findings that BRII does not interact with Ror2. Interestingly, we could also demonstrate strong ligand-independent interaction of BRIb with endogenous BRII (lane 4), reflecting the pre-formed complex (PFC) [19].

In Fig. 1B we show that phosphorylation of Ror2 in the presence of BRIb is independent of Ror2 kinase activity (lane 7). However, it does require BRIb kinase activity (compare lanes 6 and 7), further supporting the notion that Ror2 is transphosphorylated by BRIb.

Taken together, these observations indicate that Ror2 is selectively associated with and transphosphorylated by BRIb.

Formation of the Ror2/BRIb complex does not depend on N-linked carbohydrates

The apparent molecular mass of Ror2 was around 120 kDa, consistent with earlier observations. This suggested post-translational protein modifications since the deduced molecular weight is 102 kDa [1]. To assess whether this is due to *N*-glycosylation, COS7 cells transiently expressing Ror2 were cultured in the presence of tunicamycin. As outlined in Fig. 2A (lane 2), the detected molecular weight of deglycosylated Ror2 is approximately 105 kDa. In addition, we immunoprecipitated Ror2 and performed treatment with EndoH to remove N-linked carbohydrate moieties. This also resulted in a molecular weight reduction of Ror2 (lane 5).

There are three potential *N*-glycosylation sites in Ror2 located at N80, N188, and N308, whereas N188 lies within the CRD domain. To assess whether N-linked oligosaccharides are required for the interaction of Ror2 with BRIb, COS7 cells co-expressing Ror2 and BRIb were cultured in the presence of tunicamycin. As seen in Fig. 2B, a faster migrating and less diffused form of Ror2 was detected in the Western blot anti-Flag (middle panel, lanes 3 and 5), which is consistent with the loss of N-linked oligosaccharides. Phosphorylated BRIb was present in Ror2 immunoprecipitates regardless of treatment with tunicamycin (lanes 4 and 5). Hence the interaction of Ror2 with BRIb is not mediated via N-linked oligosaccharide chains within the CRD region, and also does not affect transphosphorylation of Ror2 by BRIb. Additionally, no effect of tunicamycin was observed on BRIb, consistent with observations that the receptor is not modified by *N*-glycosylation.

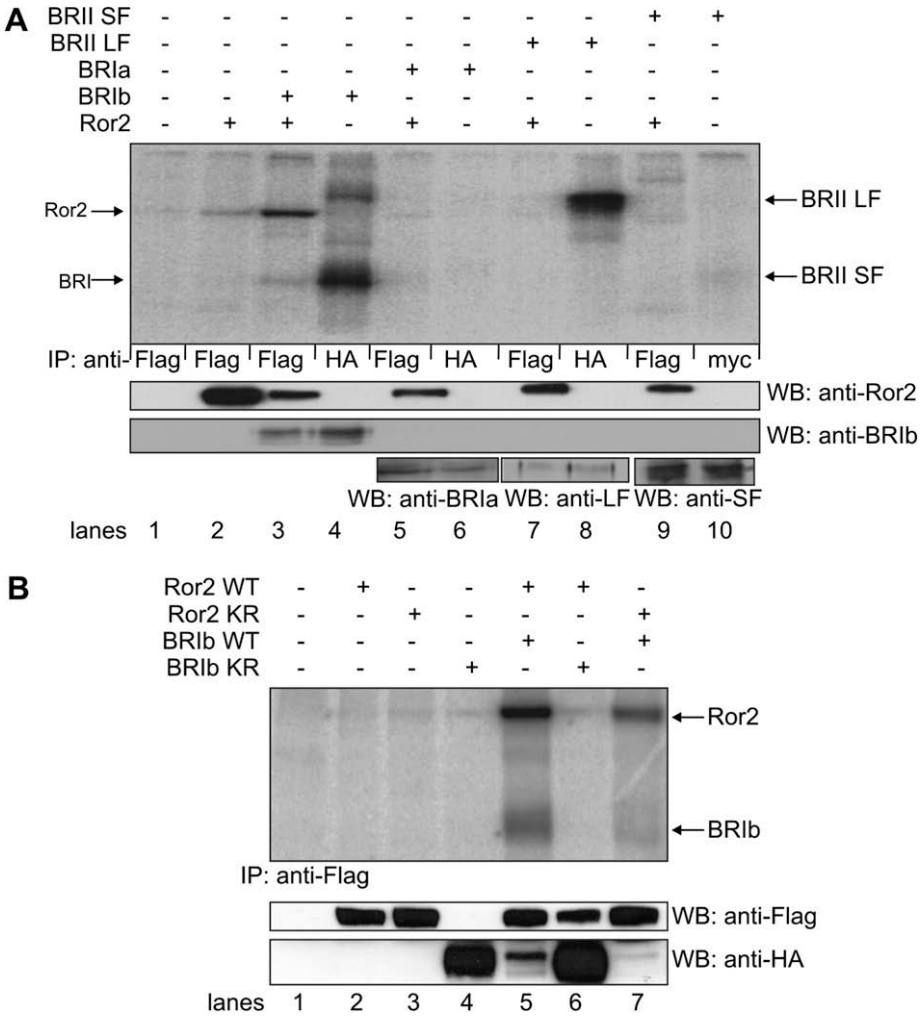

Fig. 1. Selective association of Ror2 with BRIb and transphosphorylation by BRIb. COS7 cells transiently expressing the indicated constructs were lysed and subjected to immunoprecipitation. The precipitates were subjected to an in vitro kinase assay and phosphorylated proteins were visualized by exposure to a phosphorimager screen. The expression level of the receptors is documented in the lower panels as revealed by Western blotting with the corresponding antibodies. (A) Ror2 (lanes 2, 3, 5, 7, and 9) was immunoprecipitated with anti-Flag antibody. BRIb, BRII LF, BRIa were immunoprecipitated by anti-HA (lanes 4, 6, and 8) and BRII SF by anti-myc antibody (lane 10). (B) All probes were immunoprecipitated with anti-Flag antibody.

The receptor complex forms independent of disulfide bonds

We studied the effect of DTT on the stability of the Ror2/BRIb complex. COS7 cells expressing both Ror2 and BRIb were solubilized with lysis buffer supplemented with or without 1 mM DTT (Fig. 3A, lanes 1–4 or 5–7, respectively). Ror2 was immunoprecipitated and subjected to in vitro kinase assay. As shown in Fig. 3A, BRIb is co-immunoprecipitated with Ror2 consistently in the presence and absence of DTT (compare lanes 3 and 6) and transphosphorylates Ror2 equally. These observations suggest that the receptors are not covalently linked via disulfide bonds.

High ionic conditions do not disrupt the complex

To further evaluate the stability of the Ror2/BRIb complex, we investigated the effect of increasing NaCl concentrations. Concentrations up to 1 M NaCl had no effect on the Ror2/BRIb complex (Fig. 3B, lanes 2–4). Subsequently, we tested the effect of SDS. Cells were lysed and Ror2 was immunoprecipitated with lysis buffer containing either 0.3% or 0.5% SDS (lanes 5 and 6, respectively) or RIPA buffer containing 0.1% SDS (lane 7). Concentrations of 0.3% and 0.5% SDS totally abolished the association of Ror2 with BRIb, as indicated by the extent of transphosphorylated Ror2 and the absence of phosphorylated BRIb (lanes 5 and 6). The complex is partially sensitive to RIPA conditions, due to the presence of 0.1% SDS (lane 7).

Neither high salt nor SDS affected the binding capacity of the anti-Flag antibody used for immunoprecipitation. Comparable levels of Ror2 were detected in all immunoprecipitates (data not shown), confirming the specificity of the SDS effect on disrupting the Ror2/BRIb complex.

Ror2 and BRIb co-fractionate in DRMs

These results indicate that the Ror2/BRIb complex is resistant to high ionic strength, but sensitive to SDS. As we have shown previously, BRI receptors reside primarily in DRMs, while BRII is also present in non-DRM membrane regions [14]. Since the Ror2/BRIb complex was sensitive to SDS, we investigated whether Ror2 was present in Caveolin-1α-positive microdomains, which are resistant to the detergents CHAPS, Triton or NP-40. C2C12 cells stably expressing the BRIb receptor were transiently transfected with Ror2 and membrane fractions were separated on an OptiPrep gradient. As expected, BRIb was found in Caveolin-1α-positive DRM fractions (Fig. 3C, lanes 7–10). Ror2 appeared in all protein-containing fractions (lanes 7–14), including the DRM fractions where

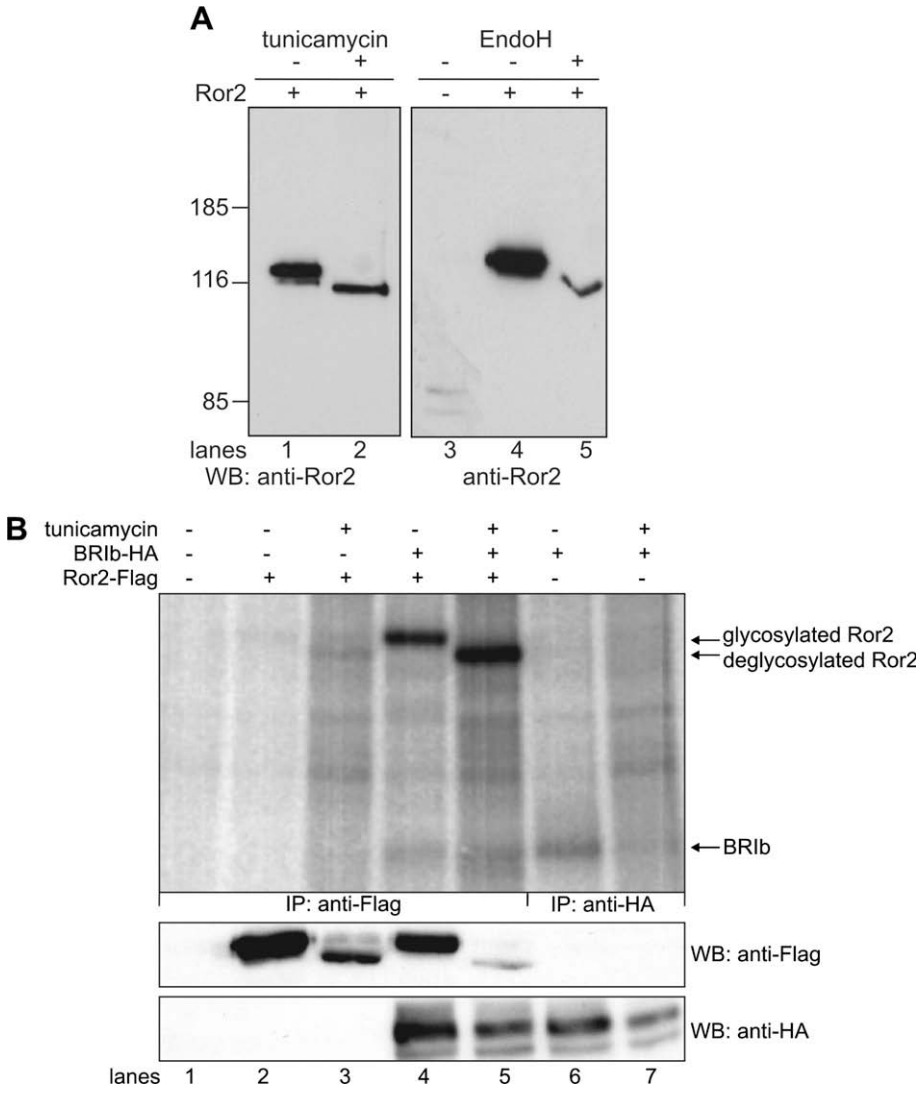

Fig. 2. Characterization of Ror2 glycosylation and its effect on association with BRIb. (A) COS7 cells transiently expressing Ror2-Flag were cultured in the presence of tunicamycin (lane 2) or DMSO as a control (lane 1). For endoglycosidase treatment Ror2 was immunoprecipitated by anti-Flag antibody and then subjected to enzymatic digestion by EndoH (lanes 3 and 4). As a control, cells transfected with empty vector were subjected to an IP anti-Flag (lane 3). The molecular mass of digested and untreated Ror2 was detected by Western blot using anti-Ror2 antibodies. (B) COS7 cells transiently transfected with Ror2-Flag and BRIb-HA constructs were cultured in the presence of tunicamycin or DMSO as a control. Ror2 was immunoprecipitated with anti-Flag antibody (lanes 1–5) and subjected to an in vitro kinase assay. The efficiency of tunicamycin on inhibiting synthesis of the N-linked carbohydrate moieties was confirmed by anti-Flag Western blotting of the total cell lysates (lower panel).

it co-localized with BRIb. This pattern reflects the distribution also seen for BRII [14].

Discussion

Preassembly of signaling receptors prior to ligand binding was described for many cell surface receptors such as the trimeric TNF [22], Epo [23], and the BMP receptors [18]. For BMP receptors we demonstrated that the oligomerization mode determines which signaling pathway these complexes are able to activate [19].

Here we characterize a preassembled receptor complex composed of a serine/threonine kinase receptor (BRIb) and a tyrosine kinase receptor (Ror2). As we have shown before, these receptors physically and functionally interact, leading to inhibition of the Smad pathway. Additionally, we were able to map the interaction site to the extracellular CRD domain of Ror2 [17]. This domain is also termed Frizzled-like domain due to sequence similarities, which had suggested Wnt proteins as potential ligands for Ror2 [24]. Indeed, it was shown that Wnt proteins bind to the CRD domain of Ror2 [25–27]. These data imply that the potential ligand binding site of Ror2 could be occupied by BRIb in a preassembled complex [17].

In this study, we further characterize the Ror2/BRIb complex and confirm that the association and phosphorylation of Ror2 is specific for BRIb (Fig. 1A). Furthermore, we can exclude that the strong interaction of BRIb with Ror2 augments Ror2 autokinase activity, since kinase active BRIb is required to phosphorylate Ror2 (Fig. 1B). These data provide additional evidence for a direct transphosphorylation of Ror2 by BRIb.

We could confirm data from Chen et al. and show that Ror2 is a highly glycosylated receptor [28]. Expectedly, both tunicamycin treatment and EndoH digestion led to a reduced molecular weight of Ror2 (Fig. 2A). As mentioned before, Ror2 carries three potential *N*-glycosylation sites. One site (N188) is located in its CRD domain. Although this domain serves as interaction site for BRIb deglycosylation of Ror2 did not abrogate the interaction. Levels of transphosphorylation appeared even stronger than compared with the native receptor (Fig. 2B, compare lanes 4 and 5), which could be due to reduced interaction of Ror2 with potential ligands, as discussed above.

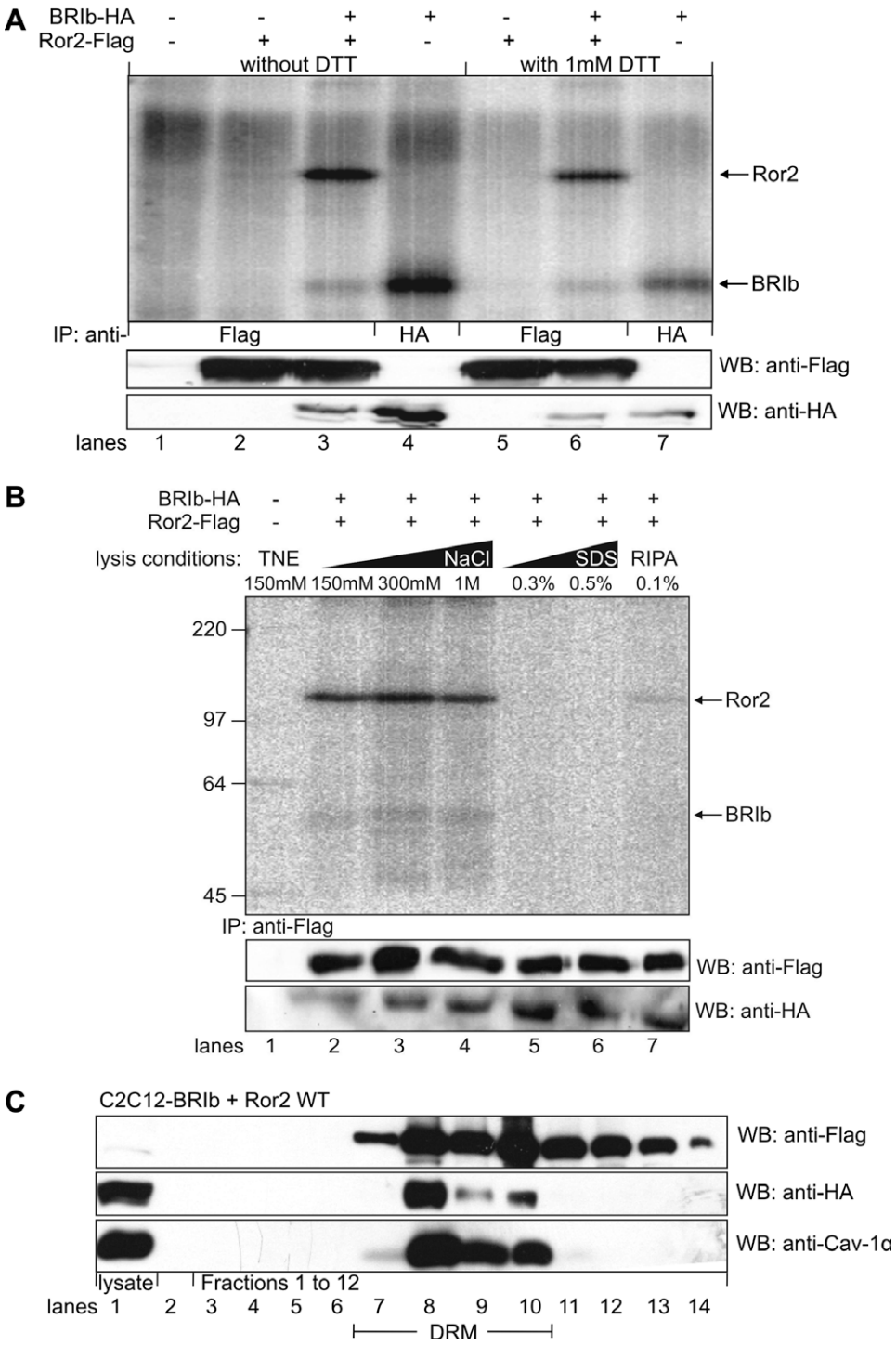

Fig. 3. Characterization of Ror2–BRIb complex stability and membrane localization. (A) COS7 cells were transiently transfected and lysed in buffer supplemented with or without DTT. Ror2 was immunoprecipitated with anti-Flag antibody and subjected to an in vitro kinase assay. The immunoprecipitated proteins were separated on SDS–PAGE. Phosphorylated Ror2 and co-immunoprecipitated phospho-BRIb were visualized with a phosphorimager. The expression of Ror2-Flag and BRIb-HA was confirmed by Western blotting with the respective antibodies as documented in the lower panels. (B) COS7 cells were co-transfected with Ror2 and BRIb. Two days after transfection, cells were lysed in buffer containing 150 mM NaCl (lanes 1 and 2), 300 mM (lane 3), and 1 M NaCl (lane 4) or with lysis buffer containing 0.3% SDS (lane 5), 0.5% SDS (lane 6) or solubilized in RIPA buffer containing 0.1% SDS (lane 7) . (C) C2C12 cells stably expressing BRIb were transiently transfected with Ror2 and lysed in the presence of the detergent CHAPS. After homogenization the lysate was subjected to an OptiPrep gradient fractionation and analyzed in a Western blot using the indicated antibodies.

Finally, we analyzed the stability of the receptor complex under different lysis conditions. While the complex remained stable after treatment with DTT (Fig. 3A, compare lanes 3 and 6) and under high ionic strength (Fig. 3B, lanes 2–4), it appeared sensitive to SDS (Fig. 3B, lanes 5–7). SDS is a strong detergent that dissolves otherwise detergent-resistant microdomains (DRMs). DRMs typically withstand treatment with milder detergents such as Triton or CHAPS. These data suggest that Ror2 and BRIb form a complex within DRMs. To support this notion we show co-fractionation of BRIb and Ror2 in Caveolin-1α-positive DRM fractions (Fig. 3C). The distribution of Ror2 depicted in Fig. 3C is not altered in the absence of BRIb. Also the extracellular truncation of Ror2 lacking the

CRD domain exhibits the same membrane distribution as the wild type (not shown), although it can no longer interact with BRIb [17]. Thus the ability of Ror2 to recruit to DRMs appears to be independent of its association with BRIb, which by itself is a major resident in these plasma membrane regions [14].

In previous studies we could show that Smad-dependent signaling requires internalization of the BMP receptor complex via CCPs [14]. Recent data suggest that BRII is localized to DRMs through its interaction with Caveolin-1 [15], where it may form a pre-formed complex (PFC) with BRI [19]. This complex is required for the initiation of Smad-dependent signaling. Ror2 on the other hand inhibits Smad-dependent signaling through its interaction with BRIb [17]. We speculate that Ror2 competes with BRII for binding of BRIb or replaces BRII in PFCs, thus inhibiting downstream Smad signaling.

Taken together, we show that the interaction of Ror2 with BRIb is specific and independent of post-translational *N*-glycosylation. Furthermore, the receptor complex appears very stable under high ionic strength, but is disrupted by low concentrations of SDS, and we provide evidence that the complex forms within DRMs. Therefore, Ror2 serves as a BMP co-receptor negatively modulating the BMP/Smad pathway by specific interaction at the site where this signaling pathway is initiated.

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