Trapping of BMP receptors in distinct membrane domains inhibits their function in pulmonary arterial hypertension

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Bone morphogenetic proteins (BMPs) are secreted signaling molecules that belong to the transforming growth factor-β (TGF-β) superfamily. BMPs play an important role during embryonic development in chondrogenesis, osteogenesis, and hematopoiesis (2, 7, 12). Three type I and three type II receptors, all of which are serine/threonine kinases, have been identified. Among the three type II receptors, BMPR2, ActR-II, and ActR-IIB, BMPR2 is specific for BMPs, whereas ActR-II and ActR-IIB are shared by activins, myostatin, and BMPs. BMP receptors exist on the cell membrane as preformed hetero- or homooligomeric complexes. To activate the signaling pathways, BMPs bind to the receptors within distinct oligomeric structures (22). After ligand binding to at least one, usually two, type I and two type II receptors, the type II receptor phosphorylates the type I receptor at the GS-box. The phosphorylated type I receptor then phosphorylates Smad1, Smad5, or Smad8. They in turn bind to Smad4, a common mediator, leading to nuclear translocation and regulation of specific genes (13, 15, 19, 23). This is the most-studied BMP signaling pathway. Previous studies also indicate that BMP receptor distributions on the cell surface are highly flexible and dynamic. The change in their state of aggregation and their interaction with cell domains, such as caveolae, which are enriched with the protein caveolin, appear to be important for their signaling (24–26). The endocytosis of transmembrane receptors by clathrin-dependent and -independent pathways plays a major role in control of the receptor density on the cell surface (9, 10). A recent study demonstrated that BMP receptor type II (BMPR2) is associated with clathrin-coated pits (CCPs) and caveolar-like structures (11). The CCPs are demonstrable by interfering with the relocalization on the plasma surface. Therefore, new treatment options for PAH should also target receptor localization, rather than just expression level.

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Bone morphogenetic protein receptors; mutants; colocalization; Smad signaling

Bone morphogenetic proteins (BMPs) are pleiotrophic growth factors that influence diverse processes such as skeletal development, hematopoiesis, and neurogenesis. They play crucial roles in diseases such as pulmonary arterial hypertension (PAH). In PAH, mutants of the BMP type II receptors (BMPR2) were detected, and their functions were impaired during BMP signaling. It is thought that expression levels of these receptors determine the fate of BMP signaling, with low levels of expression leading to decreased Smad activation in PAH. However, our studies demonstrate, for the first time, that the localization of receptors on the plasma membrane, in this case BMPR2, was misdirected. Three BMPR2 mutants, D485G, N519K, and R899X, which are known to be involved in PAH, were chosen as our model system. Our results show that all three BMPR2 mutants decreased BMP-dependent Smad phosphorylation and Smad signal morphology. As the three mutants reached the cell membrane and their expression was lower than that of BMPR2, they formed smaller clusters and associated differently with membrane domains, such as caveolae and clathrin-coated pits. The disruption of these domains restored the Smad signaling of D485G and N519K to the level of wild-type BMPR2, showing that these mutants were trapped in the domains, rather than just expressed at a lower level on the surface. Therefore, new treatment options for PAH should also target receptor localization, rather than just expression level.

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distribution on the cell membrane of BMPR2 and the three mutants differ significantly on human pulmonary artery smooth muscle cells (hPASMCs) and C2C12 cells. Their response is also different when cells are treated with an Eps15 (epidermal growth factor receptor pathway) subtype clone 15, a newly identified component of CCPs that is required for receptor-mediated endocytosis (CPZ), to disrupt the CCPs or caveolin-1 small interfering RNA (siCav-1) and nystatin to disrupt caveolae. Our findings suggest that the dispersion and, probably, mislocalization of the mutant receptors contribute to the different BMP signaling observed in PAH. New therapeutics that can rescue the clustering and redistribute the receptors may provide potential treatments for PAH.

**MATERIALS AND METHODS**

**Cell culture.** C2C12 cells (CRL-1772, American Type Culture Collection, Manassas, VA) were grown in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) at 37°C with 5% CO2. Normal hPASMCs (PromoCell, Heidelberg, Germany) were cultured in smooth muscle cell growth medium (PromoCell) supplemented with hPASMCs (PromoCell, Heidelberg, Germany) were cultured in normal human artery medium (PromoCell). A 20 mM BMP2 (Biosource, Camarillo, CA) or not stimulated. The cells were stimulated with 20 nM BMP2 for 1 h or not stimulated. To disrupt the CCPs and caveolae, 50 µM CPZ or 20 µl/ml nystatin was added to the cells during the 1-h period of stimulation or nonstimulation. Cells were lysed by 1 h by the lysis buffer (10 mM Tris, 1 mM EDTA, 1% Triton, pH 7.4, and protease inhibitor cocktail). A normalized equal amount of cell lysate was injected into 10% SDS-polyacrylamide gel to separate the proteins. After transfer and blocking with 25 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20, and 4% BSA at room temperature for 1 h, the blot was incubated with anti-phosphorylated Smad1/5/8 [phosphorylated (Ser426/428) Smad1/5/8 antibody (Santa Cruz Biotechnology)] and anti-AP-2-phycoerythrin (clone 2297, BD Biosciences) for 1 h at room temperature. The horseradish peroxidase-conjugated secondary antibodies (1:1,000 dilution; Santa Cruz Biotechnology) also were incubated for 1 h at room temperature. The colorimetric 3,3'-tetramethylbenzidine substrate (Promega) was used to detect the adsorbed antibodies. Image software (http://rsb.info.nih.gov/ij/image/) was used to normalize band intensity.

**Confocal microscopy.** Images were collected using an LSM 510 confocal microscope equipped with an inverted Zeiss microscope. For fixed hPASMCs, the images were collected at room temperature; for live C2C12 cell imaging, cells were kept at 37°C in a temperature-controlled stage. Cells expressing the wild-type receptors and mutants were selected under mercury lamp illumination with a 1×20 (1.3 numerical aperture) objective. An area of the cell, away from the nucleus, was enlarged and visualized using the confocal laser-scanning feature. For excitation, the 488- and 543-nm laser lines were used. Two scans were accumulated to produce a single image. One image was collected per cell, and 30–40 images from different cells were collected per experiment. Three independent experiments (90–120 cells) were repeated to ensure the reliability of the results. All the images were collected at identical instrument settings.

**ICS and ICCS.** ICS is based on analysis of spatial intensity fluctuations in images collected on a confocal laser-scanning microscope (5, 27, 34, 37, 38). Autocorrelation functions, \( g(\xi, \eta) \), were calculated as described elsewhere (5, 27, 34, 37, 38) and fit to a two-dimensional Gaussian function

\[
g(\xi, \eta) = g(0, 0)e^{(\xi^2 + \eta^2)R^2} + g_0
\]

where \( \xi \) and \( \eta \) are the position lag coordinates (for the x- and y-axes, respectively) of the autocorrelation function, \( R \) is the e\(^{-2}\) radius of the laser beam, and \( g(0, 0) \) is the amplitude of the autocorrelation function upon extrapolation of \( \xi \) and \( \eta \) to zero; the offset, \( g_0 \), is introduced to account for the finite sample of the images, which can result in a decay of \( g(\xi, \eta) \) to a nonzero level at large lag coordinates. The zero-lag amplitude of the autocorrelation function, \( g(0, 0) \), has been shown to be inversely proportional to the number of independently distributed particles in the observation area

\[
g(0, 0) = \frac{1}{N_p}
\]

A cluster density (CD) value, which is defined as the average number of independent fluorescent particles (clusters) per square micrometer of cell membrane, can be calculated as follows
The average intensity of an image, \( <i(x,y)> \), is proportional to the average number of fluorescent molecules in the area illuminated by the laser beam. Thus the degree of aggregation (DA), which is defined as the average number of molecules in the protein aggregate and is obtained by dividing the average total number of protein monomers, \( N_m \), by the average number of independent protein particles, \( N_p \), can be calculated

\[
DA = \frac{<i(x,y)>}{g(0,0)} \equiv \frac{N_m}{N_p} \tag{4}
\]

The constant \( c \) accounts for instrumental and experimental parameters (extinction coefficients, quantum yields and efficiency of collection of the confocal microscope). These parameters are constant for a given set of experimental conditions.

ICCS is an extension of ICS, where a cross-correlation function is generated by calculation of the spatially coincident fluorescence intensity fluctuation in images collected from two different chromophores that are attached to two different protein types (5, 27, 34, 37, 38). One protein type is labeled with a green probe and imaged to give the intensity map, \( i_g(x,y) \); the second protein type is labeled with a red probe and imaged to give the intensity map \( i_r(x,y) \). The normalized spatial cross-correlation function can be calculated as

\[
g_{gr}(\zeta, \eta) = \frac{<i_g(x,y) i_r(x + \zeta, y + \eta)>}{g_{gr}(0,0)} \tag{5}
\]

The resulting cross-correlation function is fit to a two-dimensional Gaussian function as described above to give the amplitude of the cross-correlation function, \( g_{gr}(0,0) \).

Thus we can define the CD of colocalized chromophores as

\[
CD_{gr} = \frac{g_{gr}(0,0)}{\left[g_{gr}(0,0) g_r(0,0) c \sigma^2\right]} \tag{6}
\]

The fraction of each protein type associated with the other type can be calculated by examining the ratio of CD values

\[
F(g/r) = CD_{gr}/CD_g \text{ or } F(r/g) = CD_{gr}/CD_r \tag{7}
\]

where \( F(g/r) \) represents the fraction of green-labeled protein clusters that contain red-labeled proteins and, correspondingly, \( F(r/g) \) represents the fraction of red-labeled protein clusters that contain green-labeled proteins. ICCS provides the quantitative information about the extent of colocalization of two different proteins.

The ICS and ICCS analysis was performed in the ImageJ program using available software from National Institutes of Health Image.

RESULTS

Overexpression of BMP receptor mutants (D485G, N519K, R899X) led to decreased BMP-dependent Smad phosphorylation and Smad signaling activity. Three mutants, with site mutations in the kinase domain (D485G) and in the cytoplasmic tail (N519K) and truncating mutation in the cytoplasmic tail (R899X), were identified to localize to the plasma membrane of hPASMCs; however, Smad phosphorylation and Smad signaling are impaired in response to BMP2. Figure 1 illustrates the structure and mutation sites of the BMPR2 and the three mutants of interest. In this study, C2C12 cells were transfected with plasmids encoding BMPR2 and the mutants in combination with or without pSBE and pRL-Luc (normalization) for luciferase gene reporter assay and Western blot analysis.

The normalized band intensities of phosphorylated Smad1/5/8 relative to total Smad1/5/8 illustrate that all three mutants decreased the BMP-dependent Smad phosphorylation (see Supplemental Fig. S1A in Supplemental Material for this article, available at the Journal website). Typical Western blot images of phosphorylated and total Smad1/5/8 from one of three independent experiments are shown in Supplemental Fig. S1B. The pSBE is known to be a readout for the activation of the Smad pathway (36). The pSBE readout for Smad activation is shown in Supplemental Fig. S1C (mock data) by luciferase reporter gene assay. The luciferase reporter assay results in Supplemental Fig. S1C also show the response to BMP2 and its activation of the pSBE reporter, as well as the effect of mutants on the downstream signaling. D485G, N519K, and R899X reduced Smad signaling by 90%, 70%, and 65% (P < 0.05 in each case), respectively, relative to the wild-type receptor after subtraction of the endogenous BMP-dependent Smad signal. The mutant in the kinase domain, D485G, almost completely inhibited the Smad signaling activity.

Decrease in clustering and expression of BMP receptor mutants compared with BMPR2 on the cell surface. hPASMCs were transfected with plasmids encoding BMPR2 or the BMPR2 mutants. At 24 h after transfection, confocal microscopic images were collected. As shown by the individual fluorescence spots in Fig. 2, A–D, all the receptors reach the cell membrane. However, the pattern of expression is quite different between wild-type BMPR2 and the mutants. In Fig. 2, A–D, typical confocal microscopic images at high magnification show the protein distribution on a selected region of a single cell for BMPR2, D485G, N519K, and R899X. BMPR2 is seen in particles on the surface as very bright fluorescence spots; the mutants are seen as fluorescence spots that appear much less bright. The typical autocorrelation functions of the images in Fig. 2, A–D, are shown in Fig. 2, E–H. For wild-type BMPR2, the autocorrelation function amplitude value is ~0.3, while those of the mutants are ~0.03, 10-fold less, in these examples. In ICS analysis, a smaller amplitude of the autocorrelation function corresponds to a greater average number of clusters and, therefore, a greater dispersion.

Figure 3A shows the average intensity of the fluorescence signal for BMPR2 and the three mutants. The wild-type receptor is expressed at the highest level on the hPASMC surface, while the mutant receptors are expressed at less than half this level, even though the same amount of plasmid was transfected into the cells and the same experimental conditions were set during the microscopic observation and ICS analysis.
A total of 90–120 confocal microscopic images for each of the wild-type receptors and the mutants from three independent experiments were collected and analyzed by ICS. Two parameters that describe the distribution of receptors on the cell surface (CD), the average number of clusters per unit area, and DA, the average numbers of monomers in one cluster, were obtained from this analysis.
each mutant receptor with and without BMP2 stimulation is the same. The average CD and DA values with and without BMP2 stimulation for these cells shown in Fig. 3, E and F, suggest results similar to those shown in Fig. 3, A–C, for the complete data set. At the same expression level, wild-type BMPR2 still formed larger clusters on the cell surface than all three mutants. Stimulation by BMP2 led to significant increases in aggregation, but the mutants were always more dispersed than unstimulated wild-type receptors. Additionally, aggregation of BMPR2 and mutants on live C2C12 cells at 37°C is similar to that on hPASMCs (see Supplemental Fig. S3).

Altered colocalization of BMPR2 mutants with membrane domains compared with BMPR2. Cell membrane domains, like caveolae and CCPs, play important roles in many cell functions. We have investigated the distributions of wild-type BMPR2 and the three mutants in these different cell domains.
We transfected the BMPR2 and mutant plasmids fused with GFP into the hPASMCs, labeled the cells with antibodies against caveolin-1α/β, caveolin-1α, and AP-2, and determined their colocalization using ICCS. Figure 4, A–C, shows the overlay fluorescence images of wild-type BMPR2 with caveolin-1α/β, caveolin-1α, and AP-2, and Fig. 4, D–F, shows the fraction of colocalization of wild-type receptors and mutants with caveolin-1α/β, caveolin-1α, and AP-2 obtained using ICCS.

The results in Fig. 4 show that the wild-type receptor and mutants colocalized to some extent with the cell domains enriched with caveolin-1α and -1β and in CCPs in the resting cells. Upon BMP2 stimulation, there was no significant change in the amount of the wild-type receptor colocalized with caveolin-1α/β, while that colocalized with caveolin-1α appears to increase slightly, indicating that some wild-type BMPR2 may have shifted from the region of caveolae enriched with caveolin-1β to the region enriched with caveolin-1α, as previously observed (24, 26). For the three mutants, colocalization with caveolin-1α was always equal to or greater than that with caveolin-1α/β, suggesting that none of the mutants were associated with caveolae enriched with caveolin-1β. Correspondingly, the BMP2 stimulation did not lead to a shift from caveolin-1β to -1α. Similarly, compared with the wild-type receptor, twice the amount of mutants colocalized with AP-2 in resting cells, and BMP2 stimulation did not change the distributions of wild-type receptors or mutants with AP-2. Because antibodies to caveolin-1α/β and -1α bind to some of the same domains, the fractions need not add to 1. The total expression level of wild-type BMPR2 and mutants shows no correlation with the relative association with cell domains (see Supplemental Fig. S4).

Site mutation mutants were trapped in cell domains. To investigate the potential mechanism whereby the mutants inhibit the BMP2-dependent Smad phosphorylation and Smad signaling, we used CPZ (28, 36) and a mutant of Eps15, EH29 (1), to disrupt the CCPs and nystatin (28, 29) and siCav-1 to disrupt the caveolae. Then the BMP-dependent Smad phosphorylation and Smad signaling activity were detected by Western blot and luciferase gene reporter assays before and after disruption of CCPs and caveolae.

Figure 5A shows Western blot band images of phosphorylated and total Smad1/5/8 after the CCPs were disrupted by CPZ. The C2C12 cells were transfected with wild-type BMPR2 or mutants for 17 h, and CPZ was added to the medium to disrupt CCPs with or without BMP2 stimulation for 1 h. The normalized band intensities with or without disrupted CCPs are shown in Fig. 5B. After disruption of CCPs, Smad1/5/8

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Fig. 4. Mutants decreased colocalization with caveolae composed of caveolin-1α/β (P < 0.05) but increased colocalization in caveolae composed of caveolin-1α (P < 0.05) and clathrin-coated pits (CCPs; P < 0.05) on hPASMC membrane. A–C: overlay images of wild-type BMPR2-GFP (green stain) with caveolin-1α/β, caveolin-1α, and AP-2 (2 μm, red stain). Scale bars 2 μm. D–F: localization of wild-type BMPR2 and 3 mutants with caveolin-1α/β, caveolin-1α, and AP-2 with and without BMP2 stimulation. Wild-type BMPR2 and mutants localized in cell domains enriched with caveolin-1α and -1β. With BMP2 stimulation, some wild-type BMPR2 shifted from the region enriched with caveolin-1β to the region enriched with caveolin-1α. More mutants colocalized with caveolin-1α/β (P < 0.05) and caveolin-1α (P < 0.05) with BMP2 stimulation. There is no evidence that they shifted from caveolin-1β to -1α. With AP-2, colocalization was 2-fold greater in mutants (P < 0.05) than in the wild-type receptor, and BMP2 stimulation did not change colocalization of the wild-type receptor and mutants. *Significantly different from wild-type BMPR2 (−BMP2). †Significantly different from wild-type BMPR2 (+BMP2). ‡Significantly different from −BMP2 within group. P < 0.05 was considered statistically significant as determined by ANOVA followed by Tukey’s test.
5/8 phosphorylation of wild-type BMPR2 and R899X decreased, whereas that of D485G remained the same. Smad1/5/8 phosphorylation of N519K appeared to increase slightly.

In the luciferase gene reporter assays (Fig. 5, C and D), the C2C12 cells were transfected with pSBE, pRL-Luc, and wild-type BMPR2 or mutants, and the cells were stimulated with BMP2 for 18 h or left unstimulated. To disrupt the CCPs, CPZ was added to the cells for 18 h, with or without transfection with a mutant of Eps15, EH29 plasmid, at the time of the receptor transfection. Similar results were obtained for Smad signaling activity after the CCPs were disrupted by CPZ (Fig. 5C) and EH29 plasmid (Fig. 5D). Similar to Smad phosphorylation, Smad signaling activity of the wild-type receptor also decreased, whereas the signaling activity of N519K was restored to a great degree. This result suggests that N519K may be trapped in the CCPs, so that when EH29 and CPZ disrupted the CCPs, the N519K mutant was released, and its signaling activity was restored.

Similar Western blot and luciferase gene reporter assay experiments were carried out with or without disruption of caveolae by nystatin and siCav-1 in C2C12 cells. The Western blot band images of phosphorylated and total Smad1/5/8 after the caveolae were disrupted by nystatin are shown in Fig. 6A. The normalized phosphorylated Smad1/5/8 band intensities with and without nystatin-disrupted caveolae (Fig. 6B) show that phosphorylated Smad1/5/8 of the wild-type receptor decreased after disruption of caveolae, while that of the D485G mutant increased and that of the other two mutants also decreased to some extent. The effect of nystatin and siCav-1 on downstream Smad signaling is shown in Fig. 6, C and D. Similar to our results in cells treated with CPZ and EH29, the signaling activity of the wild-type receptor decreased significantly with disruption of the caveolae by nystatin and siCav-1. The signaling activity of D485G has been enhanced to a great degree in the same experimental conditions. The signaling activity of D485G recovered after disruption of the caveolae, illustrating that the kinase domain mutant D485G may be trapped in caveolae and that disruption of caveolae released the mutant and recovered the signaling activity of the mutant receptor.

**DISCUSSION**

In our Western blot (see Supplemental Fig. S1, A and B) and luciferase reporter gene (see Supplemental Fig. 1C) assays, all three overexpressed mutants inhibit BMP-dependent Smad signaling of wild-type BMPR2 but restored signaling of N519K in the presence of BMP2 in C2C12 cells. A: Western blot bands of phosphorylated Smad1/5/8 (p-Smad1/5/8) and total Smad1/5/8 of wild-type BMPR2 and mutants with (+) and without (−) BMP2 stimulation and with chlorpromazine (CPZ) disruption of CCPs. B: normalized band intensities of phosphorylated Smad1/5/8 with BMP2 stimulation with or without CPZ disruption of CCPs (+CPZ and −CPZ, respectively). Smad phosphorylation of BMPR2 and R899X decreased with disruption of CCPs, whereas Smad phosphorylation of N519K increased slightly. C: normalized downstream Smad signaling activities of the wild-type receptor and mutants with BMP2 stimulation with or without CPZ disruption of CCPs. Smad signaling of wild-type BMPR2 (P < 0.05) and N519K (P < 0.05) was significantly different from that of untreated cells. D: normalized downstream Smad signaling activity of the wild-type receptor and mutants with BMP2 stimulation with and without EH29 plasmid transfection (+EH29 and −EH29, respectively). Smad signaling of wild-type BMPR2 (P < 0.05), N519K (P < 0.05), and R899X (P < 0.05) was significantly different from that of untreated cells. With CPZ and EH29 disruption of CCPs, Smad phosphorylation and signaling of N519K increased significantly, which suggests that N519K may be trapped in CCPs. Results are averages of 3 individual experiments. aSignificantly different from wild-type BMPR2 (−CPZ/EH29). bSignificantly different from wild-type BMPR2 (+CPZ/EH29). cSignificantly different from −CPZ/EH29 within group. P < 0.05 was considered statistically significant as determined by ANOVA followed by Tukey’s test.
phosphorylation and Smad signaling. With more mutant plasmid transfection (2.0 μg), the Smad signal of the kinase domain mutant D485G was even lower than the endogenous Smad signal (mock sample, see Supplemental Fig. S5). These results show the dominant-negative effect of D485G and are consistent with previous observations that the D485G mutant lost almost all Smad signaling ability, even though it exhibited normal ligand-binding capability (18, 20).

The ICS analysis results (Figs. 2 and 3) suggest that even though they reach the cell surface, the mutant receptors do not form highly aggregated clusters on the membrane, as wild-type receptors do. After BMP2 stimulation, the mutant receptors aggregate to some extent, but they are still highly dispersed on the cell membrane compared with unstimulated and stimulated wild-type receptors. The cytoplasmic tail mutants are slightly more responsive to BMP2 stimulation, consistent with their slightly elevated Smad phosphorylation and slightly higher Smad signaling activity. The ICCS analysis results (Fig. 4) suggest different distributions of wild-type receptors and mutants among membrane domains. BMP signaling was shown to be dependent on receptor localization with distinct membrane domains. These domains include caveolae and CCPs. Caveolae exist in two different structures on the plasma membrane: they form shallow or deep invaginations. These caveolae are composed of caveolin-1α/β or -1β. Receptor signaling is enhanced by shuttling of the BMP receptors on the plasma membrane within caveolae composed of caveolin-1β to caveolae composed of caveolin-1α/β and CCPs (3, 24–26). Here, we show that the receptors not only are expressed on the cell membrane at different aggregation states, but also their colocalization with cell-specific domains was different from that of wild-type BMPR2. A small number of BMPR2 colocalize in caveolae composed of caveolin-1β only, while the mutants do not localize in these domains. Although caveolin-1β overexpression was shown to be inhibitory to BMP signaling, BMP stimulation of A431 cells led to the shuttling of BMP receptors from domains composed of the β isoform to domains enriched with the α isoform. At the same time, shuttling of BMP receptors significantly enhanced BMP signaling (24, 26).

Structurally, BMPR2 is similar to other type II receptors of the TGF-β superfamily, such as TβR-II, ActR-IIA, and ActR-IIIB. However, BMPR2 has a long cytoplasmic tail that is not found in other type II receptors in mammals. The functions of the cytoplasmic tail of BMPR2 are not clear (24). Compared with the wild-type receptor, distributions of the truncating mutation (R899X) and the single site mutation (N519K) on the cell surface are quite different, even though they are expressed at functionally high levels. They have lost the ability to form...
highly aggregated clusters. Thus the cytoplasmic tail may have a role in maintaining an aggregated distribution of the receptor in association with domains like the caveolae. The truncating mutations in the cytoplasmic tail found in some PAH patients might prevent normal function of BMPR2 in a similar manner.

The mutation in the kinase domain (D485G) also seems to prevent aggregation of the receptor on the cell surface. The effect of this mutation is functionally more severe and suggests that other factors, in addition to the state of aggregation, contribute to the almost total loss of Smad phosphorylation and Smad signaling function.

The cysteine-substituted BMPR2 mutant C118W failed to be tracked to the cell membrane and, therefore, totally lost Smad signaling activity (21, 31). Recently, it was found that C118W was mainly retained in the endoplasmic reticulum. Treatment of the cells with some chemical chaperones, such as thapsigargin, glycerol, or sodium 4-phenylbutyrate, greatly increase the membrane expression of C118W. The increased cell surface expression of C118W also enhanced BMP-dependent Smad1/5 phosphorylation (33). In this study, disruption of caveolae and CCPs decreased the Smad phosphorylation and downstream Smad signaling response to BMP2 stimulation when BMPR2 was overexpressed. Recently, disruption of cells with CPZ or EH29 was shown to activate Smad phosphorylation (3). Therefore, the decrease in activation may be due to the overexpression of BMPR2 in these cells. In contrast, the mutants D485G and N519K recovered much of their Smad signaling activities after disruption of caveolae and CCPs, respectively, suggesting that their functions were inhibited because they were trapped in these domains. However, the R899X mutant did not show restoration after the disruption, similar to the wild-type receptor, and even showed decreased Smad phosphorylation and Smad signaling activity to some degree after disruption of caveolae and CCPs. Our ICS and ICCS results show that the three mutants have similar expression and aggregation on the cell membrane, as well as similar colocalization with cell domains, but they have different responses to BMP2 stimulation: R899X is more sensitive to BMP2 stimulation than D485G and N519K.

Taken together, the results presented here provide insight into the regulation of BMP signaling on the receptor level. Loss of ability to aggregate or colocalization in caveolae of the β-isofrom prior to BMP stimulation appears to inhibit function but is probably not sufficient to completely abolish function. Trapping of the mutants in distinct cell domains, such as caveolae or CCPs, also greatly inhibits their BMP-dependent Smad phosphorylation and Smad signaling. It is possible that the BMP receptors in PAH patients also lack the ability to aggregate and localize on the cell membrane and are trapped in cell membrane domains, which contributes to inhibition or misdirection of BMP signaling. Finding means to enhance aggregation or redistribution of the BMP receptors may provide a way to slow or eliminate the progress of PAH.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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