Bone morphogenetic proteins regulate many developmental processes during embryogenesis as well as tissue homeostasis in the adult. Signaling of bone morphogenetic proteins (BMPs) is accomplished by binding to two types of serine/threonine kinase transmembrane receptors termed type I and type II. Because a large number of ligands signal through a limited number of receptors, ligand-receptor interaction in the BMP superfamily is highly promiscuous, with a ligand binding to various receptors and a receptor binding many different BMP ligands. In this study we investigate the interaction of BMP-2 with two its two high affinity type I receptors, BMP receptors IA (BMPR-IA) and BMPR-IB. Interestingly, 50% of the residues in the BMP-2 binding epitope of the BMPR-IA receptor are exchanged in BMPR-IB without a decrease in binding affinity or specificity for BMP-2. Our structural and functional analyses show that promiscuous binding of BMP-2 to both type I receptors is achieved by inherent backbone and side-chain flexibility as well as by variable hydration of the ligand-receptor interface enabling the BMP-2 surface to adapt to different receptor geometries. Despite the high degree of amino acid variability found in BMPR-IA and BMPR-IB binding equally to BMP-2, three single point missense mutations in the ectodomain of BMPR-IA cannot be tolerated. In juvenile polyposis syndrome these mutations have been shown to inactivate BMPR-IA. On the basis of our biochemical and biophysical analyses, we can show that the mutations, which are located outside the ligand binding epitope, alter the local or global fold of the receptor, thereby inactivating BMPR-IA and causing a loss of the BMP-2 tumor suppressor function in colon epithelial cells.

Bone morphogenetic proteins (BMPs) form a subgroup within the transforming growth factor-β superfamily of cytokines that are related by structure and function (1, 2). Members of the BMP subfamily play crucial roles throughout embryonic development as well as tissue and organ homeostasis in the adult (3, 4). BMP-2, a member of this superfamily, is widely known from its ectopic bone formation properties (4, 5), but it is also involved in the regulation of angiogenesis (6), gastrulation (7), neurogenesis (8), chondrogenesis (9), and apoptosis (10).

Receptor activation by the BMP ligand is accomplished by binding and oligomerization of two types of serine/threonine kinase receptors, termed type I and type II. Transphosphorylation of the intracellular receptor domains results in phosphorylation of downstream signaling molecules, e.g. SMAD proteins, which then activate gene transcription. BMP-2 binds the type I receptors BMPR-IA and -IB with high affinity and ActR-I with lower affinity (4, 11–13). BMPR-IA and -IB can both be utilized in BMP-2-mediated cellular signaling processes (11, 14). Although BMPR-IA is ubiquitously expressed and is indispensable during development (15), inactivation of BMPR-IB results in a less severe phenotype, and its expression seems spatially restricted (4). Other ligands have been identified to also bind to these two BMP type I receptors, i.e. BMP-4 (4), GDF-5 (16), BMP-6 (17), and BMP-7 (18), indicating a high degree of binding promiscuity and/or combinatorial signaling in this system (19).

These ligands should, therefore, have common structural elements that allow them to bind to the same receptor as well as unique features allowing for discriminative recognition between different cognate receptors. In GDF-5, type I receptor specificity depends on a single residue; substitution of Arg-57 for Ala results in a loss of discriminatory binding capabilities of

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The on-line version of this article (available at http://www.jbc.org) contains supplemental. Figs. S1–S5 and Tables S1–S4.

The atomic coordinates and structure factors (code 2QJB, 2QJ9, and 2QJA) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 To whom correspondence should be addressed: Dept. of Molecular Plant Physiology and Biophysics, University of Wuerzburg, Julius-von-Sachs Plzatz 2, 97082 Wuerzburg, Germany. Tel.: 49-931-8886146; Fax: 49-931-8886158; E-mail: mueller@biozentrum.uni-wuerzburg.de.

2 The abbreviations used are: BMP, bone morphogenetic protein; GDF, growth and differentiation factor; BMPR-IA, BMP type IA receptor; BMPR-IB, BMP type IB receptor; BMPR-IA/IB; BMP type IA/IB receptor chimera; BMPR-II, BMP type II receptor; ActivR-I, activin type I receptor; ActivR-IB, activin type IB receptor; ec, extracellular domain; JPS, juvenile polyposis syndrome; CFP, cyan fluorescing protein; CAGA, activin/transforming growth factor-β responsive firefly luciferase transporter p(CAGA)_14-Luc; BRE, BMP-responsive firefly luciferase transporter; Ic50, concentration required for 50% inhibition; FACS, fluorescence-activated cell sorter; SMAD, small mothers against decapentaplegic.
GDF-5 for BMPR-IB and -IA (20). In BMP-2 alanine occupies this exact position, possibly explaining the promiscuous binding to both type I receptors. The loss of type I receptor specificity of human GDF-5 R57L leads to the disease symphalangism characterized by the fusion of the interphalangeal joints (21). This finding clearly indicates that GDF-5-induced skeletal malformation can result not only from loss in receptor binding but also from loss in receptor specificity.

Several diseases have been linked to mutations occurring in the genes encoding for BMP ligands or receptors. In primary pulmonary hypertension, inactivating mutations have been observed for the BMP type II receptor (22, 23), loss or gain of function mutations in GDF-5 and BMPR-IB are linked to skeletal malformations (24, 25), and activating mutations in ActR-I probably cause fibrodysplasia ossificans progressiva (26). Recently, missense mutations in the BMPRIA gene were linked to juvenile polyposis syndrome (JPS), an autosomal dominant disease characterized by excessive colorectal polyp growth giving predisposition for colorectal cancer (27, 28). Formation of ectopic crypts in JPS has been correlated with a loss in BMP signaling. Concordantly, inactivating mutations in as well as knock-out of SMAD4 can also lead to JPS (27, 29) due to a similar loss in the regulation of epithelial cell proliferation and apoptosis. Thus, BMP-2, via signaling through BMPR-IA, exhibits a tumor suppressor function in epithelial cells (30). Whereas the loss of BMP signaling in JPS can easily be explained for nonsense mutations in BMPR-IA, the mechanism of inactivation of the known missense mutations in the extracellular BMP-2 binding domain of BMPR-IA is not understood due to their location outside the ligand binding epitope.

In this study we present a structural and functional study of how ligand–receptor promiscuity for the BMP-2 type I receptor interaction is encoded on a molecular level. Structural analysis of a complex of BMP-2 bound to a BMPR-IB mimic indicates that limited specificity for type I receptor recognition is due to structural plasticity in the receptor as well as in the ligand binding epitope. In vitro and cell-based analyses of BMPR-IA variants with missense mutations in the extracellular part leading to JPS reveal that these mutations result in a loss of structural integrity despite the structural plasticity/flexibility of BMPR-IA found in our comparison of different BMP-2 type I receptor complexes.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**—The ectodomains of BMPR-IA and variants thereof were expressed as thioredoxin fusion proteins and are purified as described (31). Protein homogeneity was analyzed by high performance liquid chromatography, SDS-PAGE, and mass spectrometry. For preparation of ligand-receptor complexes, BMP-2 was dissolved in water, and a 2.1-fold excess of the receptor protein in the same volume of 20 mM Hepes, pH 7.4, 1.4 mM NaCl was added. The protein complex was concentrated and applied to gel filtration for the removal of excess of receptor protein.

**Crystallization and Structure Determination**—For crystallization, protein concentration of the ligand-receptor complexes was adjusted to 8 mg ml⁻¹. Crystals of the various complexes grew from 0.7 to 0.9 M potassium/sodium phosphate, pH 7.8 to 8.2, 25% ethylene glycol and appeared after 5 days. Datasets were acquired at the beamline PX14.1 at BESSY (Berlin) or on a home source (Rigaku MicroMax007 with VariMax optics) at 100 K. Data were processed with the software CrystalClear (Rigaku). Complex structures were determined employing molecular replacement using the software Phaser and the structure of BMP-2-BMPR-IA (PDB code 1REW) as template. Structures were refined with Refmac Version 5.02, and manual rebuilding was performed using the XBuild module of the software package Quanta2006 (Accelrys). Data collection and refinement statistics are outlined in supplemental Table 4.

**Circular Dichroism Spectroscopy**—Circular dichroism spectra were acquired on a Jasco J-715 spectrophotometer. Spectra were recorded from 185 to 240 nm using 30 scans, a scanning speed of 200 nm min⁻¹, and a 1-nm bandwidth. Receptor proteins were dissolved in 10 mM potassium phosphate, pH 7.2, and the protein concentration was adjusted to 0.1 mg ml⁻¹. The mean residue ellipticity [θ] was determined from the measured ellipticity θ₁₉₀ by application of [θ] = θ₁₉₀/100/(c × d × Nₐ), with c being the molar concentration, d being the path length, and Nₐ being the number of amino acids in the protein. Thermal denaturation data were acquired using a Jasco J-810 spectrometer. Proteins were heated from 20 to 96 °C at a rate of 2 °C min⁻¹. Scans from 185 to 240 nm were acquired at 5 °C temperature increments. After heating to 96 °C, the protein was cooled down to 20 °C at a rate of ~16 °C/min, and a spectrum was measured to test for refolding. The melting temperature (T_m) was determined from the wavelength showing a maximum difference between the folded and unfolded state.

**Interaction Analysis**—Biosensor-based interaction analysis was performed as described previously (20, 31). Receptor ectodomains or BMP-2 were biotinylated and immobilized on a streptavidin-coated CM5 sensor chip. BMP-2 or soluble ectodomains were perfused over the sensor chip using six different analyte concentrations. The sensorgrams were evaluated using the software BIAevaluation 2.4. Bulk face effects were corrected by subtracting a control flow cell (FC1) from all sensorgrams. Dissociation constants were obtained from the kinetic rate constants for complex formation (k₉₁₀) and dissociation (k₁₀₉). Mean values with a S.D. of 30% for k₉₁₀ and 15% for k₁₀₉ were deduced from 6–12 independent measurements.

**SMAD2/3 Luciferase Reporter-Gene Assays**—BMP type I-activin type IB receptor chimeras, which have the extracellular domain of the BMP type I receptor (BMPR-IA, BMPR-IB, BMPR-IA/IB8, BMPR-IA-P34R, BMPR-IA-Y39D, BMPR-IA-T55I) and the transmembrane helix and intracellular kinase domain of ActR-IB were constructed to measure BMP-2-induced signals uncoupled from endogenous BMP receptors. For transient transfections, HEK293T/17 cells were seeded in 24-well poly-(l)-lysine-coated plates (Greiner) at a density of 1.5 × 10⁵ cells/well. The cells were cotransfected with 50 ng/well pSV β-gal (Promega), 100 ng/well p(CAGA)12-Luc reporter plasmid, and 50–100 ng/well of the pcDNA3.1 plasmids encoding for the different BMP type I receptors using HEKfectin (Bio-Rad). After 24 h cells were treated with 5 or 10 nM BMP-2 in Dulbecco’s modified Eagle’s medium containing 0.1% fetal calf serum. β-Galactosidase activity was measured by o-nitrophenyl galactopyranoside conversion at 405 nm. Lucif-
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erase activity was determined using the Promega luciferase assay kit. All assays were performed twice with each experiment performed in duplicate.

BMPR-IA Competition Assay in C3H10T1/2 Cells—C3H10T1/2 cells stably transfected with the BRE-luciferase reporter construct were seeded in Dulbecco’s modified Eagle’s medium at a density of 3 \times 10^4 cells cm^{-2}. After 16 h the medium was exchanged, and the cells were stimulated with 5 nM BMP-2 plus increasing concentrations of BMPR-IA, BMPR-IB, BMPR-IA/IBC, and BMPR-IA variants (log 2 dilutions starting from 1 \mu M) for 72 h. Cells were lysed, and luciferase activity was determined as described above. Each experiment was performed twice, and all assays were performed in duplicate.

BMP-2 Induced Inhibition of MPC11 Cell Proliferation—MPC11 cells (ATCC CCL167) were seeded in Dulbecco’s modified Eagle’s medium in 96-well plates at a density of 2.5 \times 10^3 cells/well. For inhibition of cell proliferation 5 nM BMP2 (IC_{50} = 5 nM) was added. For competition, increasing concentrations of the receptor ectodomains (2 nM to 1 \mu M) were added. After 72 h 10 \mu l of [3H]thymidine (0.25 \mu Ci, Amersham Biosciences) was added to each well. The cells were immobilized after 24 h on fiber mats (Scatron), and the thymidine incorporation was determined using a \gamma-counter. All assays were performed in duplicate, and the experiments were repeated twice.

Cellular Localization of BMPR-IA Variants—Full-length wild type BMPR-IA and the BMPR-IA mutants P34R, Y39D, and T55I were cloned into pcDNA3.1 with CFP fused to the C terminus of the extracellular kinase domain and transiently transfected into HEK293TSA cells using Polyfect (Qiagen). As a control for plasma membrane staining, enhanced yellow fluorescent protein with the palmitylation sequence of the Lyn kinase fused to its N terminus was co-transfected. 24 h after transfection cells were transferred to a poly-(L)-lysine-coated glass slide. Cell nuclei were stained by incubation with Hoechst 33343 and analyzed using a Leica SP2 confocal microscope at 630-fold magnification. For excitation of the fluorophores, wavelengths of 350 nm (Hoechst 33343), 436 nm (CFP), and 490 nm (yellow fluorescent protein) were used. Emission was recorded at 450 nm (Hoechst 33343), 476 nm (CFP), and 535 nm (yellow fluorescent protein). Individual images were analyzed with Leica confocal software (Leica) and merged using the software package ImageJ.

For fluorescence-activated cell sorting (FACS) analysis 2 \times 10^5 HEK293TSA cells were transiently transfected with pcDNA3.1 harboring full-length wild type or mutant BMPR-IA with an N-terminal FLAG tag. The transfected cells were incubated in FACS buffer (phosphate-buffered saline, 0.1% bovine serum albumin, 0.05% NaN3) supplemented with mouse anti-FLAG M2 monoclonal antibody (1:200, Sigma) or the same amount of an isotype-specific control antibody (mouse IgG1, clone R73) for 1 h. Cells were washed with FACS buffer and incubated with phycoerythin-labeled donkey anti-mouse IgG (1:500, Dianova) for 30 min. After another washing, step cells were analyzed on a FACSscan flow cytometer (BD Biosciences). Data analysis was performed using the software CellQuest. Living cells were electronically gated according to their light scatter characteristics, which were determined by counter-staining with propidium iodide.

Coordinate Deposition—The atomic coordinates and structure factors for the structures of the complex of BMP-2-BMPR-IA/IBC, BMP-2-BMPR-IA_B1, and BMP-2-BMPR-IA_B12 have been deposited with the Protein Data Bank (accession codes 2QJB, 2QJ9 and 2QJA, respectively).

RESULTS

The BMPR-IA/IB C Chimeric Variant Represents a BMPR-IB Mimic—We have created a BMP receptor variant that has the ligand binding epitope formed by the amino acids of BMPR-IB but has a BMPR-IA scaffold (Fig. 1) because crystals of the complex of BMP-2 and BMPR-IBec suitable for structure determination could not be obtained despite great efforts. The equilibrium binding constants for the interaction of BMP-2 with either BMPR-IA or -IB differ only slightly; however, analyses of the binding kinetics reveal clear differences for the binding to both receptors (Table 1). BMP-2 binding to BMPR-IB reproducibly shows a 2–3-fold increased dissociation rate, whereas the association kinetic is almost unaffected. Thus, the parameters for binding kinetics were used to judge whether a variant exhibits BMPR-IA- or BMPR-IB-like binding character.

Exchange of residues Lys-88 to Thr-98 in BMPR-IA, located in the loop between helix \(\alpha1\) and \(\beta\)-strand 5, results in the variant BMPR-IA_B1, which already exhibits the same binding parameters for BMP-2 as observed for BMPR-IB. The additional exchange of residues Ala-74 to Tyr-80 located in \(\beta\)-strand 4 (BMPR-IA_B12) reverts the binding characteristics seen for the precursor variant BMPR-IA_B1. A BMPR-IA-like binding characteristic was then observed for all further BMPR-IA/-IB chimeras until all residues in the binding epitope were exchanged for those of BMPR-IB (Fig. 1, a and b). Consistently, all binding parameters of this chimera BMPR-IA/IB C to GDF-5 (20) and BMP-2 are indistinguishable from those of wild type BMPR-IB (Table 1).

In Vivo Signaling of BMPR-IA, BMPR-IB, and the Chimera BMPR-IA/IB C Are Similar—Cell-based assays were used to evaluate binding and signaling properties of BMPR-IA/IB C. C3H10T1/2 cells stably transfected with the BRE-luciferase reporter construct were used to determine inhibition of BMP-2 signaling by the application of type I receptor ectodomain proteins. All receptor ectodomain proteins, BMPR-IAec, BMPR-IBec, and BMPR-IA/IB C ec, exhibit a concentration-dependent inhibition of BMP-2-induced SMAD1/5/8 signaling by competing with the endogenous receptors for BMP-2 binding (Fig. 2a). The IC_{50} value of BMPR-IAec is below 500 nM, whereas that of BMPR-IBec and BMPR-IA/IB C ec is 500 nM or slightly higher.

To measure how BMPR-IB and BMPR-IA/IB C signal upon BMP-2 binding, we implemented a different luciferase-based reporter-gene assay in HEK293T cells. BMP-2-induced signaling by endogenous receptors was uncoupled from the cellular readout by fusing the extracellular part of BMPR-IA, BMPR-IB, or BMPR-IA/IB C to the transmembrane part and kinase domain of ActR-IB. Upon BMP-2 activation these BMPR-ActR-IB receptor chimeras signal via the SMAD2/3 pathway, which does not occur naturally. This assay clearly shows that BMP-2 not only binds to BMPR-IA, BMPR-IB, and BMPR-IA/
IBC but also led to signaling activities of a similar magnitude in our setup (Fig. 2).

In summary, BMPR-IA and BMPR-IB bind BMP-2 with similar affinities, but different binding kinetics indicate differences in the binding mechanism. The newly designed receptor chimeras BMPR-IA/IBC, which shares the binding epitope of BMPR-IB, exhibits binding and signaling capabilities indistinguishable from that of wild type BMPR-IB and, thus, can be used as a BMPR-IB mimic in structural and functional studies.

**Structural Plasticity in the BMP-2 Type I Receptor Complex**—We analyzed the structure of the complex of BMP-2 bound to the BMPR-IB mimic BMPR-IA/IBC and compared it to that of the BMP-2/BMPR-IAec complex (32). The asymmetric unit contains a full complex consisting of the BMP-2 dimer and two BMPR-IA/IBC molecules (Fig. 3, a and b). The complex structures of BMP-2-BMPR-IAec and BMP-2-BMPR-IA/IBCec are highly similar (root mean square deviation ~ 0.8 Å), indicating that the overall complex architecture is unchanged (sup-
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FIGURE 2. Signaling properties of BMPR-IA, BMPR-IB, and BMPR-IA/IBC in cell assays. a, inhibition of BMP-2 induced BRE reporter-activity in C3H10T1/2 cells by the addition of recombinant receptor ectodomain proteins. The lower BMP-2 binding affinities of the BMPR-IB and BMPR-IA/IBC proteins are reflected in slightly lower inhibition efficiencies. The black bar designates the stimulation by 5 nM BMP-2, and the gray bar represents BRE reporter activity in unstimulated cells. b, SMAD activation through BMPR-IA, BMPR-IB, and BMPR-IA/IBC-activin receptor type IB chimeras. cDNA of BMP type I-ActR-IB receptor chimeras were transfected into HEK293 cells, and expression of luciferase activity upon BMP stimulation was determined. The expression of luciferase activity for the three type I receptor constructs is expressed in fold induction. A negative control without transfection of a BMP type I-ActR-IB receptor chimera is indicated (CAGA). RLU, relative luciferase units.

FIGURE 3. Ribbon view of the complex BMP-2-BMPR-IA/IBC, a, the complex of BMP-2 bound to BMPR-IB mimetic BMPR-IA/IB in ribbon representation viewed from the side. The extracellular domain of BMPR-IA/IBC is shown in blue, and the BMP-2 dimer is colored in green. The 2-fold symmetry axis is indicated. b, the binary complex viewed from the top. The intermolecular central disulfide bond in the BMP-2 dimer is shown, and secondary structure elements are marked. The BMPR-IA/IBC binding loop, which exists in two different conformations, is shown in red.

The receptor ectodomain positions within the complex structure are identical for both type I receptors BMPR-IA and BMPR-IA/IBC (root mean square deviation 0.4 Å for Ca of receptor ectodomain without peripheral loops), showing that despite the 18 amino acid exchanges in the ligand binding epitopes, no displacement of the receptor in the wrist epitope is required to adapt the ligand BMP-2 to the different receptor surfaces (Fig. 1b). Thus, a possible explanation for the promiscuity in receptor binding might be in a slight structural variation within the receptor and ligand binding sites to allow binding and recognition of different receptors or ligands.

The main binding determinants established in the complex BMP-2-BMPR-IAec (31, 32) are absolutely conserved in both BMP-2-BMPR-IAec and BMP-2-BMPR-IA/IBCec complexes. The three central hydrogen bonds (H-bonds) formed between Leu-51 NH (BMP-2) and Gln-86 O (BMPR-IA/BMPR-IA/IBC) and the Asp-53 NH (BMP-2) and Cys-77 CO (BMPR-IA/BMPR-IA/IBC) are identical in length and angular parameters between the two complex structures (supplemental Table 1). This observation corroborates with our mutagenesis studies showing that mutation of Leu-51 in BMP-2 decreases the binding affinity to either BMPR-IA or BMPR-IB in a similar manner (32).

Previously, we proposed that the water molecules buried in the interface of BMP-2 and BMPR-IA play an important role in the recognition of the type I receptors and might be required to adapt to altered surface geometries of different type I receptors (32). Indeed, three of the four water molecules located in the interface nearby Leu-51 of BMP-2 are conserved between both complexes BMP-2-BMPR-IAec and BMP-2-BMPR-IA/IBCec, forming an identical H-bond network between ligand and receptor. However another water molecule, which is buried in a cleft between residues Phe-49, Leu-51, Ile-62, and Leu-66 of BMP-2 and Gln-86 of BMPR-IA is absent in the BMP-2-BMPR-IA/IBCec complex. The larger side chain of Met-62 (instead Ile-62 as in BMPR-IA) in BMPR-IA/IBC requires more space, thereby filling and precluding the solvent molecule from the cleft. In all other amino acid exchanges, larger side chains closely adapt side chain conformations that follow those of the smaller ones, thereby leaving the overall architecture and packing density unaltered.

In summary, the type I receptor interface of BMP-2 and possibly of other BMPs as well seems to have a built-in adaptability enabling limited specificity for the ligand-receptor interaction. The adaptability is based on a highly conserved polar hot spot of binding formed between two pairs of residues, Leu-51 and Asp-53 of BMP-2 and conserved Gln and Cys residues in the receptors BMPR-IA and BMPR-IB. The H-bonds between these residues contribute a large part to the binding free energy, whereas residues at the periphery of the epitope contribute only little and, thus, can be mutated without loss in affinity.

Water molecules in the core of the ligand-receptor interface modulate the packing density thereby allowing larger side chains of different receptors to be accommodated by precluding one or more of the solvent molecules from the interface. This mechanism seems to allow a high variability in the amino acid composition of the BMP-receptor interface, with almost 50% of the BMP receptor residues in the interface differing between BMPR-IA and BMPR-IB.

BMPR-IA/IBC Exhibits Two Conformations When in Complex with BMP-2—In the complex of BMP-2-BMPR-IA/IBCec, 18 direct H-bonds are present (supplemental Table 1). Compared with the complex of BMP-2 bound to BMPR-
IAec, two H-bonds are lost, possibly explaining the slightly decreased binding affinity. Closer examination reveals, however, that the two interfaces of the BMP-2, at least with respect to the H-bond network, are quite asymmetric (supplemental Tables 1 and 2). In one wrist epitope, 12 H-bonds are formed between BMP-2 and BMPR-IA/IBec, whereas in the second, only six H-bonds exist (supplemental Table 2). The latter are conserved between both interfaces of the BMP-2

\[ \text{BMP-2} \]

/H18528

BMPR-IA/IBec complex and, thus, possibly mark the minimal set of H-bonds in the BMP-type I receptor interface. Of these six H-bonds, five are also observed in the complex

\[ \text{BMP-2} \]

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BMPR-IAec. However, in the BMP-2-BMPR-

IAec complex, both interfaces are symmetrical, with 10 H-bonds in each epitope (32).

Superposition of both ligand-receptor interfaces reveals that BMPR-IA/IBec exists in two conformations in this complex (Fig. 4). Loop 5 between α-helix 1 and β-strand 5 (Arg-88 to Arg-96) deviates in the two BMPR-IA/IBec molecules in the complex with the largest Ca-Ca distance of 4 Å for Asp-89. However, not only the positions of backbone atoms differ, also the side chains of several residues within this loop show markedly different orientations. The side chain of residue Asp-89, involved in an intramolecular salt bridge with Arg-97, moves by more than 5 Å with concurrent loss of the salt bridge. Similarly, the side chain of BMPR-IA/IBec Arg-96 is rotated (distance between Cα of both Arg-96 conformations >7Å) such that the salt bridge between Arg-96 and Glu-65 is lost. This loss of both salt bridges in one BMPR-

IA/IBec conformer results in an “open” loop conformation, whereas the “closed” form of loop 5 closely resembles the conformation observed in BMPR-IA in the BMP-2-BMPR-IAec complex (Fig. 4, a and b). The loop in the open conformation seems more flexible with the temperature factors of the Cα atoms of Arg-88 to Arg-96 in the BMP-2-BMPR-IA/IBec open conformer being about 20–30Å² higher compared with those in the closed conformation (supplemental Fig. 2). As a consequence of main and side chain reorientations in this loop, four intermolecular H-bonds are lost in the interaction of BMP-2 with the open conformer of BMPR-IA/IBec. Also, the shielding of the H-bond network between the main binding determinants from the solvent seems less efficient, providing another explanation for the reduced binding affinity of BMP-2 for BMPR-IB.

The molecular cause of the loop reorientation seems due to the exchange of Ala93 in BMPR-IA to proline. Albeit the backbone torsion angles of Ala93 in BMPR-IA and Pro-93 in BMPR-

IAec are almost identical and differ by less than 10°, the larger side chain of the proline residue pushes against Arg-97 located just above. Consequently, the intramolecular salt bridge between Asp-89 and Arg-97 in BMPR-IA/IBec is destabilized (Fig. 4c). In one of the two conformers of BMPR-IA/IBec, the side chain of Asp-89 swings out into the bulk solvent. The backbone torsion angles of Asp-89 change dramatically, with the ϕ-torsion angle deviating by nearly 180° and the ψ-torsion angle changing by more than 70°. The torsion angles of the residues Asp-89 and Pro-93 also differ between both BMPR-

IA/IBec conformers, thereby adjusting the loop conformation to leave the positions of the “anchor points,” Arg-88 and His-94, unaltered.
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In the structures of two “intermediate” BMPR-IA/BMPR-IB chimeras (BMPR-IA_B1 and BMPR-IA_B12) bound to BMP-2, the loop conformation for the loop 5 is identical to that of the BMP-2-BMPR-IA/IB⁶, clearly indicating that the different conformations are directly and solely induced by the amino acids exchanges in loop 5. In conclusion, amino acid differences between BMPR-IA and BMPR-IB possibly lead to an increased flexibility on side and main chain level in BMPR-IB. The BMPR-IB like receptor chimera BMPR-IA/IB⁶ exists in two conformations in the BMP-2 receptor complex, leading to a variable number of H-bonds between ligand and receptor. The lower net number of intermolecular H-bonds and the more dynamic ligand-receptor interface probably account for the increased dissociation rate and the lower affinity of BMP-2 and BMPR-IA/IB⁶. Although we do not have direct structural data of wild type BMPR-IB, measurements of the folding stability of BMPR-IB employing a thermal melting analysis using circular dichroism spectroscopy show that the extracellular domain of BMPR-IB has a melting temperature that is 5 °C lower than BMPR-IA (data not shown). This possibly suggests a lower stability and higher flexibility of BMPR-IB ectodomain in comparison to BMPR-IA and correlates with the structural flexibility observed for the BMPR-IA mimic BMPR-IA/IB⁶.

Missense Mutations in BMPR-IA Outside the Ligand Binding Epitope Lead to JPS—Our structure analysis of the different BMP type I receptor complexes suggests that the type I receptors BMPR-IA and -IB share a common structural scaffold but residues in the ligand binding epitope are variable for modulating ligand specificity. In the case of BMP-2, both type I receptors BMPR-IA and -IB are bound with similar affinity. A comparison of the structures of BMPR-IA and the BMPR-IB mimic BMPR-IA/IB⁶ shows that differences in the amino acid composition of the epitope are compensated by backbone and side chain flexibility as well as solvent molecules modulating the interface packing. The sequence variability in the ligand–receptor interface seems, however, restricted to amino acids that neither constitute main binding determinants nor are required for structural integrity of the receptor. A hot spot of binding such as Gln-86 of BMPR-IA is also preserved in BMPR-IB, as is Phe-85 or Cys-77. Mutation of any of these residues, thus, results in a significant loss of binding affinity.

Non-sense mutations in BMPR-IA, which result basically in a knock-out of BMPR-IA through destruction of the BMPR-IA fold, have been reported in the juvenile polyposis syndrome, which is characterized by excessive colorectal polyph growth and often leads to colon cancer (27). Similarly, inactivating mutations in SMAD4 also result in JPS (33). The common mechanism of all these mutations seems to be the loss of BMP-2/-4 activity required to inhibit proliferation in the colon epithelial cells (27).

Interestingly, three missense mutations in the extracellular ligand binding domain of BMPR-IA (P34R, Y39D, and T55I) have been reported in JPS previously. These are neither main binding determinants nor constitute residues located inside the hydrophobic core but nevertheless seem to inactivate BMP-2 signaling. Two mutations, P34R and Y39D, are located outside the BMP binding epitope at the protein surface (Fig. 5, a and b). Only residue Thr-55 of BMPR-IA, which is affected in JPS via the mutation T55I, is in direct contact with BMP-2. However, BMPR-IA Thr-55 is located at the interface periphery sharing minimal surface in the contact. In addition, the H-bond of Thr-55 of BMPR-IA to Asp-53 Oδ2 of BMP-2 does not contribute to the binding affinity of the BMP-2-BMPR-IA interaction (32), and modeling of the mutation T55I does not reveal a steric hindrance for the interaction. Using the structural data provided in this study and of the complexes BMP-2-BMPR-IA, no mechanism can be proposed for how these mutations might result in a loss of BMP-2 receptor activation.

We prepared all three BMPR-IA variants and determined their BMP-2 binding properties in vitro by biosensor interaction analysis (Table 2). Whereas variant BMPR-IA-P34R shows a less than 2-fold decrease in binding affinity, the other variants, Y39D and T55I, exhibit a drastically reduced binding to BMP-2. With the receptor proteins immobilized onto the biosensor surface no binding could be detected up to a concentration of 80 nM BMP-2, indicating a dissociation constant larger than 1 μM. In a second experiment the ligand BMP-2 was immobilized on the sensor surface. Using this setup, BMPR-IA-Y39D and BMPR-IA-T55I exhibit dissociation constants of 1.5 and 2.6 μM, respectively, whereas wild type BMPR-IA binds BMP-2 with high affinity of about 30 nM. We also tested these variants in cell-based assays (Fig. 5, c and d). C3H10T1/2 cells stably transfected with a BRE-luciferase reporter-gene construct were stimulated with 5 nM BMP-2. The BMP-2-induced luciferase expression activation was then inhibited by adding increasing concentrations of recombinant receptor ectodomain proteins with wild type BMPR-IA exhibiting an IC₅₀ value of about 0.5 μM (Fig. 5c). In contrast, BMPR-IA-P34R exhibits increased IC₅₀ of 4 μM, and BMP-2 activation can only be suppressed to 50% at the highest concentration tested (Fig. 5c). In comparison, BMPR-IA-Y39D and BMPR-IA-T55I did not inhibit BMP-2-induced SMAD signaling even at high concentrations (Fig. 5c).

Effects of BMP-2 signaling on cell proliferation were tested using the mouse plasmacytoma cell line MPC11, which stops proliferating upon stimulation with members of the transforming growth factor-β superfamily like activin (34) and BMP-2 (Fig. 5d). By stimulation with 5 nM BMP-2, cell proliferation is decreased to 50%. Again, receptor ectodomain proteins of BMPR-IA, BMPR-IB, or BMPR-IA/IB⁶ reverse the BMP-2 effect in a dose-dependent manner. The addition of wild type BMPR-IA fully rescues the BMP-2-induced decrease in proliferation, with about 125 nM BMPR-IA being necessary for 50% rescue. In comparison, BMPR-IA-P34R is less efficient (IC₅₀ ≈ 4 μM) and even at high concentrations normal proliferation cannot be restored (data not shown). The BMPR-IA mutants Y39D and T55I are unable to rescue the BMP-2-induced anti-proliferative effect, similar to the inhibition of the BMP-2-induced SMAD signaling reported above. Testing the direct signaling capabilities via the BMPR-IA-AcegRI-B receptor chimera showed that both BMPR-IA-Y39D and BMPR-IA-T55I are completely inactive, whereas BMPR-IA-P34R exhibits about 30% of wild type BMPR-IA activity (supplemental Fig. 3), which is consistent with results of the inhibition and proliferation assays using recombinant receptor ectodomains (Fig. 5, c and d).
JPS Mutations Result in Unfolding of BMPR-IA—Although the in vitro measurements of the BMPR-IA variants P34R, Y39D, and T55I show that the variants are binding-deficient to BMP-2 to a variable degree, a molecular mechanism how these mutations cause their effect cannot be given. Analyses of the BIAcore data, however, showed the association kinetics being strongly affected, indicating that the mutations might affect the fold of BMPR-IA (Table 2). We, thus, used circular dichroism (CD) spectroscopy to determine possible changes in the three-dimensional structures of the BMPR-IA variants (Fig. 6a). The CD spectrum of BMPR-IA-P34R is similar to wild type BMPR-IA, suggesting that its structure is largely conserved. In contrast, the CD spectra of variants Y39D and T55I deviate clearly from wild type BMPR-IA, and a simulation of the CD-spectra
indicates an increase in random coil structures. We, therefore, performed thermal denaturation analyses using CD spectroscopy (Fig. 6b). Wild type BMPR-IA unfolds at 68 °C with a narrow transition characteristic for a stably folded globular protein. BMPR-IA-P34R unfolds at almost the same \( T_m \) (65 °C); however, the folded/unfolded transition is less sharp. Most interestingly, the two variants Y39D and T55I do not show any folded/unfolded transition. Thus, the BMP-2 binding deficiency of the latter two variants is clearly due to the loss of the BMPR-IA fold.

Because expression in eukaryotic cells might yield natively folded BMPR-IA variants due to involvement of chaperones, we performed thermal denaturation analyses using CD spectroscopy of BMPR-IA and variants reveals that wild type BMPR-IA and variant P34R unfold at comparable temperatures indicating a similar stable, globular fold for both proteins. In contrast, BMPR-IA variants Y39D and T55I show no change in their CD spectra due to involvement of chaperones, we performed thermal denaturation analyses using CD spectroscopy of BMPR-IA and variants reveals that wild type BMPR-IA and variant P34R unfold at similar temperatures indicating a similar stable, globular fold for both proteins. In contrast, BMPR-IA variants Y39D and T55I show no change in their CD spectra when heated to 95 °C, suggesting the absence of a globular fold for these two variants.

**TABLE 2**

**Binding affinities of BMPR-IA variants in juvenile polyposis syndrome**

| Receptor protein | Association rate \( k_{on} \) | Dissociation rate \( k_{off} \) | Dissociation binding constant \( K_{D}\) |
|------------------|------------------|------------------|------------------|
| BMPR-IA          | 3.4              | 2.4              | 0.7              |
| BMPR-IA-P34R     | 3.5              | 3.2              | 0.9              |
| BMPR-IA-Y39D     | ND               | ND               | ND               |
| BMPR-IA-T55I     | 2.5              | 7.0              | 2.7              |
| BMPR-IB          | ND               | ND               | ND               |

| Receptor protein (analyte) | Association rate \( k_{on} \) | Dissociation rate \( k_{off} \) | Dissociation binding constant \( K_{D}\) |
|-----------------------------|------------------|------------------|------------------|
| BMPR-IA                     | 5.6              | 1.7              | 30               |
| BMPR-IA-P34R                | 4.4              | 1.4              | 32               |
| BMPR-IA-Y39D                | 0.17             | 2.5              | 1470             |
| BMPR-IA-T55I                | 0.28             | 7.2              | 2570             |

* Dissociation binding constant \( K_D \) was derived from calculating \( K_D = \frac{k_{off}}{k_{on}} \).
* ND, no binding; due to the inability of acquiring the kinetic rate constants, the binding affinity can only be estimated to be \( >1 \mu M \).
* Dissociation binding constant \( K_D \) (eq) was obtained from a setup with the ligand BMP-2 immobilized on the biosensor surface. This setup yields binding affinities for the so-called 1:1 interaction, which does not include avidity effects of the dimeric BMP-2.

**DISCUSSION**

In this study we present a mechanism by which the promiscuous binding of BMP-2 to its high affinity type I receptors can be explained. Promiscuous binding of BMP-2 to both type I receptors BMPR-IA and -IB seems to rely on three major points. First, the majority of the binding free energy is maintained through conservation of just three H-bonds in the center of both BMP-2 type I receptor interactions with BMPR-IA and -IB. The peripheral interactions contribute little to binding affinity but are necessary to shield the polar bonds in the center from solvent access and are, thus, variable. Second, “built-in” water molecules in the interface core allow exchange of amino acids in the epitope as long as the residue type is retained. Different side chains sizes and geometries can be adapted via

![Figure 6](image-url)

**FIGURE 6. Missense mutations Y39D and T55I in BMPR-IA result in unfolded proteins.** CD spectroscopy of the receptor ectodomain proteins shows that the BMPR-IA fold is affected in the variants Y39D and T55I. a, CD spectra of wild type BMPR-IA (black) and the JPS mutants P34R (red), Y39D (blue), and T55I (green) are superimposed. b, thermal denaturation studies using CD spectroscopy of BMP-IA and variants reveals that wild type BMPR-IA and variant P34R unfold at similar temperatures indicating a similar stable, globular fold for both proteins. In contrast, BMPR-IA variants Y39D and T55I show no change in their CD spectra when heated to 95 °C, suggesting the absence of a globular fold for these two variants.
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Changes of the backbone upon ligand binding to adapt to the ligand surface (39, 42).

It is, therefore, surprising that, although various mutations in the ligand binding epitope of BMPR-IA can be accommodated without loss of BMP-2 affinity, several missense mutations in the ectodomain of BMPR-IA have been reported that lead to a loss of BMP-2 signaling activity resulting in the juvenile polyposis syndrome. Nonsense mutations like premature stop codons or mutations of structurally relevant cysteines can be easily explained; however, the three mutations described seemed not disruptive, and two, P34R and Y39D, are not even located within the binding interface. Our data, however, clearly confirm that all these mutations lead to a loss of BMP-2 binding or signaling activity, although to a variable degree (supplemental Fig. 3). The two mutations with the strongest decrease in BMP-2 binding, Y39D and T55I, result in complete unfolding of the BMPR-IA ectodomain.

Interestingly, Tyr-39 is also mutated in BMPR-IB, and the exchange does not result in a loss in BMP-2 binding affinity. To explain this apparent discrepancy, the location of Tyr-39 in a small crevice between the loop 1 and the $\beta_3$-helix of the membrane-proximal C terminus must be taken into account. The aromatic ring is placed above the peptide bond between Tyr-39 and Cys-40, possibly forming a stabilizing $\pi-\pi$ stacking interaction (Fig. 5e). In BMPR-IB Tyr-39 is replaced by a lysine residue, suggesting that possibly a hydrophobic amino acid of a certain size may be required to stabilize the loop 1. A mutagenesis series for Tyr-39 with different amino acids (Phe, Ile, Lys, and Ala) supports this hypothesis (supplemental Fig. 4 and Table 3). If Asp replaces Tyr-39, the side-chain carboxylate group might form unfavorable H-bonds with residues within the loop 1 or destabilize the loop conformation in the folded state such that the majority of the receptor protein is unfolded.

For BMPR-IA-T55I we also found that the receptor protein is unfolded in vitro. Although a mechanism explaining the unfolding of this particular mutant is not as obvious, analysis of the structures of the complexes BMP-2-BMPR-IA and BMPR-2-BMPR-IA/IBC shows that the conserved Thr-55 makes an intramolecular H-bond stabilizing the loop conformation of loop 2 (Fig. 5f). Exchange of the threonine residue with an iso-leucine first removes the H-bond, and second, the larger side chain of the isoleucine requires a particular side-chain torsion angle conformation to fit. These restraints possibly destabilize the receptor protein during unfolding, causing the unfolding of the receptor ectodomain similar to the Y39D mutation.

In contrast, it seems unfolding of the ectodomain is not the mechanism by which the mutation P34R causes decreased BMP-2 signaling. Our cell-based and in vitro studies suggest that, if folding is affected, only minor local changes are introduced. Binding affinity in the BLAcore studies is only marginally decreased. However, competition studies in cell-based experiments confirm that similar to the BMPR-IA/IBC chimera BMPR-IA-P34R cannot completely inhibit BMP-2 signaling in contrast to wild type BMPR-IA. Pro-34 is located at the N terminus of BMPR-IA with around 40% of the residue being solvent accessible (Fig. 5g). Continuous electron density is observed for Pro-34, indicating an ordered structure for this N-terminal stretch. Although residue Thr-55, which is crucial

exclusion of one or more of these interface water molecules. Third, inherent backbone and side-chain flexibility of interface residues attached onto a common scaffold allows different side-chain geometries to be adapted without major loss of interaction energy. Although the increased flexibility in the BMP-2 binding loop of BMPR-IA/IBC results in a loss of intermolecular H-bonds, their contribution to binding of BMP-2 is only marginal, and the overall binding affinity is, therefore, almost unaltered.

Similar mechanisms have been observed for other protein-protein interactions, e.g. TRAF3 (35, 36), and the requirement of flexibility for promiscuous binding has been proposed previously (37, 38). Structure analysis revealed that BMP and activin type II receptor ectodomains also exhibit inherent flexibility (39, 40). Specifically the BMPR-II receptor is highly flexible with almost all of its loops being disordered in the free state (41). The activin type II receptors are less flexible; however, several loops undergo conformational

FIGURE 7. BMPR-IA variants are transported to and integrated into the cell membrane. HEK293TSA cells were transfected with full-length BMPR-IA, BMPR-IA-P34R, BMPR-IA-Y39D, or BMPR-IA-T55I (30 ng of plasmid DNA). BMPR-IA constructs were conjugated to an N-terminal FLAG tag and a C-terminal CFP fusion. The cell membrane is stained with an enhanced yellow fluorescing protein carrying a palmytilation sequence at the N terminus for translocation at the intracellular membrane. Cell nuclei were stained with Hoechst 33342. Wild type BMPR-IA (a) and the mutants P34R (b), Y39D (c), and T55I (d) are efficiently transported to the cell membrane. ∼ we further used an anti-FLAG antibody to verify that the extracellular domains of BMPR-IA and variants P34R, Y39D, and T55I appear at the cell surface. Cells were incubated with a primary anti-FLAG antibody and a secondary phycoerythrin-conjugated antibody and sorted according to phycoerythrin fluorescence. Transfected cells (thick line) can clearly be separated from control cells (thin line), showing that BMPR-IA mutant proteins are correctly inserted into the cell membrane. Mock-transfected cells cannot be separated. The change in the mean fluorescence intensity of the low-expressing population was as follows for transfected cells: 68 (P34R), 44 (Y39D), 54 (T55I), 51 (wild type (wt)), and 21 (mock). The respective values for the isotype-specific control were between 16 and 18. The proportion of cells stained with high intensity by FLAG-specific antibody was 3% for the mock-transfected cells and between 24% (wild type) and 35% (BMPR-IA-P34R) for the receptor-transfected cells, demonstrating that expression of mutants was at least as good as that of the wild type receptor.
for correct folding, is in close proximity to Pro-34, structural changes at the Thr-55 site due to the mutation P34R seem not to cause the effects observed in the cell-based assays since this would lead to a decrease in BMP-2 affinity. Thus, we can only speculate how the BMPR-IA mutation P34R leads to the decreased activity (supplemental Fig. 3). One possibility is a reduced stability of the mutant protein in vivo. The introduction of an arginine at position 34 would create an additional cation of an arginine at position 34 would create an additional

reduced stability of the mutant protein

Thus, replacement with either receptor does not yield the same physiological function. Therefore, to allow treatment of JPS, a signaling cascade yielding similar anti-proliferative signals in colon epithelial cells as for BMP-2 must be found.

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