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Isolation of recombinant BMP receptor IA ectodomain and its 2:1 complex with BMP-2

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Abstract Bone morphogenetic protein-2 (BMP-2) is a member of the transforming growth factor β superfamily which induces bone formation and regeneration, and important steps during early embryonic development. BMP-2 signals via oligomerization of type I and type II serine/threonine kinase receptors. We report here expression of the extracellular domain of the human type IA receptor for BMP-2 (BMPR-IA) in *Escherichia coli*. This soluble form of BMPR-IA (sBMPR-IA) was purified employing a BMP-2 affinity column. Gel filtration experiments and analysis of gel filtration fractions by polyacrylamide electrophoresis and densitometry reveal that BMP-2 forms a defined 1:2 complex with sBMPR-IA that can be purified and hopefully used for crystallization studies.

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Key words: Bone morphogenetic protein-2; Bone morphogenetic protein receptor type IA; Transforming growth factor β superfamily; Binding stoichiometry

1. Introduction

Bone morphogenetic protein-2 (BMP-2) is one of the key representatives of a group of bone morphogenetic proteins that were originally defined by their bone and cartilage-inductive activity at non-skeletal sites in vivo [1,2]. Cloning of the cDNA [3] revealed that BMP-2 belongs to the transforming growth factor β (TGF-β) superfamily. The TGF-β superfamily is a group of multifunctional cytokines that play important roles in development and in the control of differentiation and proliferation. The mouse knockout mutant of the *bmp2* gene is embryonic lethal showing severe defects in cardiac development and a missing closure of the proamniotic canal [4]. BMP-2 is synthesized as a 453 residue proprotein, which becomes glycosylated, proteolytically cleaved and dimerized through a single disulphide bridge to yield the mature homodimeric protein consisting of the 114 C-terminal proprotein residues.

BMP-2 transduces signals into the cell by binding two types of receptor chains [5]. Cross-linking experiments in whole cells employing BMP-2 or the highly homologous BMP-4 revealed that BMP-2 can interact with any of three different type I receptors, as BMPR-IA (ALK-3), BMPR-IB (ALK-6) and ActR-IA (ALK-2), and with two type II receptors BMPR-II and ActR-II [6,7]. The type II receptors, however, reacted to a larger extent only when a type I receptor was present [8]. This is a clear distinction to the TGF-β system where the TGFβR-II can bind TGF-β on its own whereas TGFβR-I can not [9–13]. The specificities and affinities reported for the interactions between BMP-2 and type I receptors are still under discussion [14]. Both receptor types are membrane-bound proteins with a 110 to 130 amino acid residues long extracellular domain, a single transmembrane helix and an intracellular part which comprises a serine/threonine kinase domain. The type II receptor phosphorylates and thus activates the type I receptor. The type I receptor then phosphorylates signal transducer proteins called Smads which upon phosphorlyation translocate to the nucleus and activate specific genes.

While in whole cells homo- and heterodimeric receptors have been found in the absence of ligand [15], the stoichiometry of the complex between BMP-2 and the receptor ectodomains has not yet been determined. In principle it might be expected that a dimeric ligand like BMP-2 provides two binding epitopes for two identical receptor chains. However this 1:2 ratio of binding between dimeric ligand and receptor is not seen for interleukin-5 (IL-5); here one IL-5 dimer molecule binds only one soluble IL-5 receptor α molecule [16]. In contrast, a 1:2 stoichiometry has been demonstrated for interferon-γ, which is a homodimer, and its soluble high-affinity receptor [17,18]. Also higher ratios like 1:4 are conceivable as monomeric ligands like growth hormone or erythropoietin bind two identical receptor chains [19,20].

In this study we have expressed the extracellular domain of the human BMPR-IA (sBMPR-IA) in soluble form in *Escherichia coli* and purified it to homogeneity using a BMP-2 affinity column. This affinity chromatography step proved critical to prepare reproducibly and quantitatively a homogeneous complex with defined stoichiometry between human BMP-2 and sBMPR-IA. Gel filtration and gel electrophoresis provided direct evidence for the formation of a 1:2 complex between BMP-2 and sBMPR-IA. The purification of the defined BMP-2·BMPR-IA$_2$ complex is the prerequisite for crystallization and X-ray analysis.

2. Materials and methods

2.1. Expression and purification of recombinant BMP-2 and soluble BMPR-IA

Recombinant human BMP-2 representing the mature part of BMP-2 (residues 283–396 of the preproprotein) plus an N-terminal MA

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Abbreviations: BMP-2, bone morphogenetic protein-2; sBMPR-IA, soluble bone morphogenetic protein receptor type IA; TGF-β, transforming growth factor β; ActR-II, activin receptor type II; IL, interleukin; IFN-γ, interferon-γ; NGF, nerve growth factor; VEGF, vascular endothelial growth factor; ECD, extracellular domain; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis

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extension was expressed in *E. coli* and purified to homogeneity as previously described [21]. The molar extinction coefficient determined by means of amino acid composition analysis was $\varepsilon_{280} = 40510\ M^{-1}\ cm^{-1}$. Based on the amino acid sequence a molecular weight of 25.8 kDa was calculated for the disulphide linked BMP-2 homodimer.

The extracellular domain of human BMPR-IA (BMPR-IA ECD) comprising residues 24–152 of the preprotein [22] plus an N-terminal GSGAMA extension was expressed in *E. coli* as a soluble thioredoxin fusion protein. The gene encoding human BMPR-IA ECD was cloned into a derivative of the expression vector pET-32a (Novagen) at the *Nco*I and *Bpu*1102I restriction enzyme sites. The derivative differs from the original pET-32a through deletion of basepairs 223–297. The expression plasmid was transformed in *E. coli* strain AD494(DE3), and fusion protein synthesis was induced by addition of 1 mM isopropyl-β-D-glucopyranoside. After sonication of the cells the soluble fusion protein was prepared by chelating chromatography using a nickel column. After cleavage of the fusion protein by thrombin (2 μg thrombin per mg fusion protein) at room temperature for 4 h, the sBMPR-IA was separated from the thioredoxin by anion exchange chromatography with a fractogel EMD-TMAE column (Merck). The column was equilibrated in 20 mM Tris/HCl, pH 7.4, and bound protein was eluted with a linear NaCl gradient from 0 to 1 M NaCl. The BMPR-IA ectodomain was finally purified through chromatography on a BMP-2 affinity matrix equilibrated in 10 mM HEPES, pH 7.4, 500 mM NaCl, 3.4 mM EDTA. The matrix was prepared by binding of 10 mg BMP-2 to 10 ml BrCN activated Sepharose 6B gel (Pharmacia) according to the manufacturers instructions. Bound sBMPR-IA was eluted with 4 M $MgCl_2$. The expected N-terminus GSGAMAQNLD was confirmed by sequence analysis. The molar extinction coefficient was $\varepsilon_{280} = 4980\ M^{-1}\ cm^{-1}$ as calibrated by amino acid composition analysis. Based on the amino acid sequence a molecular weight of 14.7 kDa was calculated for sBMPR-IA.

2.2. Gel filtration chromatography and SDS–polyacrylamide gel electrophoresis

Protein samples were run at a flow rate of 0.5 ml/min on a Superdex 200 HR 10/30 column (Pharmacia). The column was equilibrated at room temperature in 20 mM Tris/HCl, pH 7.5, 700 mM NaCl. The eluant was monitored by a UV detector at a wavelength of 280 nm. Bovine serum albumin (66.4 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa) were used to calibrate the column. The void volume V_0 of the column was determined using Blue Dextran 2000. The K_{av} for the individual proteins was calculated as follows: $K_{av} = (V_R - V_0)/(V_c - V_0)$ with V_R as retention volume of the protein and V_c as the geometric bed volume in ml. A plot of K_{av} versus log MW for a standard run resulted in a straight line with a correlation coefficient $R^2 = 0.99$. Linear regression gave the equation $\log MW = -2.975 * K_{av} + 5.898$. This equation was used to calculate the apparent molecular weight. All samples were prepared in 10 mM Tris/HCl, pH 7.5, 150 mM NaCl. Mixtures of BMP-2 and sBMPR-IA were allowed to equilibrate at room temperature for 20 min before being applied to the column. All samples had protein concentrations 1.0–1.2 mg/ml and sample volumes 40–80 μl.

SDS–PAGE was performed according to the method of Laemmli [23] using 12% acrylamide gels. Densitometry of Coomassie stained gels was done using the program Scion Image, version 3b (Scion Corporation, MD, USA).

3. Results

We established an *E. coli* expression system for the extracellular domain of BMP receptor type IA (sBMPR-IA, see Section 2). After thrombin cleavage of the thioredoxin fusion protein and anion exchange chromatography we obtained routinely 2.5 mg sBMPR-IA per liter culture medium (4.2 g wet cells). Although this material is soluble and appears uniform on SDS–PAGE, reversed phase HPLC analysis showed that it is composed of several folding isomers (data not shown). Homogeneous, binding competent sBMPR-IA could only be purified by means of affinity chromatography on a BMP-2 column. The final yield is 0.5 mg sBMPR-IA per liter culture medium. The electrophoretic mobility of sBMPR-IA corresponds to an apparent molecular weight of 16 kDa under non-reducing conditions (Fig. 1). Under the same conditions (non-reducing) BMP-2 runs at about 28 kDa similar to the theoretical molecular weight of 25.8 kDa for the disulphide linked dimer. The sBMPR-IA stains 1.3-fold less intense with Coomassie than BMP-2. This can be seen by direct comparison of the sBMPR-IA band with the BMP-2 band in Fig. 1 where equal amounts were loaded on the gel (1 μg each).

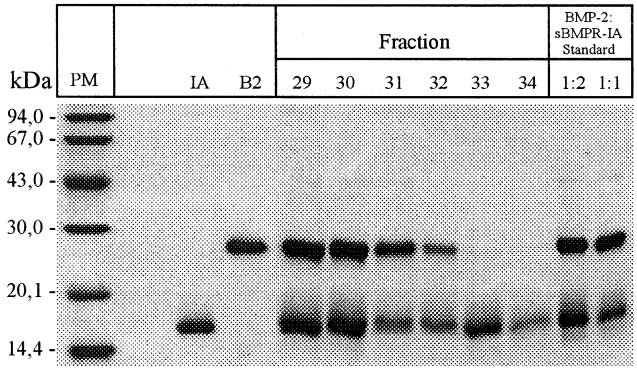

Fig. 1. SDS–PAGE under non-reducing conditions of sBMPR-IA, BMP-2 and fractions of gel filtration chromatography of a 1:3 mixture BMP-2/sBMPRIA. PM, protein marker; IA, sBMPR-IA; B2, BMP-2. Gel filtration fractions (0.5 ml) of 1:3 mixture BMP-2/sBMPR-IA are indicated with fraction number (see Fig. 2c). BMP-2:sBMPR-IA standard: standard mixtures of BMP-2/sBMPR-IA at 1:1 and 1:2 molar ratios.

This non-glycosylated sBMPR-IA provided us with suitable material for determining the stoichiometry of ligand binding and preparation of the complex with BMP-2.

The isolated components were chromatographed on the Superdex 200 HR 10/30 column. The purified sBMPR-IA protein was eluted in a uniform peak (Fig. 2a) shortly after ovalbumin (43 kDa). The apparent molecular weight of 28 kDa calculated from the elution volume is considerably larger than the theoretical molecular weight of 14.7 kDa calculated from the amino acid sequence. The possibility that sBMPR-IA forms a homodimer in solution is unlikely, because preliminary analysis of sBMPR-IA by cross-linking and by electrospray mass spectrometry did not detect an sBMPR-IA dimer (data not shown). Instead a highly asymmetric molecular shape might be responsible for the unusually large Stokes radius during gel filtration (see Section 4). Free BMP-2 was eluted shortly before the marker RNase A (Fig. 2b). Concerning the calculated molecular weight of 25.8 kDa for the disulphide linked BMP-2 homodimer its apparent molecular weight of 17 kDa was unexpected. As BMP-2 could only be recovered in maximum amount after chromatography at NaCl concentrations larger than 500 mM we suggest that the retarded elution during chromatography is due to electrostatic interactions with the gel matrix (see Section 4). We then titrated a constant amount of 1.2 nmol BMP-2 with increasing concentration of sBMPR-IA. When a 3-fold excess of sBMPR-IA over BMP-2 was present, a major peak with apparent molecular weight of 55 kDa was clearly separated from a minor peak corresponding to excess sBMPR-IA (Fig. 2c). PAGE analysis of 0.5 ml fractions taken from the gel filtration clearly shows the complex between BMP-2 and sBMPR-IA by occurrence of both proteins in fractions 29–32, whereas

the excess sBMPR-IA is seen in fractions 33 and 34 (Fig. 1). No free BMP-2 was detected, and no higher order complexes were eluted. When a 1:2 mixture of BMP-2 and sBMPR-IA was chromatographed, the whole protein eluted as a single peak (Fig. 2d). Only minor amounts of free protein were discernible as a slight shoulder. The apparent molecular weight of 53 kDa of this complex is in good agreement with the calculated molecular weight of 55 kDa for a 1:2 complex of BMP-2 and sBMPR-IA. A mixture of equimolar amounts of BMP-2 and sBMPR-IA yielded three chromatographic peaks (Fig. 2e): a major peak with apparent molecular weight of 50 kDa, a shoulder with 36 kDa and a third peak with 18 kDa corresponding to unbound BMP-2. The main protein peak eluting at $V_e = 14.4$–14.6 ml with an apparent molecular weight of 50–55 kDa is compatible with the molecular weight of the 1:2 complex. Whereas the apparent molecular weight of 36 kDa of the shoulder observed only after separation of the 1:1 mixture is similar to the molecular weight of a 1:1 complex (41 kDa). The third peak is identified as excess BMP-2 because its apparent molecular weight of 18 kDa is very close to the 17 kDa obtained for BMP-2 alone and PAGE analysis reveals that it migrates as BMP-2 (data not shown). In summary, a surplus of free receptor was obtained in the 1:3 mixture and a surplus of free BMP-2 in the 1:1 mixture. Only at the 1:2 molar ratio both proteins were completely bound into the complex.

The quantitative protein composition of the chromatographed fractions was determined by PAGE analysis and subsequent densitometry of the Coomassie stained proteins (Table 1). On the same gel 1:1 and 1:2 mixtures of BMP-2 and sBMPR-IA were analyzed. PAGE of the gel filtration fractions of the 1:3 BMP-2/sBMPR-IA mixture reveals the complex formation by coelution of BMP-2 and sBMPR-IA in fractions 29–32, corresponding to $V_e = 14.0$–16 ml (Fig. 1). The ratio of the stain of the BMP-2 band relative to the sBMPR-IA band clearly shows that the complex eluting with $V_e = 14.4$–14.6 ml (MW = 50–55 kDa) is compatible with the 1:2 complex. E.g. densitometrical analysis of the protein bands in fraction 30, which is the peak fraction, gives a ratio of 1.14 for the peak integrals of the BMP-2 band relative to the sBMPR-IA band. This is very close to the ratio of 1.13 measured for the 1:2 molar ratio of BMP-2 and sBMPR-IA. If the complex was formed with a 1:1 stoichiometry, we would expect a ratio of 2.19, which is clearly not the case (Table 1). This is a third evidence that BMP-2 forms a 1:2 complex with sBMPR-IA, besides the evidences arising from the gel filtration chromatography. The composition of the peak with shoulder in Fig. 2e is clearly different from the 1:2 complex. The ratio lies between the 1:1 and the 1:2 stoi-

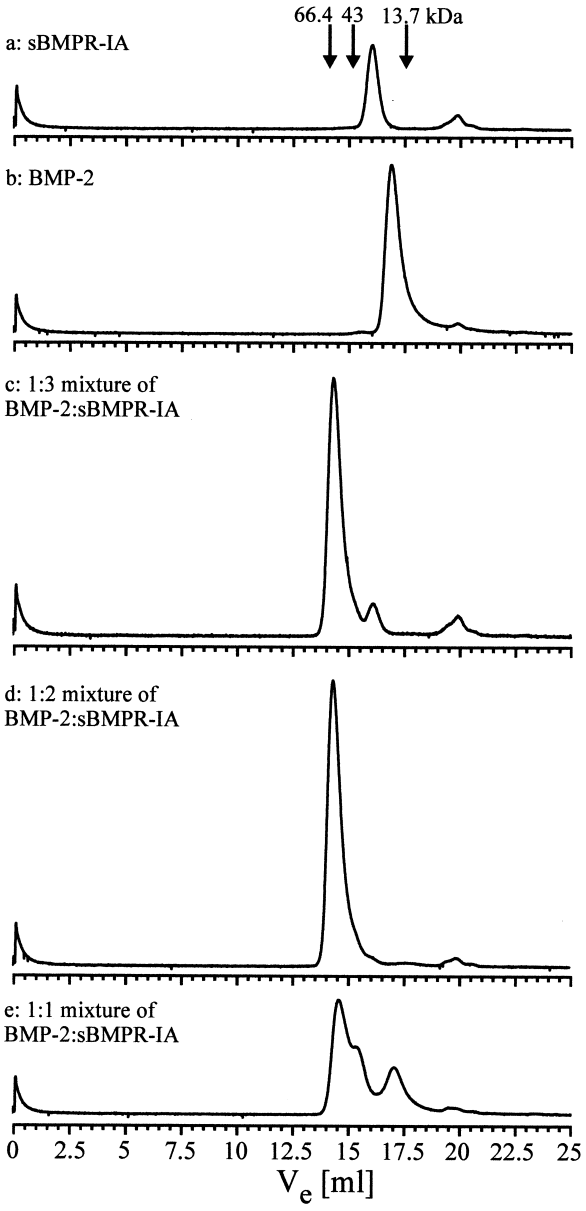

Fig. 2. Chromatographs of the gel filtration runs with A_{280} on the y-axis and elution volume V_e on the x-axis. a: sBMPR-IA; b: BMP-2; c: 1:3 mixture of BMP-2 and sBMPR-IA; d: 1:2 mixture of BMP-2 and sBMPR-IA; e: 1:1 mixture of BMP-2 and sBMPR-IA. The positions of the protein molecular weight standards albumin (66.4 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa) are indicated in panel a.

Table 1
1:2 stoichiometry of BMP-2/sBMPR-IA complex derived from densitometry of chromatographic fractions after SDS–PAGE

Run	BMP-2:sBMPR-IA ratio of peak integrals				
	Fraction			1:2 BMP-2:sBMPR-IA	1:1 BMP-2:sBMPR-IA
	29	30	31		
c: 1:3 mixture BMP-2:sBMPR-IA	1.23 ± 0.05	1.06 ± 0.02	1.46 ± 0.20	1.05 ± 0.07	1.85 ± 0.05
d: 1:2 mixture BMP-2:sBMPR-IA	1.10 ± 0.02	1.14 ± 0.07	1.50 ± 0.07	1.13 ± 0.05	2.19 ± 0.08
e: 1:1 mixture BMP-2:sBMPR-IA	1.46 ± 0.50	1.44 ± 0.09	1.71 ± 0.10	1.06 ± 0.08	2.44 ± 0.39

0.5 ml fractions of gel filtration experiments c–e presented in Fig. 2 were subjected to SDS–PAGE. The protein amount of the bands in each lane was determined by densitometry of the Coomassie blue stained gels. The ratio between the peak integrals of BMP-2 and sBMPR-IA bands was calculated. Each gel was calibrated using internal standards of 1:2 and 1:1 molar ratios of BMP-2:sBMPR-IA (see Fig. 1). All reported values are the mean of two measurements ± mean deviation.

chiometry (Table 1). The quantitative analysis is difficult because the two peaks in Fig. 2e with $V_e = 14.6–15.3$ ml display no baseline separation. Probably the major peak is the 1:2 complex and the shoulder represents the 1:1 complex.

4. Discussion

We have established an *E. coli* expression system for the extracellular domain of human BMP receptor type IA (sBMPR-IA). Due to the affinity chromatography step in the preparation the sBMPR-IA is fully binding competent. The K_D for the binding of BMP-2 to sBMPR-IA is about 1 nM as determined by biosensor measurements using immobilized sBMPR-IA [24]. A homogeneous and functional protein is critical for performing binding studies and for the preparation of a homogeneous complex with BMP-2. We also tested sBMPR-IA expressed with the baculovirus system in *Sf*9 cells. The sBMPR-IA from *Sf*9 cells is heavily glycosylated and migrates as a diffuse band with apparent molecular weight between 24 and 31 kDa, which does not permit separation from BMP-2 on PAGE (data not shown). In contrast the different electrophoretic mobilities of *E. coli* sBMPR-IA and BMP-2 clearly separate the two components during PAGE which makes densitometrical analysis possible (Fig. 1). Gel filtration on a Superdex 200 HR 10/30 column allowed the separation of BMP-2-bound sBMPR-IA from free sBMPR-IA (Fig. 2c).

Up to now structural data are only available for several TGF-β like ligands [25–30] and the free activin type II receptor ectodomain [31]. It would be very informative to have a structure of the BMP-2 ligand bound to its type I receptor. This goal has been hampered by the lack of a powerful bacterial expression system for the receptor ectodomain. Up to now only a silkworm expression system for a glycosylated mouse sBMPR-IA has been described [32]. Our *E. coli* expression system for the BMPR-IA ectodomain supplies us with milligram quantities of non-glycosylated sBMPR-IA. With the isolation of the BMP-2·sBMPR-IA_2 complex we have now established the basis for crystallization trials in order to determine the three-dimensional structure.

The present results provide 3-fold evidence that BMP-2 binds to sBMPR-IA in a defined 1:2 ratio forming a tight trimeric complex BMP-2·sBMPR-IA_2. First, titration of BMP-2 with sBMPR-IA shows that only at a 1:2 ratio of BMP-2 to sBMPR-IA there is neither excess sBMPR-IA nor excess BMP-2 detected by gel filtration analysis (Fig. 2c–e). Second, the calibration of the Superdex 200 HR 10/30 column with BSA, ovalbumin and RNase A as molecular weight standards leads to an apparent molecular weight of 50–55 kDa for the complex eluting after 14.4–14.6 ml. This is in good agreement with the theoretical value of 55 kDa for the BMP-2·sBMPR-IA_2 complex. Third, quantitative analysis of the relative amounts of BMP-2 and sBMPR-IA in this isolated complex by PAGE and densitometry confirms a 1:2 ratio of BMP-2:sBMPR-IA (Table 1). As BMP-2 is a member of the cystine knot growth factor superfamily [33,34], it is interesting to compare the receptor binding stoichiometries of other members of this protein family. For VEGF the structure of its complex with domain 2 of the Flt-1 receptor has been solved [35]. The dimeric VEGF is bound by two receptor molecules. The same holds true for the complex of NGF with domain 5 of the TrkA receptor [36]. We propose that this stoichiometry of two identical receptor chains bound by the homodimeric ligand is also shared by other members of the TGF-β superfamily.

The BMP-2 system shows similarities to the IFN-γ system. As the BMP-2 system the IFN-γ system displays two different receptor types. The structure of a complex between IFN-γ and its soluble high-affinity receptor has been solved [18]. In this complex two receptor molecules bind the IFN-γ homodimer. The binding sites place the receptor molecules apart from the 2-fold symmetry axis of the complex. We propose that this holds also true for the 1:2 complex of BMP-2 with sBMPR-IA. The X-ray structure of BMP-2 revealed two cavities at the symmetry axis of the molecule and two identical symmetry-related finger-helix cavities remote from the symmetry axis [30]. It is intriguing to speculate that the binding epitope for sBMPR-IA is near this finger-helix cavity and thus distant from the symmetry axis. In contrast, we would expect a location of the binding epitope near the symmetry axis of the dimer if a dimeric ligand binds only one receptor chain. This seems to be the case for the IL-5 system which shows binding of a single receptor chain by the homodimeric IL-5 [16].

The BMP-2·sBMPR-IA_2 complex appears to have a globular like shape. This is indicated by the good agreement between the apparent molecular weight of 50–55 kDa during gel filtration and the theoretical molecular weight of 55 kDa. Besides the size complementarity a charge compensation between BMP-2 and sBMPR-IA is observed. The sBMPR-IA has a calculated p*I* of 4.8 compared to BMP-2 with a p*I* of 8.2. So the negatively charged receptor ectodomain neutralizes or shields the positive charges of the basic BMP-2 thus preventing unspecific ionic interactions with the column material. Remarkably the chromatographic mobility of the free proteins differs from the nearly ideal behavior of the complex. The sBMPR-IA elutes too early, most likely due to an asymmetric molecule shape. The extracellular domain of the related activin type II receptor is about twice as long as thick leading to an increased Stokes radius compared to a globular protein of the same molecular weight [31]. The N-protein of phage λ shows a similar behavior during gel filtration which is due to shape asymmetry [37]. We thus predict that the structure of sBMPR-IA displays a similar shape asymmetry. The chromatographic mobility of the highly basic BMP-2 is retarded compared to the calibration proteins. As we have observed that BMP-2 is completely bound to the Superdex 200 column at a physiological salt concentration of 150 mM (data not shown) but elutes at NaCl concentrations of 700 mM, we conclude that BMP-2 makes ionic interactions with sulfate groups of agarose in the column matrix, probably through its heparin binding site [21].

It will be interesting to investigate the architecture of the BMP-2·sBMPR-IA_2 complex to determine which kind of interactions are responsible for the molecular recognition between BMP-2 and sBMPR-IA. With the isolation of the stable and homogeneous complex assembled from *E. coli* expressed recombinant proteins we have settled the basis for crystallization trials to open the door for X-ray analysis of the structure.

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