Bone morphogenetic proteins (BMPs)\(^2\) are secreted growth factors of the TGF-\(\beta\) superfamily, which play important roles during embryonic development in pattern formation and tissue specification (1). In adult tissues, BMPs are essential for tissue repair and homeostasis (2, 3). The effects of dimeric BMP depend on binding to and signaling through a receptor complex consisting of two type I and two type II serine/threonine kinase receptor chains. The type II chains transactivate the type I chains, which transduce the signal to nucleus through Smad proteins (4–6).

BMPs and other TGF-\(\beta\) like proteins have two kinds of epitopes for receptor binding. The so-called "wrist" epitope comprises residues from both monomers and binds type I receptor ectodomains (ECD). The "knuckle" epitope is constituted by one monomer only and binds type II receptor ectodomains (7, 8). Both epitopes are promiscuous, and each of them can functionally interact with several different receptor chains. Furthermore, the epitopes can have low or high affinities, depending on the ligand and the receptor chain. For instance, the BMP-2 wrist epitope binds with high affinity the type I receptors BMPR-IA and BMPR-IB, whereas at the BMP-2 knuckle epitope, type II receptors BMPR-II, ActR-II, and ActR-IIB are bound with low affinity. Remarkably, the same ActR-II and ActR-IIB receptors interact at high affinity with the corresponding epitope of activin A (ActA), whereas ActR-IB is the low affinity chain specifically for ActA (9).

The residues of BMP-2 determining affinity and specificity for type I and type II receptor binding have been identified by submitting a large array of BMP-2 mutants to Biacore interaction analysis with receptor ectodomains. The binding interface of the wrist epitope for the most part is hydrophobic (7). Of 10 hydrogen bonds occurring in the BMP-2-BMPR-IAbcd contact, only two in the center contribute to affinity. A BMP-2 L51P mutant, which has lost one of the central hydrogen bonds due to the introduced proline, lacks high affinity binding to BMPR-IA but has retained wild type affinity for BMP modulator proteins, like noggin and gremlin (10).

The binding interface of the BMP-2 knuckle epitope is also hydrophobic. A central hydrogen bond with type II receptors ActR-II and ActR-IIB can be disrupted in the BMP-2 S88A mutant with a minor effect on binding affinity (11). This central hydrogen bond is highly conserved and exists also in the ActA–ActR-IIB contact. In the activin receptor, this hydrogen bond determines high affinity binding (12). Remarkably, some BMP-2 mutants at the border of the knuckle epitope (L100K and L100K/N102D) exhibit strongly increased affinity specifically for ActR-IIB (11). In contrast, other mutations (e.g. BMP-2 A34D) disrupt binding of type II receptors, resulting in antagonistic variants (8).
The BMP signaling is subject to stringent regulation at multiple levels (13). Intracellular cofactors, such as inhibitory Smads and Smurfs, act as modulators. At the cell surface, pseudoreceptors like BAMBI can attenuate BMP signaling. Finally, a large number of modulator proteins exist in the extracellular space, which inhibit and/or enhance the receptor-mediated activity of BMPs. These proteins include noggin, follistatin, members of the Dan family proteins, and chordin-like proteins, which contain a von Willebrand factor type C (VWC) domain. The three-dimensional structure of the noggin-BMP-7 complex provides a paradigm for the mechanism of BMP inhibition by a secreted antagonist. Noggin binds as a dimer with very high affinity to the dimeric BMP-7, occluding all four binding sites of the BMP for the type I and type II receptors (14). However, for the interaction of BMPs and noggin, the functional epitope(s) (i.e. the residues determining binding affinity and specificity) are still poorly understood. In follistatin-activin complex, two follistatin monomers surround the dimeric ligand and bury the binding sites for type I and type II receptors (15, 16). Mutational analysis revealed that only residues at the type II receptor-binding surface of activin are critical for high affinity follistatin binding, and the interaction surfaces of activin for type II receptors and follistatin are overlapping but not identical (17). Furthermore, a dissection of the follistatin domain structure revealed that the unique N-terminal Fs0 domain, which as a pseudo-type I receptor contacts the wrist epitope of activin, appears to be dispensable for activin interaction (16).

Noggin, chordin, follistatin, and Dan family proteins have different specificities and affinities for different BMPs (13). Therefore, different binding epitopes are likely to exist on the BMPs. To better understand the regulatory mechanisms of these proteins, it is necessary to know how these binding epitopes are constructed, how they overlap with the receptor-binding wrist and knuckle epitopes, and whether inhibitors could be generated that are specific for each type.

In this study, we concentrated on the VWC-domain-containing proteins chordin, CHL2 (chordin-like 2), and CV2 (crossveinless 2). In addition, Tsg (twisted gastrulation), which does not contain the VWC domain but participates in the regulation of BMP functions by chordin-like proteins (Fig. 1A), was also analyzed. The VWC domain, also called the cysteine-rich domain, typically contains less than 100 residues and has in common the conserved CXCCXC and CCXXC consensuses. Otherwise, the sequences of the VWC domain are highly diverse. The VWC exists in about 500 extracellular proteins from Drosophila to human (82 proteins in Homo sapiens and 85 proteins in Mus musculus). Many VWC-containing proteins act as extracellular modulators in the BMP/TGF-β signaling pathway (18, 19). The proteins chordin, CHL2, CV2, and Tsg function via the direct binding to BMPs and play important roles in development and diseases (20–25). Chordin and Tsg regulate the dorsoventral patterning in early embryogenesis (20). CV2 plays essential pro-BMP roles in mouse organogenesis (26). CHL2 is expressed preferentially in chondrocytes of developing cartilage and osteoarthritic joint cartilage and may play negative roles in the (re)generation and maturation of articular chondrocytes in the hyaline cartilage of both developing and degenerated joints (21). Studies showed that chordin and CHL2 inhibit BMP signaling (20, 21). Tsg and CV2 are BMP-modulating proteins for which both anti- and pro-BMP activities were reported (22–24). Tsg forms a ternary complex with chordin and BMP, making chordin a better BMP inhibitor. On the other hand, Tsg facilitates the cleavage and inactivation of chordin by zinc-metalloproteinases of the Tld (Tolloid)/Xld (Xolloid) family. In this context, Tsg behaves as a pro-BMP factor (27). For Drosophila, a model has been suggested in which chordin homologue Sog (short gastrulation) and Tsg act to transport BMPs (Dpp/Scw) to the dorsal midline of the embryo. The diffusion of BMPs in the embryo in this way establishes a graded BMP activity (28, 29). The BMP/Tsg/chordin/Xolloid system has been recognized to be more complex than we understood. Several chordin-like proteins have been identified (18), Tsg activity independent of chordin and BMP binding has been found (30, 31). Recently, the sizzled protein has been found to inhibit Xolloid enzyme activity and thereby enhances chordin function (32).

In contrast to the extensive studies on the functions of chordin/Tsg and other modulator proteins in development and diseases, the structural basis for the mechanisms of BMP regulation by these proteins remains uncertain. We show in this study that the BMP-2 binding epitopes for chordin and CV2 are mainly overlapping with the knuckle epitope of BMP-2, CHL2 binds to both the wrist and knuckle epitopes of BMP-2, and Tsg binds only to the wrist epitope of BMP-2 for type I receptor. The three VWC domain-containing proteins chordin, CHL2, and CV2 regulate BMP-2 signaling via different recognition mechanisms. Furthermore, their binding to BMP-2 differs from the binding mode established for noggin/BMP7 or follistatin/activin interaction.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—All of the modulator proteins and VWC domains were expressed with a C-terminal thrombin cleavage site (LVPRGS) plus a His$_{6}$ tag in SF9 insect cells according to the manufacturer’s instructions (PharMingen). The full-length proteins included mouse chordin (amino acids 1–922), CHL2 (amino acids 1–401), and Tsg (amino acids 1–198 and zebrafish CV2 (amino acids 1–651). The VWC domains of chordin were expressed with 20 amino acids in the N- and C-terminal flanking regions (before the first and after the last cysteine, respectively; see supplemental Fig. 1). CHL2 VWC domains were expressed as follows: 7-VWC1-15, 15-VWC2-20, and 20-VWC3-16 (the numbers indicate numbers of residues in flanking sequences; see supplemental Fig. 1). Chordin-VWC1 and CHL2-VWC3 were also expressed with 5 residues in flanking sequences (supplemental Fig. 1). To determine the specificity of chordin domains for BMPs, constructs containing chordin VWC1 plus the large segment between VWC1 and VWC2 (VWC1-P) and VWC2, -3, and -4 (VWC2-3-4; Fig. 1A) were also generated. The zebrafish CV2-VWC domains are spaced very closely to each other and were therefore expressed as follows: 16-VWC1-2, 2-VWC2-2, 2-VWC3-13, 13-VWC4-11, and 11-VWC5-6. Later, a construct consisting of CV2 VWC2-3-4-5 (CV2-VWC2-5) was also expressed. The proteins were isolated from the culture medium of infected SF9 cells by standard procedures involving Ni$^{2+}$/nitrilotriacetate/
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agarose (Qiagen), directly purified or digested with thrombin (Sigma), and then purified by TMAE (Merck) or SP-Sepharose (Amersham Biosciences) ion exchange chromatography, BMP-2 affinity column (for proteins with BMP-2 binding activity), and gel filtration chromatography through a Superdex 200 HR 10/30 column (Amersham Biosciences) to apparent homogeneity.

BMP-2, GDF-5, and BMP receptor ectodomains (with His6 tag) were expressed as described (8, 11). BMP-7 was purchased from R&D.

Biosensor Interaction Analysis—The binding of BMPs/modulators was recorded on a BLAcore 2000 system (Amersham Biosciences Biosensor) as described (8, 10). For dissociation constants ($K_D$) of $<50$ nM, kinetic constants ($k_{on}$ and $k_{off}$) were evaluated. For $K_D$ values of $>50$ nM, dose-dependent equilibrium binding was evaluated. $K_D$ values or rate constants $k_{off}/k_{on}$ were evaluated from one experiment determined for 6–9 different concentrations of the analytes. Mean values of $K_D$ and their S.D. values were calculated from the values of at least three different experiments. S.D. values for the obtained affinities were less than 50%. For the comparison of the BMP-2 wild type and mutants, differences between mean values of more than 2 times S.D. were considered significant.

Co-immunoprecipitation—Immunoprecipitation was performed as described (21, 33). Briefly, the modulator proteins containing His6 tag were incubated with BMP-2 in 1 ml of binding buffer, followed by 2–5 µg/ml anti-His tag antibody (Invitrogen). The complex precipitated with 20 µl of protein A-Sepharose beads (Amersham Biosciences) was subjected to SDS-gel electrophoresis under reducing conditions, and BMP-2 was detected with anti-BMP-2 monoclonal antibody (R&D). As the controls, the modulator proteins were detected by anti-His tag antibody in parallel. To demonstrate the inhibitory effects of the modulator proteins on BMP-2 binding to the receptors, BMPR-IA and ActR-IIB ectodomains containing His6 tag were bound to BMP-2 and competed with modulator proteins, where the His6 tag has been cleaved off by thrombin (see above). The complex was co-precipitated by protein A-Sepharose.

Gel Filtration Chromatography and SDS-PAGE—The experiments were performed as described previously (34) except that β-galactosidase (130 kDa) was used additionally to calibrate the Superdex 200 column, and the equation $M_r = -2.5524 \times K_w + 5.6528$ was used to calculate the apparent molecular weight. To avoid insolubility, BMP-2 was diluted to 0.1 mg/ml, and the injected sample value was 500 µl.

Biological Activity in Cell Lines—Alkaline phosphatase (ALP) activity was determined in serum-starved C2C12 cells as described (8, 10). Inhibition of BMP-2-induced ALP activity by modulators was assessed by incubating C2C12 cells with different concentrations of modulators plus 10 nM BMP-2. Relief of CHL2, CV2, and Tsg inhibition of BMP-2-induced ALP activity was assessed by incubating C2C12 cells with 10 nM BMP-2 plus 50 nM CHL2 or CV2 or 500 nM Tsg and increasing concentrations (10–40 nM for CHL2, 6.3–25 nM for CV2, and 31–250 nM for Tsg) of BMP-2 mutants. Results are given as mean values from six determinations done in parallel for each condition.

RESULTS

Binding Affinities and Specificities of BMPs for Modulator Proteins—The binding affinities of chordin for various BMPs and GDFs have been described before (20, 33). Now we compared the binding of chordin, CV2, CHL2, and Tsg to representatives of three subfamilies of BMPs (i.e. BMP-2, BMP-7, and GDF-5) by biosensor-based interaction analysis. The $K_D$ values of BMPs/GDF to immobilized modulator proteins were in the nanomolar level (Fig. 1B). BMP-2 bound to CV2 and CHL2 with high affinities, which are similar to those of BMP-2 for its high affinity type I receptor (8). BMP-7 bound to chordin and CV2 with similar affinity as BMP-2, but it bound to CHL2 with about 20 times lower affinity than BMP-2. GDF-5, a more distant relative member of the BMP subfamily, bound to the three VWC domain-containing proteins with lower affinities than BMP-2 and BMP-7, but binding affinity to Tsg was comparable with those of BMP-2 and BMP-7. The discriminating affinities demonstrate that binding specificity exists in ligands of the BMP/GDF subgroup and the analyzed modulator proteins.

Binding Affinities and Specificities of BMPs for VWC Domains—The interactions of BMP-2 with the VWC domains in the modulator proteins were analyzed by Biacore and further confirmed by co-immunoprecipitation (Fig. 1). As shown before (33), chordin VWC domains VWC1 and VWC3 were found to bind to BMP-2 (Fig. 1, B and C). Surprisingly, however, BMP-7 bound to immobilized VWC1 with an affinity about 5 times higher than that of BMP-2 to VWC1. It bound to VWC3 very weakly but bound to VWC4 with 100-fold higher affinity (23 nM) than that of BMP-2 to VWC4 (Fig. 1B). The binding of BMP-2 to VWC4 could not be visualized in co-immunoprecipitation, probably due to the low affinity (Fig. 1, B and C); see also below, the affinity of VWC4 to immobilized BMP-2). GDF-5 bound to VWC1 with relatively lower affinity (180 nM) and to VWC3 with very low affinity (~1 µM, Fig. 1B), leading to the weaker binding of GDF-5 to whole chordin (220 nM). No binding was found between chordin VWC2 and BMP-2/-7 and GDF-5 (Fig. 1C) (data not shown). These results suggest that BMP-2 preferentially binds to VWC1 and VWC3 of chordin, BMP-7 does so to VWC1 and VWC4, and GDF-5 binds weakly to VWC1 and VWC3.

We found comparable binding affinities of BMPs when VWC domains of chordin were expressed with flanking regions of different sizes (i.e. VWC1 or VWC1-P, VWC3 or VWC2-3-4, VWC4 or VWC2-3-4 (Fig. 1B), and VWC1 with 5 or 20 residues in the flanking region (data not shown)). These results indicate that the binding epitopes are located within the VWC domains, and the connecting peptide between VWC1 and VWC2 and the other adjacent non-VWC sequences do not contribute to the binding affinities and specificities of chordin VWC domains to BMPs.

CHL2 and CV2 contain three and five VWC domains, respectively (Fig. 1A). We found that CHL2-VWC1 and CHL2-VWC3 bound to BMP-2, BMP-7, and GDF-5, but VWC2 of CHL2 did not (Fig. 1, B and C) (data not shown). Similar to the chordin-VWC1s of different sizes, the CHL2-VWC3s with 5 or 20 amino acids in the flanking region showed also the same
affinity for BMPs/GDF (data not shown). VWC1 and VWC3 of CHL2 bound to BMP-2 separately with about 100 and 20 times lower affinity compared with full-length CHL2. This indicates that the affinity of CHL2 to BMP-2 is the sum of the cooperative binding of two VWC domains. Interestingly, VWC1 and VWC3 of CHL2 bound to BMP-7 with affinity similar to or slightly weaker than that of full-length CHL2. In contrast, in the full-length CHL2, VWC1 seems to account for most of the binding affinity for GDF-5.

The binding of CV2 to BMP-2/-7 and GDF-5 occurs via its N-terminal segment containing VWC1–5 (24), but which of the individual domains was involved was not known. To our surprise, now we found that the VWC1 domain of CV2 was the only one of the five existing VWC domains that bound to BMPs (Fig. 1, B and C). CV2-VWC1 bound to BMP-2/-7 and GDF-5 with affinities similar to those of full-length CV2 (Fig. 1B) (24). The VWC domains 2, 3, 4, and 5 and the fragment VWC2-5 had no binding affinity for these ligands (data not shown and Fig. 1C). This indicates that the binding occurs only via CV2-VWC1.

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FIGURE 1. Binding affinities and specificities of BMPs for modulator proteins. A, domain composition of VWC-containing proteins and Tsg. The blue boxes indicate signal peptide. VWD, Von Willebrand factor type D domain; TIL, trypsin inhibitor-like cysteine-rich domain; N and C, the N- and C-terminal domain of Tsg, respectively. B, binding affinities of BMPs for modulator proteins immobilized on the Biosensor chips. Values of the apparent $K_D$ are classified into five categories. Full, full-length protein; VWC1-P, chordin VWC1 plus the fragment between VWC1 and VWC2; VWC2-3-4, chordin fragment consisting of VWC2, -3, and -4. a, the dissociation constant of GDF-5 for VWC3 of CHL2 was estimated from equilibrium binding using the equation, $K_D = (RU_{\text{max}} - RU)RU/\text{concentration}$. B, Western blot analysis of BMP-2 bound to the individual VWCs after immunoprecipitation (IP) with anti-His tag antibody. BMP-2 (200 ng in 1 ml of IP buffer) was mixed with VWC-His proteins at the indicated concentrations. The co-immunoprecipitates were separated into two sets; one was loaded on a SDS-gel under reducing condition to visualize BMP-2 (top) and the other was loaded to detect VWC-His (bottom). BMP-2 standards (the first and last lanes in the upper panel) were loaded directly. As controls, full-length CV2 (Full) and CV2-VWC2-5 were also analyzed. The full-length CV2 protein was a cleavage-resistant mutant in which the original proteolytic site was destroyed (CV2-CM). This mutant has the same binding property as wild-type CV2 (see Ref. 24).
structure of the ternary complex of BMP-2-BMPR-IA-ActR-IIB (11), six variants mutated in the wrist epitope and seven variants mutated in the knuckle epitope (8, 10) (Fig. 2A) were selected for analyzing the interaction with modulator proteins by Biacore. The binding characteristics of the five wrist and three knuckle epitope mutants for different receptor ectodomains have been described elsewhere (8, 10). The additional mutants S88P, V98P, and L100P bound type II receptors with reduced affinities (supplemental Table 1). The M106A mutant exhibited a reduced affinity for type I receptors (supplemental Table 1). It has been shown that the corresponding mutant of M108A in activin behaved as an activin antagonist, possibly due to its disrupted binding to type I receptor ActR-IB (35).

The decreases of the binding affinities between BMP-2 mutants and immobilized modulator proteins indicated the influences of the amino acid substitutions in the wrist and knuckle epitopes for binding. This influence could be grouped into three categories (Fig. 2B): first, affected by wrist but not knuckle epitope mutation (Tsg); second, affected mainly by knuckle epitope mutation but also by the mutation of M106A (chordin and CV2); third, affected by both wrist and knuckle epitope mutations (CHL2). The mutants F49A, P50A, A52R, and S69R showed 2.4–6.1-fold lower affinities to Tsg compared with wild type BMP-2. M106A-BMP-2 exhibited a 36-fold lower affinity to Tsg. All of the mutants in the knuckle epitope bound to Tsg normally. These results suggest that Tsg uses determinants in the wrist epitope of BMP-2 for binding. Met106 could be a main determinant for Tsg binding.

Many of the knuckle epitope mutants bound to chordin and CV2 with reduced affinity, suggesting that the binding epitopes of BMP-2 for chordin and CV2 overlap with the knuckle epitope. M106A, a wrist epitope mutant, also exhibited 4.1- and 11-fold lower affinities to CV2 and chordin, respectively. These results suggest that the epitopes of BMP-2 for chordin and CV2 binding are also partially overlapping with the wrist epitope. Remarkably, the variant L100K exhibited a dramatic decreased affinity for CV2 compared with that of L100P. This effect was even more pronounced for a L100K/N102D double mutant. When variants L100K and L100K/N102D were immobilized on the chip, the binding of CV2 to these mutants was not measurable (data not shown).

Together, these results demonstrate that CV2 binds to the BMP-2 knuckle epitope mainly by hydrophobic interactions, and Leu100 of BMP-2 is a hot spot for CV2 binding.

FIGURE 2. Mutational analysis of BMP-2 interaction with modulator proteins. A, residues in the wrist (blue) and knuckle (red) epitopes of BMP-2, which were selected in the mutational analysis. The small ribbon model presents a view of BMP-2 along the 2-fold axis and shows the location of the wrist and knuckle epitopes. B, relative affinities showing the influence of the mutations on binding. The binding affinities (in parentheses) of wild-type BMP-2 (WT) for full-length modulator (Full) or their VWC domains are set to 1. Values of relative $K_D$ are classified into six categories. W, wrist epitope; K, knuckle epitope. a, the original dissociation constants of BMP-2 A34D for chordin and its two VWC domains were estimated from equilibrium binding using the equation $K_D^\text{app} = (RU_{\text{max}} - RU)/RU \times$ concentration.

Table 1). It has been shown that the corresponding mutant of M108A in activin behaved as an activin antagonist, possibly due to its disrupted binding to type I receptor ActR-IB (35).
The binding epitope of BMP-2 for CHL2 seems to be different from those of BMP-2 for chordin and CV2. CHL2 bound many of the tested wrist and knuckle epitope mutants with reduced binding affinity. Although the changes of affinity for the wrist epitope mutants were mild, the results clearly indicated that CHL2 bound to both the knuckle and wrist epitopes of BMP-2.

The epitopes of BMP-2 for single VWC domains of chordin and CV2 are similar to those of BMP-2 for the whole molecules of these proteins (Fig. 2B). However, the situation for CHL2 is different. The relative affinity of BMP-2 mutants for VWC1 and VWC3 of CHL2 clearly indicated that the residues in the wrist epitope bound to CHL2-VWC3 and the residues in the knuckle epitope were involved in CHL2-VWC1 binding. The decreases of affinities of the variants to full-length CHL2 were lower than those to its VWC1 or VWC3 domain. This might be due to the compensating effects of two cooperative VWC domains in the CHL2 molecule. This assumption is supported by the observation that CV2-VWC1 showed similar decreased affinities to some of the BMP-2 variants like those for full-length CV2, because CV2-VWC1 alone governs all of the binding affinity in CV2 (Fig. 1B).

The VWC domains of chordin, CHL2, and CV2 exhibited different binding characteristics. Most of them bound mainly to the knuckle epitope, and only VWC3 of CHL2 bound to the wrist epitope. The binding determinants of BMP-2 for the VWC domains were also different. For example, Ala100 and Leu100 of BMP-2 were hot spots for CHL2-VWC1 and CV2-VWC1 binding, respectively, whereas Met106 was a hot spot for the binding of chordin-VWC1. Met106 was also an important residue for CHL2-VWC3 binding but played only a minor role in CV2-VWC1 binding. M106A-BMP-2 bound to chordin-VWC1 with 10 times lower affinity than to chordin-VWC3, although the other determinants of the BMP-2 knuckle epitope for chordin-VWC1 and -VWC3 were very similar. In addition, the findings that Met106 was an important binding determinant for both chordin-VWC1 and Tsg in the binary interactions of BMP-2/chordin-VWC1 and BMP-2/Tsg raise the possibility that the assembly of BMP-2-chordin-Tsg in the ternary interaction is different from that in the binary interactions.

**FIGURE 3. Stoichiometry of BMP-2/modulator interaction.** A, binding affinities of full-length modulator proteins (Full) or their domains for immobilized BMP-2 (in nM). The discrepancy between the affinities measured with immobilized BMP-2 and immobilized modulator (see Fig. 1B) is shown as theKD ratio. The binding affinities and ratios are classified into four and three categories, respectively. B, scheme illustrates the 1:1 (a, b, d, and f) and 2:1 (c and e) interactions of modulators and BMP-2. BMP-2 binds to chordin by a 1:1 interaction, irrespective of whether BMP-2 or chordin is immobilized on the chip (a and b). The immobilized CV2 or CHL2 molecules are “coupled” that bind to the BMP-2 dimer by 2:1 interaction (c and e). The CV2 or CHL2 molecules in solution are not “coupled” that bind to immobilized BMP-2 separately by 1:1 interaction. The second CV2 or CHL2 molecule in solution is omitted to emphasize the 1:1 interaction (d and f).

The hypothesis that chordin binds to BMP-2 in a 1:1 interaction fits to our findings that both VWC1 and VWC3 of chordin bound mainly to the knuckle epitope of BMP-2. The 2:1 interaction model of CV2/BMP-2 binding also fits to the notion that CV2 bound to the knuckle epitope of BMP-2 via its single VWC1 domain. Our findings that VWC1 and VWC3 of CHL2 bound to the knuckle and wrist epitopes of BMP-2, respectively,
fit to the model of 2:1 interaction of CHL2/BMP-2 in Fig. 3B, because two wrist and two knuckle epitopes exist in the BMP-2 dimer. The 2:1 stoichiometry of the CHL2-BMP-2 complex was also confirmed by gel filtration chromatography and SDS-PAGE (see supplemental Fig. 2).

Competition of Modulator Proteins with Receptors for BMP-2

Binding—It has been shown that CHL2, CV2, chordin, and Tsg inhibited BMP activity in a competitive manner in vitro (21, 25, 33, 36). Co-precipitation experiment showed that CHL2 and chordin competed with type I receptors (BMPR-IA or BMPR-
IB) for BMP-4 binding (21, 33). However, their competing capabilities for type II receptors are unknown. Since chordin, CV2, Tsg, and CHL2 bind specifically to the knuckle or wrist epitope of BMP2 or both of them, we investigated by means of Biacore methodology whether they inhibit binding of BMP-2 to type I or type II receptors.

As shown in Fig. 4, A and B, and summarized in Fig. 4C, 100–150 nM chordin, CV2, or CHL2 prevented completely the binding of 50 nM BMP-2 to immobilized BMPR-IA (Fig. 4A, a–c) or ActR-IIB (Fig. 4B, a–c), indicating that chordin, CV2, and CHL2 inhibited the binding of BMP-2 to both type I and type II receptors. In contrast, Tsg did not inhibit the binding of BMP-2 to ActR-IIB (compare Fig. 4B, d and j). It inhibited the binding of BMP-2 to BMPR-IA, but only at a 20-fold molar excess (Fig. 4A, d). The effects of CHL2 and Tsg were in good agreement with the results of the mutational analysis (Fig. 2B).

It was unexpected that chordin and CV2 inhibited binding of BMP-2 to both BMPR-IA and ActR-IIB. The results of the mutant array analysis predicted an overlapping of their binding epitopes mainly with the knuckle epitope for type II receptors. In contrast, Tsg did not inhibit the binding of BMP-2 to BMPR-IA and ActR-IIB, but only at a 20-fold molar excess (Fig. 4A, d). The inhibitory effects of CHL2 and Tsg were in good agreement with the results of the mutational analysis (Fig. 2B).

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FIGURE 5. Inhibitory properties of modulator proteins and their VWC domains. Inhibition of the 10 nM BMP-2-induced alkaline phosphatase (ALP) activity by increasing doses of modulator proteins was assayed in serum-starved C2C12 cells. Dose-response curves were determined for CHL2 (open circles), CV2 (filled squares), CV2-VWC1 (open diamond), Tsg (filled triangles), chordin-VWC1 (open triangles), chordin-VWC3 (filled circles), CHL2-VWC3 (open squares), and CHL2-VWC1 (filled stars). The dotted lines indicate the IC$_{50}$ values.

This suggests that CHL2-VWC3 and the type II receptor can simultaneously bind to BMP-2.

Together, these results indicate that the single VWC domains are able to inhibit the binding of BMP-2 to type I and/or type II receptors in vitro, and their inhibitory efficiency correlates with the binding affinities for BMP-2.

Inhibition of BMP-2 Signaling by Modulator Proteins and Their Domains—The inhibitory capability of the modulator proteins was assayed in C2C12 cells (10). CHL2 and CV2 inhibited alkaline phosphatase (ALP) activity induced by 10 nM BMP-2 with IC$_{50}$ values of 9 and 11 nM, similar to that of chordin (21). The IC$_{50}$ value of Tsg was about 8 times higher (80 nM) than those of CHL2 and CV2 (Fig. 5). This is consistent with the observations that Tsg had the lowest potency in inhibiting BMP-2/BMPR-IA interaction and could not inhibit the BMP-2/ActR-IIB binding (see Fig. 4).

The five tested VWC domains exhibited different inhibitory capabilities. CV2-VWC1 was able to inhibit BMP-2 signaling, but VWC1 and VWC3 of chordin and VWC1 and VWC3 of CHL2 showed no inhibitory activity up to a concentration of 1 mM (Fig. 5). The IC$_{50}$ value of CV2-VWC1 was about 40 nM. This is 4 times higher than that of full-length CV2 (9 nM). In Biacore interaction analysis, CV2-VWC1 bound to BMP-2 with the same affinity as CV2 and showed similar inhibitory activity in the in vitro receptor competition experiment (Fig. 4). Therefore, the different inhibitory capabilities of the CV2-VWC1 domain and the full-length CV2 suggested that other parts of the whole CV2 molecular played a role in the inhibition.

Interestingly, the chordin-VWC1 domain, which at a concentration of 1 mM inhibited BMP-2/BMPR-IA and BMP-2/ActR-IIB interactions in the in vitro competition experiment (Fig. 4), could not inhibit BMP-2-induced ALP activity at the corresponding concentration (Fig. 5). Thus, also for the single VWC domain, the in vitro and in vivo activities were not strictly correlated. The reason might be that the equilibrium binding of
BMP-2-chordin-VWC1 in solution perfused over the receptors on the Biacore chip was in steady state, and the same interaction in the cell culture was a dynamic process during 3 days. The receptors exist in the cell as a quaternary receptor complex (two type I and two type II receptors). There are also potential coreceptors on the cell surface. Therefore, the overall binding affinity of BMP-2 and receptors in the cell would be much higher than that of BMP-2 and the individual receptor on the Biacore chip. The longer binding half-life of BMP-2 receptor, the ligand-receptor internalization, and the receptor recycling process could shift the equilibrium of BMP-2/chordin-VWC1 binding (in low affinity) to the direction of BMP-2/receptor binding and signaling during the 3 days.

BMP-2 Variants Inhibiting Modulator Proteins—In previous experiments, a “receptor (BMPR-IA)-dead” mutant BMP-2-L51P has been generated (10). This mutant did not bind BMPR-IA but bound to type II receptors normally. It did not induce measurable ALP activity in C2C12 or ADTC5 cells and has no dominant-negative inhibitory effect. The L51P mutant bound normally to noggin (10), chordin, CHL2, CV2, and Tsg (Fig. 2B). It could release inhibition of BMP-2-induced ALP activity by noggin (10) and also by CHL2, CV2, and Tsg in a dose-dependent manner as shown in Fig. 6. Now, five double or triple mutants of BMP-2 were generated, in which the knuckle epitope mutants A34D, L90A, V98P, L100P, and L100K/N102D were introduced in the background of the L51P mutation. These mutants showed a dramatic reduced affinity for BMPR-IA; decreased affinities for BMPR-II, ActR-II, and ActR-IIB; normal or decreased affinities for CHL2 and CV2; and normal affinity for Tsg in Biacore measurement (data not shown), as expected from the properties of the single mutants (8, 10) (Fig. 2B and supplemental Table 1). The inhibition of BMP-2 activity by CHL2 could be partially relieved to different extents by the five tested mutants (Fig. 6A). The biological activity of the mutants (i.e. release of CHL2 inhibition) correlates with their binding affinities to CHL2 (see Fig. 2B). When CV2 was tested in the same way, 25 nM L51P could relieve completely the inhibition of 10 nM BMP-2-induced ALP activity by CV2; this release was also complete for the L51P/V98P double mutant, slightly weaker for L51P/L90A, significantly weaker for L51P/
L100P and L51P/A34D, and completely lost for L51P/L100P/K102D mutant (Fig. 6B). This pattern is in good agreement with the binding analysis (see Fig. 2B). Similarly, the Tsg inhibition of BMP-2-induced ALP activity could be rescued by the L51P mutant, but only partially rescued by the P50A mutant, which alone did not induce ALP activity and bound to Tsg with 6 times lower affinity (Figs. 2B and 6C) (8). Together, these results open the possibility that BMP-2 mutants can be constructed that specifically block single modulator proteins.

**Tsg Forms a Ternary Complex with BMP-2 and Makes CHL2 a Better BMP-2 Antagonist**—We found that Tsg bound to CHL2 with high affinity (Table 1), and a Tsg/CHL2/BMP-2 complex could be isolated by gel filtration chromatography (Fig. 7A). A biological assay showed that Tsg and CHL2 synergistically inhibited BMP-2-induced ALP activity (Fig. 7B). Therefore, similar to chordin, CHL2 can form a ternary complex with Tsg and BMP-2, and this makes CHL2 a better BMP-2 antagonist. Interestingly, only the BMP binding VWC domains of chordin and CHL2 bound to Tsg (Table 1 and data not shown), indicating that the binding sites of chordin and CHL2 for BMP and Tsg are very close, and this might be the basis for generating a high affinity ternary complex. Tsg bound to CHL2 with a 60 times higher binding affinity (24 nM) than to chordin (1200 nM). It had a 10–30 times higher affinity to full-length CHL2 than to the single VWC1 or VWC3 domain of CHL2, whereas Tsg bound with similar affinities to full-length chordin or to the single VWC1 or VWC3 domain of chordin (see Table 1, $K_D$ values for perfused Tsg). The probable 1:2 interaction of chordin with immobilized Tsg showed a 25 times higher affinity (50 nM) than that of the 1:1 interaction of Tsg with immobilized chordin (1200 nM), whereas the binding affinity of CHL2 to Tsg was similar in both experimental setups (Table 1). These results suggest that Tsg binding to VWC1 and VWC3 domains in full-length CHL2 is cooperative, and Tsg binding to the VWC domains in full-length chordin is independent (see model in Fig. 8). In addition, we did not observe binding of Tsg to CV2 or its single VWC domains (data not shown).

**DISCUSSION**

Chordin-like proteins play important roles in development and diseases. To understand the recognition mechanisms of VWC domain-containing proteins for BMPs, we analyzed in detail the binding affinities and specificities of BMP-2 for chordin, CHL2, CV2, some of their VWC domains, and Tsg and identified the binding epitopes of BMP-2 specifically for the modulator proteins.

We found that three of the four VWC domains of chordin, two of the three VWCs of CHL2, and one of the five VWCs of CV2 bind to BMPs. CV2 binds to BMP-2 via its VWC1 domain with high affinity. CHL2 and chordin bind to BMP-2 with similar affinities, but these affinities are the sum of the binding of at least two VWC domains to BMP-2. Thus, the VWC domain, like CV2-VWC1, governs all of the binding affinity of a VWC-containing protein to BMP. VWC domains like chordin and CHL2 VWCs, which bind separately to BMP with relatively low affinity, cooperate in binding to generate a high affinity.

Chordin, CV2, and CHL2 all bind to BMPs via their VWC domains, but not all of the VWC domains are involved in BMP binding. It is still unknown if these “silent” VWCs exert a purely
structural role or if they interact with other not yet identified proteins, but in any case, the present results indicate that the VWC domains are versatile binding modules that can exhibit multiple binding characteristics. They bind not only to BMPs but also to Tsg. They show different affinities and specificities to different BMPs. Many VWC domains bind mainly to knuckle epitope of BMP-2, but CHL2-VWC3, for instance, binds preferentially to the wrist epitope of BMP-2. Moreover, even for the same epitope of BMP-2, different determinants are found for different VWCs. It will be interesting to know how the epitopes of the VWC domain for BMP binding are constructed, because there are more than 500 VWC domain-containing proteins, and besides the cysteine pattern, no clear homology is found for these domains in alignment. It is tempting to speculate that the numerous disulfide bonds of VWC provide a scaffold for the attachments of a diversity of loops that can generate different binding specificities similar to an immunoglobulin. In the conotoxin superfamily comprising many hundreds of members with a disulfide-rich core, a similar principle has been established (38, 39).

Our in vitro results showed that the binding affinity and specificity of BMPs to different forms of chordin VWC domains are not influenced by the adjacent non-VWC sequences (Fig. 1B). A previous in vivo genetic study revealed that different combinations of the Sog (chordin homologue in Drosophila) VWC domains and/or VWCs with adjacent non-VWC fragment had three types of Sog activity. The intact Sog inhibited the signaling of BMP-7 homologue Gbb. The so-called Supersog, consisting of VWC1 and part or complete segment between VWC1 and VWC2, blocked the effects of both Gbb and BMP-2/4 homologue Dpp. A combination of VWC1-segment-VWC2 promoted BMP activity (40). It seems that the VWC domains interact with adjacent non-VWC sequences to create forms of Sog with distinct BMP modulatory activities in vivo. The possibility that the VWC domains alone bind to BMPs with little selectivity and that the surrounding non-VWC sequences may provide specificity by interacting with only a subset of BMPs (40) is not supported by our results. We found that binding specificities are determined on the level of individual VWC domains. BMP-2 preferentially binds to VWC1 and VWC3, and BMP-7 does so to VWC1 and VWC4. These results are consistent with the findings of genetic studies with Sog showing that VWC1 of Sog inhibited Dpp and VWC4 interfered selectively with Gbb (40). Therefore, the effect of adjacent non-VWC sequences on in vivo Sog activities seems not to be generated by their binding specificities for BMPs. Rather, it may be that the adjacent sequence interacts with other proteins involved in BMP/chordin regulation (e.g. heparan sulfate proteoglycan or integrins) (41, 42). How the specificity of VWCs for BMPs contributes to the regulation of BMP function by chordin in vivo remains to be investigated in future studies.

Our experiments showed that CV2 recognizes BMP-2 by a mechanism that differs from chordin/BMP-2 and CHL2/BMP-2 interaction in two aspects (Fig. 8). First, CV2 binds to BMP-2 via its first VWC domain with high affinity, which is comparable with the cooperative binding of two VWC domains in complete chordin or CHL2. Second, Tsg does not participate in CV2/BMP-2 interaction. Similar to full-length CV2, CV2-VWC1 could simultaneously block the binding of BMP-2 to both type I and type II receptors. The 4-fold lower inhibitory capacity of CV2-VWC1 in the cell assay compared with complete CV2 was probably caused by the lack of the steric hindrance from other parts of CV2. Previous studies showed that CV2 could be a pro- or anti-BMP factor, depending on different contexts (24–26, 43, 44). In vitro, CV2 at excessive doses inhibited the BMP-dependent differentiation of osteoblast and chondrocyte in cell culture (25). The transfection of 293T cells with a CV2 cDNA-containing plasmid reduced cellular response to BMP-4 in a BMP-responding luciferase reporter assay (43). These results are consistent with our observation that excessive doses of CV2 inhibited BMP-2-induced ALP activity. However, in vivo studies showed that CV2 is an essential pro-BMP regulator of BMP signaling during zebrafish gastrulation (24) and in mouse organogenesis (26). Although the mechanism of the in vivo pro-BMP activity of CV2 remains uncertain, a model for positive feedback is intriguing (45). In this model, molecules like CV2 provide positive feedback to produce a spatial bistability in which BMP binding and signaling capabilities are high in the dorsal-most cells and low in

![FIGURE 8. Models of different modes of BMP2-modulator protein complex formation. A, BMP2 signaling complex with type I and type II receptors (as referred to in Ref. 8). B, BMP2-chordin-Tsg complex (as referred to in Ref. 27). C and D, BMP2-CHL2-Tsg complex. E, BMP2-CV2 complex. The cyan box in E indicates the C-terminal domain of CV2. VW, von Willebrand factor type C domain.](image-url)
lateral cells of Drosophila (46). The positive feedback could be reached when the cell surface-associated CV2 helps to present ligand to the signaling receptors. Indeed, CV2 is associated with the cell surface via the binding to heparin (24). Our findings that CV2 binds mainly to the knuckle epitope of BMP-2 with an affinity similar to that of the BMP-2/BMPR-IA binding support this model in that the membrane-associated CV2 could first present BMP-2 to type I receptor and then be replaced by the type II receptor, which is recruited into the receptor complex. Additionally, the functions of the four other VWC domains and the long C terminus of the large CV2 molecule are still unknown. Elucidation of their functions may help to explain the complex anti- and pro-BMP activities of CV2 in early embryogenesis.

The binding mode of CHL2/BMP-2 seems to follow the follistatin/activin model, in which two CHL2 monomers surround the BMP-2 dimer (Fig. 8C). However, we cannot exclude the possibility that each CHL2 monomer binds to one side of the butterfly-shaped BMP-2 (Fig. 8D), but in any case, Tsg, which binds to VWC1 and VWC3 of CHL2 in a cooperative way, plays a role in strengthening the binding affinity. A study (21) showed that CHL2 inhibited BMP functions in vitro and in vivo. Our result indicates that this inhibition must be enhanced by the presence of Tsg. Recent results from Tsg-deficient mice indicated a critical role of Tsg in skeletal development of vertebrates (47–50). Tsg has also been shown to be an important modulator of BMP-regulated cartilage development and chondrocyte differentiation (51). However, whether Tsg exerts its regulating function in cartilage through the interaction with chordin remains questionable, because chordin is absent from resting chondrocytes (51). In situ hybridization studies of the developing mouse skeleton showed the absence of chordin from cartilage proper (52, 53). Thus, at least in the regulation of BMP activity by Tsg in cartilage, other modulator proteins might be at work. CHL2 could be one of these proteins, since CHL2 is co-expressed with Tsg in cartilage (21, 51) and they bind each other with high affinity. Further studies will have to show the in vivo relevance of the Tsg/CHL2 interaction and whether it leads to a pro- or anti-BMP activity. It seems unlikely that CHL2 is cleaved by Xolloid protease like chordin, since no Xolloid cleavage site is found in the CHL2 sequence. This could mean an important difference between the BMP-2/Tsg/chordin and BMP-2:Tsg-CHL2 complexes. It remains to be seen whether CHL2 could be cleaved by other proteases. Otherwise, the formation of a BMP-CHL2:Tsg complex would only result in a strong anti-BMP activity differing from the formation of the BMP-chordin:Tsg complex, which plays a role as transporter, and thereby promote BMP activity in this context (20, 28, 29).

The results in this study open the possibility of generating BMP-2 mutants as specific antagonists for VWC domains (Fig. 6). Studies showed that some of the VWC-containing proteins are involved in the pathological process. For example, CTGF, which inhibits BMP signaling and promotes TGF-β activity via the binding of its VWC domain to BMP-4 and TGF-β (19), has been shown playing a very important role in fibrotic diseases (54, 55). The multi-VWC domain-containing protein Kielin could attenuate the pathology of renal fibrotic disease by enhancing BMP signaling while suppressing TGF-β activation (56, 57). The functions of the VWC-containing proteins in these processes must be properly regulated. The antagonist specifically for the defined VWCs might be used in the therapeutic intervention of the VWC-containing protein-related diseases.

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