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The anti-Müllerian hormone prodomain is displaced from the hormone/prodomain complex upon bivalent binding to the hormone receptor

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ABSTRACT

Noncovalent complexes of TGF-β family growth/differentiation factors with their prodomains are classified as latent or active, depending on whether the complexes can bind their respective receptors. For the anti-Müllerian hormone (AMH), the hormone/prodomain complex is active, and the prodomain is displaced upon binding to its type II receptor, AMHR2, on the cell surface. However, the mechanism by which this displacement occurs is unclear. Here, we used ELISA assays to measure the dependence of prodomain displacement on AMH concentration, and analyzed results with respect to the behavior expected for reversible binding in combination with ligand-induced receptor dimerization. We found that, in solution, the prodomain has a high affinity for the growth factor ($K_d = 0.4\ pM$). Binding of the AMH complex to a single AMHR2 molecule does not affect this $K_d$ and does not induce prodomain displacement, indicating that the receptor binding site in the AMH complex is fully accessible to AMHR2. However, recruitment of a second AMHR2 molecule to bind the ligand bivalently leads to a 1000-fold increase in the $K_d$ for the AMH complex, resulting in rapid release of the prodomain. Displacement occurs only if the AMHR2 is presented on a surface, indicating that prodomain displacement is caused by a conformational change in the growth factor induced by bivalent binding to AMHR2. In addition, we demonstrate that the BMP-7 prodomain is displaced from the complex with its growth factor by a similar process, suggesting that this may represent a general mechanism for receptor-mediated prodomain displacement in this ligand family.

INTRODUCTION

The transforming growth factor (TGF)-β superfamily of growth factors regulate many aspects of cell growth and differentiation (reviewed in 1). Consistent with the important roles played by these factors, a number of regulatory mechanisms have evolved that control access of TGF-β family members to their receptors (2). One important regulatory mechanism involves post-translational proteolytic processing. TGF-β family ligands are translated as dimeric precursor proteins comprising two polypeptide chains, each containing an N-terminal prodomain and a smaller C-terminal growth factor domain, which must undergo cleavage at dibasic or monobasic sites located between the two domains to generate the mature growth factor. After proteolytic processing at this site, the prodomain and growth factor remain noncovalently associated in a complex. In some TGF-β members, the prodomains block access of the growth factors to their type I and II receptors and render the complexes latent: TGF-β (3,4), GDF-8 (5) and BMP-2 (6). In other TGF-β members, the prodomains do not block access to receptors: The noncovalent complexes of BMP-7 (7) and BMP-9 (8) have been shown to be active in biological assays.

The X-ray structure of the latent TGF-β1 complex has revealed that it is in a cross-armed conformation and that the prodomain shields the growth factor from recognition by receptors and alters its conformation (9). In contrast, the non-latent BMP-9 complex was found to have an open-armed conformation, with a number of different interactions between the prodomain and the growth factor that may permit stepwise displacement of the prodomain by the type I and II receptors (10). Subsequently, the structures of complexes of other TGF-β family members have indicated that the relationship between the conformation of the complexes and latency is more complicated. The non-latent activin A complex has an intermediate conformation between cross-armed and open-armed, with some interactions between the prodomain and
growth factor more similar to those in TGF-β1 (11). On the other hand, the latent GDF-8 complex is in an open-armed conformation, with latency conferred by a number of distinct features that stabilize the complex (12).

Anti-Müllerian hormone (AMH), also called Müllerian inhibiting substance (MIS), is a member of the TGF-β family involved in male and female reproductive development (13). AMH is responsible for the regression of Müllerian ducts in the male fetus; in the female fetus, the Müllerian ducts develop into the uterus, Fallopian tubes, and vagina (14). In the adult, AMH plays a role in Leydig cell differentiation and function (15,16) and follicular development (17). AMH also has been shown to have potential roles within the nervous system (18,19) and the hypothalamic-pituitary-gonadal axis (20,21). AMH is synthesized as a large homodimeric precursor, which undergoes an obligatory proteolytic cleavage, generating a noncovalent complex that, similar to the BMP-7 and BMP-9, is biologically active (22,23). Furthermore, the AMH complex can bind its type II receptor, AMHR2, which leads to displacement of the prodomain (24) (Fig. 1A). Prodomain displacement by receptors has also been shown for BMP-7 (7) and BMP-9 (25). AMHR2 is one of only five type II receptors that must accommodate the thirty plus TGF-β ligands, so sharing receptors is a common feature of the TGF-β family. Uniquely for the family, AMH and AMHR2 have a monogamous relationship. The crystal structure of AMH bound to AMHR2 has revealed the basis for this specificity (26).

In the present study, we have investigated the nature of the interaction between the AMH complex and AMHR2 and the requirements for displacement of the prodomain. We find that binding of AMH complex to AMHR2 when it is on a surface, increases the $K_d$ for dissociation of the complex by a factor of over 1000, leading to rapid release of the prodomain. Displacement of the prodomain correlates with bivalent binding of the AMH complex by two molecules of AMHR2. By fitting our data to the relationships that have been derived for ligand induced receptor dimerization (27), we are able to obtain values for the affinity of the initial, monovalent binding of AMH to AMHR2 ($K_1$) and for the subsequent recruitment of a second AMHR2 molecule to form a bivalent interaction (driven by $K_2$), as well as for the dissociation constants for the AMH complex when bound monovalently and bivalently by the receptor ($K_{mono}$ and $K_{bi}$; Fig. 1A).

RESULTS

Surface-captured AMHR2 discriminates between intact AMH complex and the mature growth factor, and promotes prodomain displacement. We have previously shown that when the cleaved noncovalent AMH complex binds to AMHR2 on cells, or to soluble AMHR2 receptor coupled to Sepharose, the prodomain is displaced from the complex leaving the mature growth factor (GF) bound to receptor (24). To quantitatively characterize these events, we developed assays to assess the binding affinity of AMH for AMHR2 under various conditions, and to measure the $K_d$ for dissociation of the AMH complex into separate pro- and GF domains in solution or when bound to AMHR2. For these experiments we used a recombinant form of AMH that is plasmin-cleaved to give 100% noncovalent complex (referred to as c-AMH; Fig. 1B). We employed a soluble receptor construct that contains the extracellular domain (ECD) of human AMHR2 fused to the Fc portion of human IgG. This construct is functionally monovalent, as it comprises a heterodimer of one Fc-only chain disulfide
bonded to one AMHR2-Fc chain (referred to as AMHR2-Fc/Fc; Fig. 1C) (28). This heterodimeric receptor construct was used because the AMHR2-Fc dimer we initially generated was found to contain an intermolecular disulfide bond between ECDs that affected AMH binding. For these assays we also used four anti-AMH mAbs, two that bind the N-terminal prodomain (10.6 and 11F8), hereinafter referred to as mAb-N1 and mAb-N2, and two against the C-terminal GF (22A2 and F2B/12H), referred to as mAb-C1 and mAb-C2.

Two different ELISA formats shown in Figs. 2A and C were used to measure the K_d for the AMH/AMHR2 interaction. When the soluble receptor was captured on the assay plate surface (Fig. 2A), the dissociation constants of the GF and c-AMH were found to be 0.03 and 0.3 nM, respectively (Fig. 2B). In contrast, when the GF and c-AMH were captured on the surface and the soluble receptor was presented in solution (Fig. 2C), the dissociation constants were almost identical at 3-4 nM (Fig. 2D). Thus the GF and c-AMH have higher affinities for AMHR2 when the receptor is on a surface. As demonstrated in the next section, the higher affinity is the result of an avidity effect due to a bivalent interaction of the homodimeric AMH with neighboring AMHR2 molecules on the surface. Although two separate receptor molecules can also bind AMH when the receptor is in solution, under these conditions each interaction is independent and no avidity-enhancement of binding is expected. The results in Fig. 2B and D also show that the prodomain weakens AMH binding to the receptor, but that this effect is only seen when the receptor is presented on the solid surface. To test whether the AMH complex remained intact during the experiment shown in Fig. 2C, D, we performed a similar experiment but used mAb-N1 to detect whether binding of the soluble receptor caused dissociation of the AMH complex and loss of the prodomain (Fig. 2E). The results (Fig. 2F) show that no displacement of the prodomain occurred when c-AMH captured on mAb-C1 was incubated with a high concentration of AMHR2-Fc/Fc (33 nM) prior to detection with biotinylated mAb-N1.

To further investigate the effect of receptor binding on dissociation of the AMH complex, the ELISA assays shown in Fig. 3A and C were performed. In these experiments, c-AMH was captured via mAb-N2, or via the soluble receptor, and the bound AMH was detected either using biotinylated mAb-N1 to detect presence of the prodomain or using mAb-C1 to detect the GF domain. The results show that similar signals were detected with the two different biotinylated detection antibodies, mAb-N1 and mAb-C1, when the c-AMH was captured via mAb-N2 (Fig. 3B), indicating that the AMH complex remains intact. However, when the c-AMH was captured via the plate-bound receptor (Fig. 3D), much less signal was detected by biotinylated mAb-N1, relative to mAb-C1, indicating that the N-terminal prodomain has been displaced. The ratios of the signals obtained with mAbs C1 and N1 in Figs. 3B and D provide an estimation of how much AMH complex remains intact at each AMH concentration. Fig. 3E shows that, while no dissociation of the complex was observed when the c-AMH was captured on the mAb, significant dissociation was seen when the c-AMH was captured on the plate-bound receptor. However, this dissociation only occurred at low concentrations of c-AMH; at higher concentrations an increasing proportion of the AMH complex remained intact.

To confirm that the assay in Fig. 3D was indeed detecting dissociation of the AMH complex, the experiment in Fig. 3F was performed. In this case, the c-AMH itself was directly biotinylated on the prodomain, allowing detection with streptavidin-HRP.
This experiment exploited the adventitious observation that, upon biotinylation of c-AMH, most (>95%) of the biotin is incorporated into the N-terminal prodomain, as shown by the western blot in the inset to Fig. 3G (24). When the biotinylated c-AMH was captured on either mAb-N₁ or mAb-C₁, similar signals were obtained (Fig. 3G). However, when the biotinylated c-AMH was captured via the plate-bound receptor, much less signal was observed at low AMH concentrations, again indicative that under these conditions the AMH complex dissociates and the biotinylated prodomain is released from the plate. The ratios of the signals measured after capturing the biotinylated c-AMH via the mAbs versus the soluble receptor provide an estimate of how much AMH complex is still intact at each AMH concentration (Fig. 3H). Consistent with the results obtained above using the antibody detection, the c-AMH remained intact when captured on the plate via the antibodies, but considerable dissociation of c-AMH was observed when captured via the plate-bound receptor, although again only at low AMH concentrations.

In previous experiments with COS cells expressing AMHR2, we showed that AMH complex bound at low concentration was detectable with mAb-C₁, but not with mAb-N₁, consistent with the prodomain being displaced (24). This result is confirmed in Fig. 4, when c-AMH was bound at a low concentration (0.5 nM) to COS cells expressing AMHR2. However, when c-AMH was bound at a high concentration (200 nM), both mAbs could now detect the bound AMH, indicating that the prodomain was still present. Thus, the phenomena observed in the ELISAs of prodomain displacement occurring at low AMH concentration but not high concentration, could also be recapitulated on cells.

**Dissociation of the AMH complex correlates with bivalent binding to AMHR2.** The simplest hypothesis that accounts for the concentration dependence of the dissociation of the c-AMH complex is that it can bind to the plate-captured receptor in two different states, one that is prone to dissociation and one that is not. Specifically, we hypothesized that, at low AMH concentrations, this homodimeric protein can bind to two neighboring molecules of the monovalent AMHR2 on the plate, whereas at high AMH concentrations mass action requires that each AMHR2 molecule on the assay plate will bind a separate AMH dimer, interacting with only one of the polypeptide subunits of the GF domain. This concentration-dependent shift from bivalent to monovalent binding is a well-known feature of systems in which a bivalent ligand binds to a surface-presented monovalent receptor (27,29). If this hypothesis is correct, the observation that prodomain displacement is seen predominantly at low AMH implies that a bivalent interaction with the receptor is required for dissociation of the AMH complex. To test this hypothesis, we fitted our ELISA data using the equations that have been previously derived for ligand-induced receptor dimerization (27,29) (see Experimental Procedures). The proposed mechanism for AMH induced dimerization of AMHR2 is shown in Fig. 5A, with monovalent and bivalent interactions labeled RL or RLR, respectively; the equations of Perelson and DeLisi allow the calculation of [RL] and [RLR] over a range of c-AMH concentrations at specific values of K₁, the affinity of the initial monovalent binding of ligand to the first receptor molecule, and K₂, the affinity of the subsequent recruitment of the second receptor molecule into the bivalent complex. As Fig. 6A shows, the form of the AMH complex detected by mAb-C₁ can be related to [RL + RLR], as this antibody will detect all forms of bound AMH.
whether or not the prodomain remains associated. Meanwhile, if our hypothesis that binding the receptor bivalently promotes dissociation of the prodomain is correct, the species detected by mAb-N1 can be related to [RL], as the monovalyantly bound RL will experience little loss of the prodomain, while there will remain little or no prodomain to be detected in RLR (Fig. 6A).

To generate suitable data for comparison to this mechanistic model, we developed ELISA methods to detect bound AMH using non-biotinylated mAbs and a secondary anti-mouse-Fc Ab conjugated to HRP, added either together as a preformed complex or individually in two steps (Fig. 5B). We found that, when mAb-C1 is added as a preformed complex, only one detection antibody complex binds per bound AMH molecule, whether the AMH is bound monovalyantly (RL) or bivalently (RLR). Thus, monitoring bound AMH in this manner gives a signal that is proportional to the sum of [RL + RLR]. In contrast, when mAb-C1 is added alone, two molecules of this Ab can bind per AMH dimer, one to each polypeptide, whether the AMH is present as RL or RLR. However, while the subsequently added HRP-conjugated anti-mouse-Fc Ab can detect both mAb-C1 molecules bound to the RLR complex, only one of the bound mAb-C1 molecules is detected in the case of the RL complex. The reasons for this two-to-one bias in detecting RLR versus RL, when the antibodies are added separately, are explained in Supplementary Figs. S1 and S2. Thus the signal obtained by this method is proportional to [RL + 2RLR]. A third way of detecting bound AMH was by using the prodomain-targeted antibody mAb-N1 together with the anti-mouse-Fc Ab. According to our hypothesis this combination will predominantly recognize RL but not RLR, because in the latter case the prodomain has mostly dissociated from the complex. Thus, the signal given in such assays should be approximately proportional to [RL] (Supplementary Fig. S3). The data obtained using mAb-N1 was unaffected by whether the mAb-N1 and the anti-mouse-Fc Ab were added sequentially or as a pre-incubated complex, consistent with the expectation that this method detects only a single species. Overall, therefore, we can use mAb-C1/anti-mouse-Fc added together to measure [RL + RLR], which corresponds to the total amount of bound AMH, mAb-C1 and anti-mouse-Fc added sequentially to measure [RL + 2RLR], which corresponds to the level of receptor occupancy, and mAb-N1/anti-mouse-Fc to measure primarily [RL], which corresponds to c-AMH bound monovalyantly to receptor (summarized in Fig. 5A).

We performed experiments similar to the one in Fig. 3D, except using the nonbiotinylated mAbs detected using anti-mouse Fc, as described above. The data (Table 1) were globally fitted to the equations of Perelson and DeLisi, giving values for K1 of 1.1 nM and [R0]/K2 of 3.7 (Fig. 5C). Note that, because in our experiments the receptor is captured on a solid surface, it is not appropriate to treat K2 as a molar equilibrium constant. Instead, [R0]/K2 is a dimensionless number that represents the ratio of receptor involved in bivalent versus monovalent complexes at low receptor occupancy (i.e. when bivalent binding is most favored because there is no shortage of unoccupied receptor available to form bivalent complexes). Importantly, almost identical results were obtained with two different prodomain- and GF-specific mAbs, which bind to different epitopes in AMH (Supplementary Fig. S4A). Also, an ELISA that detected RL by means of biotinylated c-AMH, performed as in Fig. 3F, gave results similar to those obtained using mAb-N1 (Supplementary Fig. S4B).
Values for [R]/[R₀] and [RLR]/[R₀] were calculated from the experimental data sets as described in Table 1. Fig. 5D shows how the concentrations of RL, RLR, and free R vary as a function of AMH concentration. Global fitting of these three data sets to the equations of Perelson and DeLisi gave values of K₁ = 1.1 nM and [R₀]/K₂ of 4.9 which are in close agreement with the best fit values obtained from Fig. 5C. As expected, the relationship of [RLR]/[R₀] to AMH concentration is a bell shaped curve, with the value of K₁ – the dissociation constant for the monovalent interaction of the AMH complex with AMHR2 – corresponding to its midpoint. This value of K₁ = 1.1 nM agrees well with the value of 3-4 nM obtained for the monovalent AMH/AMHR2 interaction measured in solution (Fig. 1D). The best-fit value obtained for the ratio [R₀]/K₂ was 4.9 (Fig. 5D). Fig. 6B shows how the RLR curves are predicted to look at different values of [R₀]/K₂. Taken overall, the close fit of the data to the predicted curves in Fig. 5D strongly supports the notion that bivalent interaction of the AMH complex with the receptor results in displacement of the prodomain, while little or no dissociation of the AMH complex occurs when bound monovalently.

Bivalent binding to AMHR2 increases the Kₐ for dissociation of the AMH complex by at least 1000 fold. To learn more about how receptor binding affects the interaction between the AMH prodomain and GF domain, we first measured the Kₐ for dissociation of the AMH complex in solution. To do this, c-AMH was diluted to a concentration of 1 ng/ml (8.8 pM), and incubated for 10 d at RT. As a control, a preparation of recombinant AMH that contains a mutation at the cleavage site and thus is not susceptible to proteolytic cleavage and therefore cannot dissociate (u-AMH, Fig. 1B), was treated in a similar way. At the end of the incubation, the AMH levels were determined using the ELISA shown in Fig. 7A, which detects only intact complex. After incubation for 10 d, the mean level of c-AMH measured was 80% of the mean level measured in samples diluted immediately before the assay (Fig. 7B); an unpaired t-test indicated that this difference was significant at a confidence level of p = 0.0016. In contrast, no significant reduction in mean AMH concentration was observed in the u-AMH samples after 10 d compared to samples diluted immediately before the assay. The fraction of intact c-AMH complex observed after the 10 d incubation was used to calculate an estimated Kₐ for the complex, giving a value of 0.4 pM.

To measure the dissociation constants of the AMH complex when bound monovalently (Kₐ mono) or bivalently (Kₐ bi) to AMHR2, we modeled our experimental data using the quadratic equation for a reversible dissociation/association reaction in combination with the equations of Perelson and DeLisi (Fig. 7C). In our experimental design, it is not possible to directly measure Kₐ mono or Kₐ bi. However, it is possible to measure the overall amount of receptor-bound AMH, which corresponds exactly to the species detected by mAb-C₁, [RL + RLR], as well as the total amount of bound AMH that has remained intact, which corresponds exactly to the species detected by mAb-N₁ (see Fig. accompanying Table 1). The signal detected by mAb-N₁, in principle, could include intact AMH bound either monovalently (NCₐ mono) or bivalently (NCₐ bi). Thus, mAb-N₁ detects the sum of these species, [NCₐ]₁, where [NCₐ]₁ = [NCₐ mono] + [NCₐ bi]. By subtracting [NCₐ]₁ from the total AMH bound, [RL + RLR], we can also quantify the amount of bound AMH that no longer has an associated prodomain, which we term [Cₐ]₁ = [Cₐ mono] + [Cₐ bi]). This treatment does not require the approximation, made in the preliminary analysis described
above, that mAb-N₁ detects only RL. The results of these calculations are shown in Table 1.

The total distribution of bound AMH between prodomain-dissociated and intact forms can be described by the following equations:

\[ K_T = \frac{[N_{bi}][C_{bi}]}{[NC_{bi}] + [NC_{mono}]} \]

\[ K_T = \frac{[N_{bi} + N_{mono}][C_{bi} + C_{mono}]}{[NC_{bi} + NC_{mono}]} \]

The quantity \( K_T \) is not a dissociation constant, but corresponds to the weighted average of \( K_{mono} \) and \( K_{bi} \) that pertains under particular experimental conditions. Values for \( K_T \), calculated from the experimental data measured at each AMH concentration tested, are given in Table 1 and Fig. 7C. The results show that \( K_T \) has low values at both low and high AMH concentrations, indicating that there is less or little dissociation of the AMH complex at these extremes of concentration, but passes through a maximum value indicating that substantial release of prodomain occurs at intermediate \([AMH]\).

To investigate whether this observed bell-shaped relationship between \( K_T \) and AMH concentration is expected for our proposed mechanism from Fig. 5A, we modeled how the value of \( K_T \) would vary as a function of \([c-AMH]\) for this mechanism, using the equations of Perelson and deLisi to model the distribution of bound AMH between monovalent and bivalent receptor engagement, and the quadratic equation for reversible binding to determine the extent of dissociation of prodomain from the plate-bound AMH. The parameters that determine the behavior of this model are the values of \( K_1, [R_0]/K_2, K_{mono} \) and \( K_{bi} \), as shown in Fig. 6A. Specifically, \( K_1 \) determines the overall extent of receptor occupancy that will be seen at a given AMH concentration, \([R_0]/K_2\) determines the distribution of bound AMH between monovalent (RL) and bivalent (RLR) complexes, and \( K_{mono} \) and \( K_{bi} \) determine the extent to which the monovalently bound (RL) or bivalently bound (RLR) AMH complexes will dissociate to release the prodomain into solution. If \([R_0]/K_2\) is a large number (Fig. 6C), then at low \([AMH]\), when there is plenty of unbound receptor available, a substantial fraction of the bound ligand will be recruited into RLR complexes. Consequently, the \( K_T \) value at low ligand concentrations will approximate \( K_{bi} \). On the other hand, at high AMH concentrations that force the system into a predominantly monomerically bound state, \( K_T \) will approximate \( K_{mono} \). Moderate AMH concentrations, at which there is a mix of bivalently and monovalently bound AMH, will lead to \( K_T \) values that lie between \( K_{bi} \) and \( K_{mono} \). In contrast, if \([R_0]/K_2\) is small (Fig. 6C), such that there is no significant recruitment of bound AMH into RLR complexes at any AMH concentration, then \( K_T \) will equal \( K_{mono} \) at all concentrations of ligand. The model reveals that if \([R_0]/K_2\) has an intermediate value, such that there are significant amounts of both monovalently bound and bivalently bound AMH even at low AMH concentrations where bivalent binding is most favored, the \( K_T \) value will vary from \( K_{mono} \) at high ligand concentrations, through a maximum at moderate ligand concentrations, to a value at the lowest ligand concentrations that is small but still intermediate between \( K_{bi} \) and \( K_{mono} \).

Fig. 7C and Table 1 show that the experimentally determined \( K_T \) values range from a high of around 45 pM at low \( c-AMH \) concentration, to a low of 0.8 – 1.0 pM at high \( c-AMH \) concentration. The latter range of values provides a measure of \( K_{mono} \), and is very close to the \( K_d \) value determined for the AMH complex in solution. This finding indicates that monovalent binding of \( c-AMH \) to a single AMHR2 molecule does not significantly promote \( c-AMH \) dissociation and release of the prodomain. Values for \( K_{bi} \) of \( \geq 5 \) nM give the best fit between the model
and the experimental data, with values for $K_{\text{b}}$ less than 0.5 nM being clearly inconsistent with the data. Thus, engagement of a second molecule of AMHR2 to form a bivalent complex decreases the affinity of the prodomain for the GF by at least 1000-fold, compared to unbound c-AMH, from $\sim$0.4 pM to $\geq 0.5$ nM.

Fig. 7D represents another test of the hypothesis that a bivalent interaction induces dissociation of the AMH complex. Curves were generated by plotting the fraction of intact AMH complex, given by $[\text{NC}_T]/([\text{RL}+\text{RLR}]$, against the fraction receptor occupancy, $[\text{RL}+2\text{RLR}]/[R_0]$. Fig. 6D shows how the model predicts this relationship will differ for low, high, and intermediate values of $[R_0]/K_2$. At low values of $[R_0]/K_2$, the affinity for recruiting a second receptor molecule is low and there is essentially no bivalent binding at any ligand concentration. Thus, there is little or no release of prodomain, except at very low levels of receptor occupancy where the ligand concentration is below even the very low $K_d$ for dissociation of monovalently bound complex. At high values of $[R_0]/K_2$, under which conditions bivalent binding is strongly favored except at extremely high ligand concentrations, loss of prodomain is almost complete at all levels of receptor occupancy except the very highest, because only at extremely high ligand concentrations will the equilibrium favor RL. Finally, at intermediate values of $[R_0]/K_2$, such as we see experimentally in this study, the model predicts a gradual increase in the fraction of ligand that remains intact from low to high receptor occupancy, as the proportion of RL to RLR gradually increases. Fig. 7D shows the experimental values for the fraction of bound c-AMH that is intact at different AMH concentrations from Table 1 (column G) plotted against the level of receptor occupancy determined from the signal measured using mAb-C$_1$ (column A), together with the best fit to the model. Fitting to the model gives values for $K_{\text{mono}}$ and $K_{\text{b}}$ of 1 pM and $\geq 0.5$ nM, respectively, consistent with the results obtained from Fig. 7C.

**Displacement of the prodomain from the BMP-7 complex.** Because the activation mechanism we observed for AMH differs, in key respects, from the activation mechanisms reported for other TGF-β family members (7,25), we decided to directly compare prodomain displacement from the AMH complex to prodomain displacement from another TGF-β family ligand. We chose BMP-7 for this comparison because, for this ligand too, it has been shown that prodomain release is induced by binding to its type II receptor (BMPR2). Unlike for AMH, however, for BMP-7 it was reported that the soluble receptor can achieve this activation (7). Experiments on BMP-7 that exactly parallel those shown in Figs 5 and 7 were not possible, because the mAb against the BMP-7 prodomain that at one time was commercially available has only low affinity, while the mAb against the GF is neutralizing and thus may block interaction with the type II receptor. We therefore prepared BMP-7 that was biotinylated on just the prodomain or just the GF domain for our measurements. These materials were prepared by biotinylating the BMP-7 complex, separating the biotinylated protein into prodomain and GF fractions, and then combining each biotinylated component into a complex with its non-biotinylated counterpart.

To probe the mechanism of prodomain displacement, the prodomain-biotinylated or GF-biotinylated BMP-7 complexes were captured on an ELISA plate either using an anti-BMP-7 GF mAb or via BMPR2-Fc, shown schematically in Figs. 8A and C. When captured on the anti-BMP-7 mAb, reduced signals were observed for the prodomain relative to the GF domain at low
concentrations of BMP-7 (Fig. 8B), indicating that a significant amount of complex dissociation has occurred. Assays were also performed in which the biotinylated prodomain preparation was added as a preformed complex with streptavidin-HRP (SA-HRP) in order to eliminate the incubation step with SA-HRP after binding of the biotinylated prodomain protein to the mAb. As expected, less dissociation was observed (Fig. 8B). Assays were also performed in which the biotinylated prodomain preparation was added as a preformed complex with streptavidin-HRP (SA-HRP) in order to eliminate the incubation step with SA-HRP after binding of the biotinylated prodomain protein to the mAb. As expected, less dissociation was observed (Fig. 8B). When captured on BMPR2-Fc, almost no signal was detected with the biotinylated prodomain preparation, when added individually or as a complex with SA-HRP (Fig. 8D). The ratios of the signals obtained with the two biotinylated complexes in Figs. 8B and D provide an estimate of how much BMP-7 complex remains intact at each concentration (Fig. 8E). In contrast to the AMH complex, considerable dissociation was observed when the BMP-7 complex was captured on the mAb, an indication that the $K_d$ of the BMP-7 complex is higher than that of the AMH complex; modeling indicates a value of $K_d = 0.1–0.2$ nM. An even higher level of complex dissociation was observed when the BMP-7 complex was captured on the plate-bound receptor, indicating that interaction with receptor further weakens the complex, consistent with the prior literature (7). Displacement of the prodomain at low concentrations of complex is most likely caused by bivalent interactions with BMPR2. The decrease in displacement at the highest complex concentrations, observed more prominently when the biotinylated prodomain preparation was added as a preformed complex with SA-HRP, suggests that a monovalent interaction with BMPR2 may not cause dissociation of the BMP-7 complex. However, to prove this by going to higher BMP-7 complex concentrations was not possible, due to the limited availability of the biotinylated prodomain complex.

To assess whether an interaction between the complex and receptor in solution can induce displacement, biotinylated prodomain complex was captured using an anti-BMP-7 GF mAb and then incubated with or without a high concentration of BMPR2-Fc for 2 h (Fig. 8F). As shown in Fig. 8G, no prodomain displacement was observed with soluble receptor. The experiment in Figs. 8H and I shows that BMPR2-Fc, added in solution, binds to the biotinylated prodomain complex with a $K_d$ value similar to that of the biotinylated GF complex and unbiotinylated BMP-7 complex. Together, the experiments in Figs. 8G and I indicate that BMPR2 can bind to the BMP-7 complex without inducing further displacement of the prodomain. This finding suggests that, as was the case with AMH, prodomain displacement from the BMP-7 complex requires a bivalent interaction with surface-bound BMPR2. However, we cannot rule out the possibility that, in Fig. 8G, the bound BMP-7 can bind soluble BMPR2-Fc only monovalently, if receptor binding to the other monomer in the homodimeric GF is blocked by the interaction with the anti-BMP-7 GF mAb. Thus, it remains possible that two BMPR2 molecules binding to the BMP-7 complex in solution might induce prodomain displacement. These results therefore indicate that displacement of the BMP-7 prodomain occurs by a mechanism similar to that seen with AMH, involving binding of type II receptor molecules to both GF polypeptides within the complex, but leave open the question of whether surface presentation of the receptor is absolutely required in the case of BMP-7.

**DISCUSSION**

The regulatory mechanisms that control access of TGF-β family members to their receptors are of central importance to
understanding the physiology of TGF-β signaling (2). An important mechanism for regulating the maturation of some TGF-β ligands is the association of the proteolytically cleaved prodomain with the growth factor. In some cases, this noncovalently-bound prodomain prevents interaction with receptors and renders the complex latent. However, for other family members the cleaved ligand complexes are not latent and can interact with their receptors. The latter is true for the cleaved AMH complex, which can bind its type II receptor in an interaction that then induces displacement of the prodomain (24). In this report, we have investigated the effect of AMHR2 on the prodomain/growth factor interaction in the AMH complex, and the effect of the prodomain on the AMH/AMHR2 interaction. We show that binding of the AMH complex to a single AMHR2 molecule has no detectable effect on the stability of the prodomain-GF complex, but recruitment of a second AMHR2 to form a bivalent complex with ligand increases the $K_d$ for the ligand complex by a factor of over 1000, resulting in rapid dissociation of the prodomain. Importantly, this effect is only observed when AMHR2 is presented on a surface; binding of soluble AMHR2 to c-AMH did not lead to prodomain displacement.

It is usually difficult to discriminate monovalent and bivalent interactions between receptors and their ligands, or to obtain precise measurements for the affinity of the receptor dimerization step. In this study we exploited the fact that, for bivalent ligands binding to an identical monovalent receptor on a surface, the relative abundances of monovalently bound and bivalently bound ligand vary in a predictable manner, with the abundance of bivalently bound ligand showing a characteristic bell-shaped dose-response (27). Using this feature, and antibodies that allowed us to detect whether the bound AMH molecules did or did not contain prodomain at each condition, we were able to determine the affinity constants for all steps in the reaction. In particular, we were able to determine the value for the affinity for the initial monovalent binding of c-AMH to the first equivalent of AMHR2, $K_1$, as well as the value for the quantity $[R_0]/K_2$ that reflects the affinity for recruitment of a second AMHR2 to form the bivalently bound RLR complex. $[R_0]/K_2$ is a dimensionless quantity that corresponds to the preponderance of bivalently compared to monovalently bound receptor that exists at conditions of low receptor occupancy where all nearby receptor molecules are available for recruitment into a bivalent complex. The value we obtained for $[R_0]/K_2 = ~6$ indicates that, under the conditions of our experiments, at low levels of receptor occupancy, approximately six times as many receptor molecules are engaged in bivalent complexes (RLR) compared to monovalent complexes (RL). The numerical value of $[R_0]/K_2$ will depend not only on the affinity of the final step in bivalent binding ($K_2$), but also on the density of AMHR2 present on the surface ($[R_0]$). In the current work, the significance of this value is mainly that it was large enough for us to observe a substantial degree of bivalent binding, which allowed us to characterize how this bivalent receptor engagement affected the affinity of prodomain binding. However, in previous work we have shown that binding of c-AMH to AMHR2 on cells similarly results in displacement of the prodomain (24), establishing that receptor-induced prodomain displacement also occurs in a cellular context. In the current work, characterizing these events in vitro has allowed us to establish the detailed mechanism by which this process occurs.

The bell-shaped curve observed for bivalently bound AMH in Fig. 5D (RLR) spans about 4 logs of ligand concentration.
Other homodimeric receptors are known to exhibit bell shaped dose responses. For example, hGH has an effective dose range of 4-6 logs, while erythropoietin and prolactin display broad effective dose ranges of 6 logs and >6 logs, respectively (30). These very large effective dose ranges indicate that the affinity for the initial, monovalently-bound ligand complex on the cell surface to recruit a second receptor molecule to form a bivalent, functional complex is very high. Consequently, these bivalent complexes form at very low ligand concentration, and require extremely high concentrations of ligand to outcompete the bivalent binding to force the system into a state where each receptor is occupied by a separate ligand molecule in a nonfunctional monovalent complex (30). The similarity between the bell-shaped curve for the bivalently bound AMH complex (RLR) and the bell-shaped effective dose ranges of these other cytokines suggests that AMHR2 has also evolved a high interaction affinity for recruitment of a second molecule of AMHR2 after the initial monovalent binding of AMH to the first AMHR2 molecule. The affinity enhancement that results from bivalent binding may have particular importance in extra-gonadal targets of AMH action such as the nervous system (19,31), where AMH levels may be low. It should be noted that determination of an exact value of $[R_\text{d}]/K_2$ for the reaction as it occurs on cells expressing a given level of AMHR2 would require experimentation on those cells (30).

The similarity between the $K_d$ for dissociation of the AMH complex in solution (0.4 pM) determined directly, versus $K_{\text{mono}}$ (0.8–1.0 pM) determined by fitting our data to the equations of Perelson and DeLisi, indicates that monovalent binding to AMHR2 does not substantially affect the dissociation affinity of the AMH complex. The low $K_d$ for the AMH complex in solution indicates that most of the complex is intact in biological fluids, an unsurprising result given that the mature growth factor is relatively unstable in the absence of the prodomain (23). AMH levels are present in the range of 25-50 pM in human female serum (age 5-30 years; (32)), a concentration at which 84-87% of AMH would exist as the intact complex. Even higher AMH levels (> 250 pM) are present in male serum prior to puberty (33), corresponding to a level of intact complex of >94%. On the other hand, the dissociation constants for the BMP-4, BMP-5, BMP-10, and GDF-8 complexes have been reported to be in the range of 4-13 nM (34), while $K_d$ for the activin A complex is 5 nM (11). Most of these factors show bioactivity and are present in biological fluids at concentrations below 1 nM, such that a significant level of dissociation would be expected. For example, activin A levels range from a value of 10 pM in female serum during the menstrual cycle to as high as 1 nM during the third trimester of gestation (35). Even at the higher level, at a $K_d$ of 5 nM only ~15% of the complex would remain intact at equilibrium absent some other mechanism for preventing dissociation. The low $K_d$ of the AMH complex is most likely due to an avidity effect since, unlike many members of the TGF-beta family, the AMH prodomain has been shown to exist as a disulfide-linked dimer (22,23).

The $K_d$ for the release of the prodomain from the AMH complex bound bivalently to AMHR2, $K_{\text{bd}}$, was found to be ≥ 500 pM, indicating that bivalent engagement of the AMH complex by AMHR2 increases the dissociation constant by a factor ≥1000. An important property of allosteric interactions is that they are reciprocal in nature (36-38). Therefore, since AMHR2 can bind to the growth factor in the AMH complex and displace the prodomain, the prodomain must have a reciprocal effect on the binding affinity of the growth factor to AMHR2. In previous experiments we failed to find an effect of the prodomain on binding affinity for AMHR2; similar affinities were
observed for the mature domain and the AMH complex binding to AMHR2 (24). However, in these earlier experiments, the AMH was presented on a surface and the receptor protein was presented in solution. As shown in Fig. 2, when the receptor is presented on a surface, the C-terminal growth factor does indeed have a higher affinity for AMHR2 than does the AMH complex. Thus, the prodomain and AMHR2 have reciprocal allosteric effects on the growth factor when the receptor is presented on a surface.

The fact that only surface-presented AMHR2 induces release of the prodomain suggests that it is not occupancy of the second AMHR2 binding site on c-AMH, per se, that drives the allosteric effect. Rather, the proximity or mutual orientation of the AMHR2 molecules within the bivalent RLR complex must also be important. We therefore propose a model in which the c-AMH molecule in its solution conformation is not optimally organized to bind simultaneously to two surface-presented AMHR2 molecules, and that some conformational rearrangement is required for bivalent binding to occur. The >1000-fold effect of this conformational change on prodomain binding suggests that, in c-AMH that is unbound or bound only monovalently, this change in the structure of the AMH complex is energetically unfavorable by at least 16.5 kJ/mol (4 kcal/mol). It is the binding energy generated by the interaction of AMH with the second AMHR2 molecule that pays for this unfavorable conformational change. Thus, the value of $[R_0]/K_2$ that is observed experimentally, though relatively high, in fact underestimates the true strength of interaction of monovalently bound c-AMH with a second AMHR2 presented nearby on a surface. This is because a substantial amount of this binding energy is not expressed as affinity, but rather is used to force the ligand into a significantly less stable conformation that promotes prodomain displacement to generate the fully mature growth factor (39).

X-ray crystal structures of complexes of TGF-β ligands (TGF-β1 (9); BMP-9 (10); Activin A (11); and GDF-8 (12)) have revealed how the prodomains of these hormones interact with their growth factors, showing that certain elements within the prodomains come into close proximity with the binding sites for both the type I and type II receptors (reviewed in 2). In these systems, the so-called “latency lasso”, α2-helix, and β1-strand of the prodomain form close associations with the type II receptor binding site (in TGF-β1 this interaction is predominantly with the latency lasso alone), while the α1- or α5-helix forms a close association with the type I receptor binding site. However, the extent to which these associations actually block access to receptors is an open question. Latent complexes clearly cannot bind their receptors, but in these cases the growth factors are confined by their prodomains in a more restricted state, which requires either torsional force for the TGF-β1 complex (9) or proteolysis for the GDF-8 complex (12) to liberate the growth factor and allow interaction with receptors. On the other hand, non-latent complexes can bind their receptors without the prodomain dissociating. The BMP-9 complex can form a ternary complex with the type I receptor ALK1 (40) despite the association of the α5-helix with the type I receptor binding site (10). In fact, the X-ray structure of ALK1 with the BMP-9 complex shows that ALK1 displaces the α5-helix from the type I receptor binding site, but does not affect the interaction of the α2-helix and β1-strand with the type II binding site on the GF (40). During proteolytic activation of the latent GDF-11 complex, a fragment of the prodomain containing the α1- and α2-helices remains associated with the growth factor without affecting binding to the type II receptor ActRIIA or bioactivity, and is not
displaced after binding of ActRIIA (41). Fig. 9 provides a summary of how the TGF-β1, GDF-8, and BMP-9 complexes access their receptors.

A sequence alignment of AMH with other TGF-β ligands (10) indicates that the β1-strand and α2-helix are conserved in the AMH prodomain, so it is likely that these elements also form an association with the type II receptor binding site on the growth factor. AMH is missing a feature that may stabilize the α2-helix interaction with the type II binding site in the BMP-9 complex: Arg-128 at the center of a π-cation cage formed by Trp-179, Phe-230, and Tyr-65 (10). In AMH, the residue homologous to Arg-128 is replaced by leucine, and two residues corresponding to those within the cage are replaced with non-aromatic amino acids. Because the $K_d$ for the AMH complex is around $10^{-12}$ M, and given that the association rate constant between two proteins is unlikely to exceed $10^7$ M$^{-1}$s$^{-1}$ (42), the dissociation rate constant is likely to be no greater than $10^5$ s$^{-1}$ and consequently the half-life for the dissociation of the complex (in the presence of AMHR2) will be in the range of $\geq$20 hours. This time range is not consistent with the kinetics of the AMH complex binding to AMHR2, which is on the order of minutes. Thus, binding to the receptor must precede prodomain displacement. Furthermore, we have shown here that binding of AMHR2 to c-AMH does not require prior displacement of the prodomain. Fig. 2F indicates that AMHR2, AMH prodomain, and AMH growth factor can form a stable ternary complex at a very high concentration of AMHR2. For this to be possible, it appears that the association between the β1-strand and the α2-helix of the prodomain and the type II binding site of the growth factor, if it occurs in the AMH complex, must be in equilibrium with a different conformational state that allows access of AMHR2 to its binding site on the growth factor. This picture of events is supported by our observation that $K_{mono}$ is essentially identical to the $K_d$ of the unbound complex, indicating that the binding of one AMHR2 molecule to the complex does not lead to any significant destabilization of the complex.

There are a number of differences in the mechanism of AMH prodomain displacement by AMHR2 and the mechanisms of prodomain displacement used by other TGF-β family members. For example, dissociation of the BMP-9 complex can be induced by the type I receptor ALK1, type II receptors BMPR2, ActR2A, ActR2B, and the co-receptor endoglin (25). In addition, antibodies to either the prodomain or growth factor domain can induce dissociation of the BMP-9 complex. The finding that the BMP-9 complex can form a ternary complex with the type I receptor ALK1 (40) appears to be in conflict with the finding that ALK1 can displace the prodomain (25), but it should be noted that the former experiment was done in solution, while the latter experiment was performed with an ALK1-Fc fusion protein on a biosensor chip where bivalent engagement by the receptor is possible. Another family member for which prodomain displacement can be induced by binding to its receptor is BMP-7. It was previously reported that BMPR2-Fc can induce BMP-7 complex dissociation when added as a soluble reagent, based on the sedimentation properties of the BMP-7 complex when combined with BMPR2-Fc in sucrose gradients (7). This result differs from our findings with AMH, for which prodomain displacement requires that AMHR2 is presented on a surface. In our own experiments on BMP-7 we found that binding to surface bound receptor induces prodomain displacement for BMP-7 similarly to AMH, though the intrinsic affinity of the BMP-7 complex is $\geq$250 fold weaker. We did not observe enhanced
prodomain displacement when we used the BMPR2 as a soluble reagent, suggesting that surface presentation of the receptor is required for BMP-7 as it is for AMH. If so, this finding would suggest a similar allosteric mechanism, requiring a constrained geometry between two receptor molecules, could be operative for both of these ligands. However, we were not able to formally exclude the possibility that BMPR2 binding can induce prodomain displacement when in solution if both receptor binding sites in the GF domain of BMP-7 are simultaneously occupied.

In the AMH system, dissociation of the AMH complex strictly requires bivalent interaction with AMHR2 on a surface, which we conclude is the result of an allosteric effect. In considering the potential biological utility of this mode of receptor activation for AMH signaling, we note that the large reduction in prodomain affinity for the GF after bivalent binding to AMHR2 is likely to render prodomain displacement essentially irreversible, thus driving the receptor activation process forward. Such a mechanism additionally ensures that prodomain release will occur only when the cleaved ligand binds to cells that express AMHR2 at sufficient levels to favor formation of a c-AMH/(AMHR2)₂ ternary complex (43). Ligand binding to cells that express only low levels of AMHR2 will form few such ternary complexes, with most ligand molecules instead simply dissociating from the cell surface as the intact GF/prodomain complex. Thus, we speculate that this mechanism may serve to ensure that the mature GF is generated only at the site of action, with a greater selectivity for cells having high receptor expression than would be expected for alternative prodomain release mechanisms, and under conditions where receptor engagement by AMH is predicted to become kinetically irreversible.
EXPERIMENTAL PROCEDURES

Reagents. AMH proteins were previously described (23,24). AMH produced in CHO cells expressing a wild type AMH gene and purified from the culture medium is referred to as secreted-AMH (s-AMH). It contains about 95% AMH precursor and 5% cleaved AMH. Completely cleaved AMH (c-AMH) was prepared by plasmin digestion of s-AMH and has been assessed as 100% cleaved by SDS-PAGE. Completely uncleaved AMH (u-AMH) was produced in CHO cells expressing an AMH cDNA with a mutation (R451T) at the monobasic cleavage site and has been assessed as 0% cleaved by SDS-PAGE. c-AMH was biotinylated as previously described (24). The AMH GF was prepared as previously described (23). The AMHR2-Fc/Fc fusion protein was produced in HEK293E cells as previously described (28) and was purified from conditioned medium using Protein A Sepharose and size-exclusion chromatography. The mAbs used in this study have been previously described: mAb-C1 (22A2; (44)), mAb-C2 (F2B/12H; (45)), mAb-N1 (10.6; (23)) and mAb-N2 (11F8; (44)).

ELISAs. Conditions for ELISAs with mAb-N1 and mAb-C1 coated on plates have been previously described (24). Conditions for an ELISA with AMHR2-Fc/Fc have also been previously described (28,33). Briefly, ELISA plates (Nunc Maxisorp) were coated with a goat anti-human Fc antibody (10 µg/ml; Jackson ImmunoResearch; catalog #109-005-098) overnight at 4°C in 50 mM sodium bicarbonate, pH 9.6. After washing with water and blocking with 1% BSA (Sigma-Aldrich; A-7906) and 1% goat serum (Invitrogen; 16210064) in PBS, AMHR2-Fc/Fc was added at a concentration of 0.75 µg/ml and incubated for 1 h. After washing with PBS, AMH samples were serially diluted down the plate by a factor of 2-3 and incubated for 2-4 h. After washing with PBS/0.05% Tween-20, biotinylated mAb-N1 or mAb-C1 (0.5 µg/ml) were added and incubated for 1 h, followed by a 1h incubation with streptavidin conjugated to HRP (1:3000; Jackson ImmunoResearch). After washing, 3,3',5,5' tetramethylbenzidine (TMB) was added to each well, reactions were quenched by the addition of 2M sulfuric acid and absorbances were read at 450 nm. When non-biotinylated mAbs were used for detection, the subsequent incubation was with goat anti-mouse-Fc conjugated to HRP (1:3000; Jackson ImmunoResearch). Preformed complexes between mAbs and the goat anti-mouse-Fc conjugated to HRP were generated by mixing the mAbs and secondary Ab at a 1 to 2 ratio, with mAbs at a concentration of 50 nM. After incubation at 90 min at RT, the complexes were diluted 1:20 and added to the plate. When biotinylated-c-AMH was used, it was directly detected with streptavidin conjugated to HRP. c- or u-AMH captured on mAbs-N1 and N2 were detected as described above with biotinylated mAb-N1 or mAb-C1. All antibodies were coated as described above for the goat anti-human Fc antibody.

Binding of AMH to AMHR2 on COS cells. COS cells were transfected with the human AMHR2 cDNA as previously described (24). After washing with DMEM, cells were incubated for with c-AMH (0.5 or 200 nM) in DMEM/1% FBS for 4 h at 37°C, then washed with DMEM and incubated with mAb-N1 or mAb-C1 (3 µg/ml) in DMEM/1% FBS for 2 h at 37°C. After rinsing with PBS, cells were incubated 1 h with a goat anti mouse IgG Ab conjugated to Alexa 488 in DMEM, washed with PBS and fixed 5 min in methanol/acetone (vol/vol). After hydration, slides were mounted in Vectashield containing DAPI (Vector Laboratories) and cells were examined with an Olympus IX83 microscope.
Biotinylation of BMP-7. Full-length human BMP-7 was expressed in CHO cells and purified from the medium using sequential chromatography steps on Zn chelating Sepharose and SP-Sepharose as previously described (41). The isolated BMP-7 was >98% pure and eluted as a homogeneous peak when analyzed by size exclusion chromatography. ~90% was cleaved at the junction of the pro- and mature domains to form the active mature domain/prodomain soluble BMP-7 complex and the remainder was not fully processed. Two different methods were used to biotinylate the soluble BMP-7. In method 1, the soluble BMP-7 was reduced with 5 mM sodium meta periodate and biotinylated through the sugars using 3 mM Easy-link hydrazide PEG4 Biotin (Thermo Fisher). In method 2, soluble BMP-7 was biotinylated through amines using 0.3 mM EZ-Link NHS-PEG4-Biotin. The 3 preparations with and without biotin were loaded onto Zn Chelating Sepharose columns at 4 mg BMP-7/mL resin. Mature BMP-7 was selectively eluted from the columns with 6 M urea and the prodomain was then eluted with 50 mM imidazole. By SDS-PAGE, the mature BMP-7 was >95% pure and the prodomain was 90% pure containing about 10% unprocessed BMP-7. Biotinylated soluble BMP-7 complexes were reconstituted by mixing equimolar concentrations of mature BMP-7 that had been biotinylated through the glycan with unmodified prodomain, and prodomain that had been biotinylated through amines with unmodified mature domain. Samples were incubated 2 h at room temperature in 6M urea, then diluted to 2M urea in 20 mM HEPES pH 7.0, 160 mM NaCl, 0.1% Tween 20 both at final protein concentrations of the reconstituted complexes of 2.7 µM. ELISAs with BMP-7 proteins were performed similar to AMH above, except that Tween-20 was added to all incubation steps at 0.1%. The BMPR2-Fc (Catalog # 811-BR) and BMP-7 GF mAb (Catalog # MAB3542) were from R&D Systems.

Modeling with the equations of Perelson and DeLisi.

Data were treated as described in the text and fit to the equations for ligand induced receptor dimerization (27).

\[
[R]/[R_0] = (1-\beta)(-1+\lambda)/2\delta
\]

\[
[RL]/[R_0] = \beta(-1+\lambda)/2\delta
\]

\[
[RLR]/[R_0] = (1+2\delta-\lambda)/4\delta
\]

\[
\beta = ([L]/K_1)/(1+[L]/K_1)
\]

\[
\delta = \beta(1-\beta)[R_0]/K_2
\]

\[
\lambda = (1+4\delta)^{1/2}
\]

[L] = ligand concentration

[R_0] = total concentration of receptor sites

[R] = concentration of free receptor sites at equilibrium

[RL] = concentration of monovalently bound ligand at equilibrium

[RLR] = concentration of bivalently bound ligand at equilibrium

K_1 = dissociation constant for the initial, monovalent binding step

K_2 = dissociation constant for the second, bivalent binding step
Modeling of AMH complex dissociation in RL and RLR.

To model the equilibria of AMH complex dissociation established during the binding of c-AMH to AMHR2-Fc/Fc, where c-AMH bound is bound either monovalently (in RL; \(K_d = K_{\text{mono}}\)) or bivalently (in RLR; \(K_d = K_{\text{bi}}\)), it is necessary to take into account the total amount of prodomain released from both RL and RLR complexes as well as the prodomain released from free c-AMH \((K_d = K_{\text{free}})\). This is because all three complexes are present in the experiment, and as a common product of dissociation the total prodomain concentration will determine the position of the equilibria between intact and dissociated complex in all cases. To do this, the modeling was divided into two stages (Supplementary Fig. S6). In the first stage the amount of released prodomain \((N_{\text{free}}, N_{\text{mono}^*}, \text{or} N_{\text{bi}^*})\) was calculated at each c-AMH concentration; in the second stage the level of intact complex, \(N_{\text{C mono}}\) or \(N_{\text{C bi}}\), was determined after the association of growth factor \((C_{\text{mono}}\) or \(C_{\text{bi}}\)) with prodomain \((N_{\text{mono}}\) or \(N_{\text{bi}}\)) in the presence of prodomain from other sources.

1) The amount of the prodomain released from c-AMH, RL or RLR was determined using the quadratic equation for a dissociation/association reaction for the case where the initial concentration of the undissociated complex is known and the complex is allowed to dissociate and come to equilibrium. Specifically, at equilibrium,

\[
[N] = \frac{-K + (K^2 + 4K[N_C^0])^{1/2}}{2}
\]

where \(K\) is the dissociation constant for the complex, \([N]\) is the concentration of the released prodomain, and \([N_C^0]\) is initial complex concentration.

For the dissociation of free c-AMH, \([N_C^0]\) was set to \([c-AMH_0]\), and \(K\) was set to the value of \(K_{\text{free}} = 0.4\) pM that we determined separately, as described in the main text. For the subsequent analysis, the concentration of prodomain that results from dissociation of free c-AMH was termed \([N_{\text{free}}]\). For dissociation of c-AMH bound monovalently to the receptor, as the RL complex, \([N_C^0]\) was set to the value of \([RL]\) calculated using the Perelson and DeLisi equation. As described above, the concentration of prodomain released (before correction for the presence of prodomain from other sources) was termed \([N_{\text{mono}^*}]\), and the unknown dissociation constant was termed \(K_{\text{mono}}\). Similarly, for c-AMH bound bivalently in an RLR complex, \([N_C^0]\) was set to the value of \([RLR]\) calculated using the Perelson and DeLisi equation, the concentration of prodomain released (before correction for the presence of prodomain from other sources) was termed \([N_{\text{bi}^*}]\), and the unknown dissociation constant was termed \(K_{\text{bi}}\).

To calculate \([RL]\) and \([RLR]\) values from \([RL]/[R_0]\) and \([RLR]/[R_0]\), it is necessary to specify a concentration for \([R_0]\). For this purpose, we used a value of \([R_0] = 0.1\) nM, which was determined through experimentation. However, as shown in Supplementary Fig. S5, values of \([R_0]\) twice as high \((0.2\) nM) or half as high \((0.05\) nM), give similar results when fitting the data to the model, indicating that the results of the analysis described herein are relatively insensitive to errors in the value of \([R_0]\).

2) Using the results from (1), the level of association between growth factor and prodomain, in the presence of prodomain from other sources, was then determined using the quadratic equation for
a dissociation/association reaction for the case where the initial concentrations of the two components are known and are allowed to associate and come to equilibrium. At equilibrium,

\[ [NC] = \frac{\{(\left[N_0\right] + \left[C_0\right] + K) - ((-\left[N_0\right] - \left[C_0\right] - K)^2 - 4\left[N_0\right]\left[C_0\right])^{1/2}\}}{2} \]

where \([N_0]\) and \([C_0]\) represent the initial concentrations of prodomain and growth factor domain present in the system, \(K\) is the dissociation constant for the complex, and \([NC]\) is the concentration of intact complex present at equilibrium.

For c-AMH bound monovalently in RL complexes: \([C_0] = [RL]\) calculated from the Perelson and DeLisi equation as described; \([N_0] = [RL + N_{\text{free}} + N_{\text{bi}*}]\); \(K = K_{\text{mono}}\); and \([NC]\), the equilibrium concentration of complex present at equilibrium, is termed \([NC_{\text{mono}}]\). For c-AMH bound bivalently in RLR complexes: \([C_0] = [RLR]\) calculated from the Perelson and DeLisi equation as described; \([N_0] = [RLR + N_{\text{free}} + N_{\text{mono}*}]\); \(K = K_{\text{bi}}\); and \([NC]\) is termed \([NC_{\text{bi}}]\).

The levels of C-terminal GF without a prodomain, in the RL and RLR complexes, and the N-terminal prodomain released from the RL and RLR complexes were then derived as followed:

\([C_{\text{mono}}] = [N_{\text{mono}}] = [RL] - [NC_{\text{mono}}]\) and \([C_{\text{bi}}] = [N_{\text{bi}}] = [RLR] - [NC_{\text{bi}}]\)

And the fraction intact complex, \(F\), is given by:

\[ F = \frac{[NC_{\text{mono}}] + [NC_{\text{bi}}]}{[RL + RLR]} = \frac{[NC_T]}{[RL + RLR]} \]

3) The quantities defined in (1) and (2) were then used to define a notional composite equilibrium constant for the combined dissociation of RL and RLR, which we termed \(K_T\).

\[ K_T = \frac{[N_{\text{mono}} + N_{\text{bi}}][C_{\text{mono}} + C_{\text{bi}}] + [NC_{\text{mono}} + NC_{\text{bi}}]}{[N_T][C_T][NC_T]} \]

\(K_T\) is not a real equilibrium constant, but instead reflects the overall distribution of intact versus dissociated complex for a given mixture of RL and RLR. However, because \(K_T\) relates directly to measured experimental quantities, we could fit the measured values of \(K_T\) at each total AMH concentration against the above equation to determine the best-fit values for \(K_{\text{mono}}\) and \(K_{\text{bi}}\).

**Calculation of \(P_a\).**

The fraction of AMH in the RL and RLR complexes bound by mAb-C1 (\(P_a\)) was calculated using the quadratic equation for a reversible association reaction:

\[ P_a = \frac{[mAb-C1-AMH\ complex][RL+RLR]}{[RL+RLR]} = \frac{-b - (b^2 - 4c)^{1/2}}{2[RL+RLR]} \]

where \(b = -[RL+RLR] - [mAb-C1] - K_d\) and \(c = [RL+RLR][mAb-C1]\)

The \(K_d\) for the AMH/mAb-C1 interaction is 0.1 nM.
DATA AVAILABILITY
All data described in the manuscript are contained within the manuscript.

SUPPORTING INFORMATION
This article contains supporting information

ACKNOWLEDGEMENTS
RLC would like to thank all members of the Whitty laboratory over the last six years for help, advice, and feedback concerning this project. A special thanks to Thomas E. Roche (Kansas State University) for insightful discussions and to Samantha Muellers (Boston University) for assistance with PyMol.

AUTHOR CONTRIBUTIONS
RLC and NdC designed and performed experiments. CR assisted with the immunofluorescence experiments. RBP and NPG provided insight on experimental design and the generation of key reagents. RLC and AW developed the models for testing the hypothesis that bivalent binding of AMH by AMHR2 is responsible for prodomain displacement. All authors were involved in analyzing the data and results. RLC and AW wrote the manuscript with contributions from all authors.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

ABBREVIATIONS: The abbreviations used are: AMH, anti-Müllerian hormone; AMHR2, AMH type II receptor; TGF, transforming growth factor; BMP, bone morphogenetic protein; GDF, growth and differentiation factor; mAb, monoclonal antibody; ALK, activin receptor-like kinase; ActR2, activin type II receptor; BMPR2, BMP type II receptor; ECD, extracellular domain; GF, growth factor; SA, streptavidin; HRP, horseradish peroxidase.
FIGURE LEGENDS

Figure 1. Interaction of AMH with its type II receptor AMHR2. (A) The AMH precursor undergoes an obligatory cleavage resulting in the noncovalent AMH complex that can bind to AMHR2, the type II receptor. After binding, the prodomain is released, but it has not been determined whether this is the result of a monovalent or bivalent interaction with AMHR2, or either interaction. In the current study, values for $K_{\text{mono}}$ and $K_{\text{bi}}$ have been derived showing that prodomain release occurs only after a bivalent interaction, which induces a change in the conformation of the GF. Both the GF and prodomain contain intermolecular disulfide bonds. (B) Reagents used for experiments in the current study. c-AMH was produced in Chinese hamster ovary cells as mostly precursor and converted to completely cleaved noncovalent complex by treatment with plasmin. u-AMH contains an R451T mutation at the cleavage sites between the N-terminal prodomain and the C-terminal growth factor domain. The numbering corresponds to amino acid residues in UniProtKB accession number P03971 (human AMH). (C) AMHR2-Fc/Fc was generated by co-expressing a cDNA encoding an AMHR2-Fc fusion protein together with a cDNA encoding the Fc portion of IgG1 in HEK293 cells. The numbering corresponds to amino acid residues in UniProtKB accession numbers Q16671 (human AMHR2) and P01857 (human Ig γ-1 chain constant region). The representations of c-AMH and u-AMH (shown in B) and AMHR2-Fc/Fc (shown in C) are used to depict these molecules in ELISA formats.

Figure 2. A negative effect of the prodomain on AMH complex binding to AMHR2 is observed when AMHR2 is on a surface but not in solution. (A, B) ELISA format and results for comparing binding of c-AMH or GF to AMHR2 when the receptor is on a surface. The AMHR2-Fc/Fc was captured on a goat anti-human Fc Ab and incubated with various concentrations of AMH; bound AMH was detected with biotinylated anti-C-terminal AMH mAb-C1 and streptavidin conjugated to horse radish peroxidase (HRP). (C, D) ELISA format and results for comparing binding of c-AMH or GF to AMHR2 when the receptor is presented in solution. AMH was captured on mAb-C1 and incubated with various concentrations of AMHR2-Fc/Fc; bound AMHR2-Fc/Fc was detected with an anti-human Fc Ab conjugated to HRP. (E, F) ELISA format and results for assessing whether AMHR2-Fc/Fc in solution can induce displacement of the prodomain. c-AMH, after capture on mAb-C1, was incubated with AMHR2-Fc/Fc (33 nM) or buffer for 2 h, prior to detection with mAb-N1. Data in B and D were fit to the quadratic equation for a reversible association reaction. Error bars show standard deviations. Multivalent interactions are not shown in the diagrams depicting ELISA formats.

Figure 3. The AMH complex undergoes dissociation after binding to AMHR2-Fc/Fc on a surface but only at low concentration. (A, B) ELISA used for assessing AMH complex dissociation when bound to anti-N-terminal mAb-N2. Bound c-AMH was detected with either mAb-N1 or mAb-C1. Similar signals were observed with both mAbs indicating that the AMH complex has not dissociated. (C, D) ELISA used for assessing AMH complex dissociation when bound to AMHR2-Fc/Fc. Less signal was obtained with mAb-N1 than mAb-C1 at low c-AMH concentrations indicating that the AMH complex has dissociated. (E) The ratios of the signals produced by mAb-N1 and mAb-C1 in B and D are shown for a range of c-AMH concentrations and provide an estimation of the amount of intact AMH complex. (F, G) ELISA used for assessing dissociation of biotinylated c-AMH when bound to mAb-N1, mAb-C1 or AMHR2-Fc/Fc. The inset in G shows a western blot of biotinylated c-AMH showing that most of the biotin was incorporated into the N-terminal prodomain. (H) The ratios of signals produced after binding biotinylated c-
AMH to the two mAbs (C1/N1) or to AMHR2-Fc/Fc and mAb-N1 are shown for a range of c-AMH concentrations. The numbers in red in E and H show the actual ratios at these low c-AMH concentrations. Values of 100 have been used in their place so as to not skew the slopes of the trend lines. Error bars show standard deviations.

**Figure 4. The prodomain can be detected after binding of c-AMH to AMHR2 on COS cells at high concentration but not after binding at low concentration.** COS cells were transfected with AMHR2 and incubated with c-AMH at 0.5 or 200 nM. AMH bound to cells was detected with either mAb-C1 or mAb-N1 as described in the Experimental Procedures.

**Figure 5. Dissociation of the AMH complex correlates with bivalent binding to AMHR2.** (A) A mechanism for the receptor induced dimerization of AMHR2 by c-AMH; monomeric and bivalent interactions are labeled RL or RLR, respectively. The relationships between mAb-N1 or mAb-C1 and RL and RLR are shown below the diagram. (B) Schematic diagrams showing the two experimental conditions for testing whether bivalent binding of AMH by AMHR2 induces prodomain displacement. On the left, the mAbs and secondary Ab are added individually and on the right they are added as a complex. (C) Data from Table 1 obtained with mAb-N1 and mAb-C1 under the two experimental conditions shown in B, fit to the equations of Perelson and DeLisi. (D) Dependence of [R]/[R0], [RL]/[R0], and [RLR]/[R0] on AMH complex concentration. In C and D, K1 and [R0]/K2 values were determined by simultaneously fitting all data to the predicted curves and minimizing the RMSE. Error bars show standard deviations.

**Figure 6. The effect of the [R0]/K2 ratio on the level of ligand bound bivalently and on complex dissociation.** (A) The two equilibrium states for the AMH complex interacting with AMHR2 monovalently or bivalently are shown in relation to the species detected by mAb-N1 and mAb-C1. The species detected by mAb-N1 (shown in the green dashed rectangle) are related to [RL], while the species detected by mAb-C1 (shown in the red dashed rectangle) are related to [RL + RLR] or [RL + 2RLR], depending on experimental conditions as described in the text. The effect of varying the [R0]/K2 ratio is shown for [RLR]/[R0] (B), K_T (C), and the relationship between complex dissociation and receptor occupancy (D). Curves were generated using the equations of Perelson and DeLisi alone (B), or in combination with the quadratic equation for a reversible dissociation/association reaction (C, D) and the indicated values of K1, [R0]/K2, K_mon, and K_hi as described in the Experimental Procedures.

**Figure 7. Bivalent binding of AMHR2 to the AMH complex increases the K_a of the complex by a factor of 1000.** (A) ELISA used to measure intact AMH complex. (B) c-AMH and u-AMH were diluted in PBS containing 1% BSA and 1% goat serum to 1 ng/ml (8.8 pM) and the level of intact AMH complex was assessed after 10 d (t10; N = 21); samples diluted immediately before the ELISA are labeled t0 (N = 9). (C) Concentration dependence of the apparent dissociation constant (K_T) determined for the AMH complex bound to AMHR2. K_T values are from Table 1. (D) Relationship between complex dissociation and receptor occupancy. Data for intact AMH complex [NC_T]/[RL+RLR] and receptor occupancy [RL+2RLR]/[R0] are from Table 1. In C and D, the curves were generated using the quadratic equation for a reversible dissociation/association reaction in combination with the equations of Perelson and DeLisi. K1 and K_hi were fixed at 1.1 nM and 5 nM, respectively, and values for K_mon and [R0]/K2 were determined by minimizing the
RMSE as described in the Experimental Procedures. The effect of varying $K_{bi}$ is shown. Error bars show standard deviations.

**Figure 8. Displacement of the prodomain from the BMP-7 complex.** (A, B) ELISA format and results for assessing BMP-7 complex dissociation when bound to BMP-7 GF mAb. BMP-7 complexes containing either biotinylated prodomain or GF were captured on the BMP-7 mAb and the amount of bound prodomain or GF was assessed using streptavidin-HRP. (C, D) ELISA format and results for assessing BMP-7 complex dissociation when bound to BMPR2-Fc. In B and D, assays were also performed in which the biotinylated prodomain preparation was added as a preformed complex with streptavidin-HRP (SA-HRP). (E) The ratios of the signals produced by biotinylated prodomain and GF in B and D are shown for a range of BMP-7 concentrations and provide an estimation of the amount of intact BMP-7 complex. The concentration dependence of the dissociation of the BMP-7 complex captured on the BMP-7 mAb indicates that the $K_a$ of the complex is around 0.1-0.2 nM. The data for the biotinylated prodomain protein added as a complex with SA-HRP are not shown, only the curve fitted to the data. Much lower levels of intact complex were detected after capture on BMPR2-Fc, indicating that the prodomain has been displaced by the receptor. (F, G) ELISA format and results for assessing whether BMPR2-Fc in solution can induce displacement of the prodomain. BMP-7 complex containing biotinylated prodomain was captured on the BMP-7 mAb and incubated with BMPR2-Fc (60 nM) or buffer for 2 h, prior to detection with streptavidin-HRP. No prodomain displacement was observed relative to the buffer control. (H, I) ELISA format and results for assessing binding of BMPR2 to the two biotinylated proteins and the unbiotinylated BMP-7 complex. Similar $K_a$ values were obtained for the three proteins. Error bars show standard deviations.

**Figure 9. Summary of how various TGF-β family ligands access their receptors.** The latent TGF-β1 and GDF-8 complexes require either torsional force or additional proteolysis to liberate their growth factors and allow access to their receptors. The GF of GDF-11, which is closely related to GDF-8, is shown bound to its receptors. The non-latent BMP-9 complex can bind to its type I receptor, ALK1, without inducing prodomain displacement. Prodomains are shown in green, growth factors in red, type I receptor ECDs in yellow, and type II receptor ECDs in cyan. The PDB file names are shown beneath each structure. The $α_1$-, $α_2$-, and $α_5$-helices and latency lasso (LL) in the prodomains are labeled; these elements are located in close proximity to receptor binding sites on the GFs.
TABLE LEGENDS

Table 1. Data from ELISA with mAb-N₁ and mAb-C₁ shown in Figure 4C. Calculations were performed as shown in schematic diagram below the table (concentrations of species are shown) and at the bottom of the table (ratios of concentrations of species relative to [R₀] are shown). Errors are standard deviations. N refers to the number of experiments; each experiment contained 2-4 replicates. *Values of [RL]/[R₀] and [RLR]/[R₀] are estimates based on assumption that [RL] = [mAb-N₁] when Kₘₙₐₒ is small and Kᵦ is large (see Supplementary Figure 3). #Experimental and theoretical values for Kₕ were calculated using a value of [R₀] = 0.1 nM, determined from experimentation. Values of [R₀] twice as high (0.2 nM) or half as high (0.05 nM), give similar results when fitting the data to the model (Supplementary Fig. S5), indicating that having an exact value of [R₀] is not essential for this analysis.
<table>
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<tr>
<th>[c-AMH] (nM)</th>
<th>mAb-C(_1)/mAb-C(<em>1)(</em>{\text{max}}) (mAb-C(_1) &amp; 2nd Ab added alone)</th>
<th>mAb-C(_1)/mAb-C(<em>1)(</em>{\text{max}}) (mAb-C(_1) &amp; 2nd Ab added as complex)</th>
<th>mAb-N(_1)/mAb-N(<em>1)(</em>{\text{max}})</th>
<th>Calculated from values in columns A, B, or C</th>
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<td></td>
<td>([\text{RL} + 2\text{RLR}] / [\text{R}_0])</td>
<td>Fraction receptor occupancy</td>
<td>([\text{RL} + \text{RLR}] / [\text{R}_0])</td>
<td>Fraction maximal AMH binding</td>
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<td>52.83</td>
<td>1.000 ± 0.000</td>
<td>1.026 ± 0.087</td>
<td>0.941 ± 0.022</td>
<td>0.086 ± 0.090</td>
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<td>26.41</td>
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<td>0.871 ± 0.041</td>
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<td>6.60</td>
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<td>0.83</td>
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REFERENCES


**A**

AMH precursor

Protease

AMH Complex (c-AMH)

N-terminal prodomain

C-terminal growth factor (GF)

AMHR2

Monovalent interaction

Bivalent interaction

**B**

AMH Complex (c-AMH)

Uncleaved AMH (u-AMH)

Used to depict AMH in ELISA formats

**C**

ECD hinge- CH2-CH3

AMHR2-Fc/Fc

hinge- CH2-CH3

Used to depict AMHR2-Fc/Fc in ELISA formats
\textbf{A} \quad \text{Capture Ab: anti-human-Fc-Ab}

\textbf{B} \quad \text{biotinylated-mAb-C}_1

\textbf{C} \quad \text{Detection: anti-human-Fc-Ab-HRP}

\textbf{D} \quad \text{Fraction maximal signal}

\begin{align*}
\text{GF} & \quad K_d = 0.03 \text{nM} \\
\text{c-AMH} & \quad K_d = 0.3 \text{nM}
\end{align*}

\textbf{E} \quad \text{Detection: streptavidin-HRP}

\textbf{F} \quad \text{Absorbance (X 1000)}

\begin{align*}
\text{Buffer control} & \quad \text{AMHR2-Fc/Fc} \\
\text{AMHR2-Fc/Fc} & \quad \text{c-AMH}
\end{align*}
**A** Detection: streptavidin-HRP
- biotinylated-mAb-N₁
- biotinylated-mAb-C₁
- c-AMH
- Capture Ab: mAb-N₂

**B** AMH captured on mAb-N₂
- mAb-C₁
- mAb-N₁
- Absorbance (X 1000)

**C** Detection: streptavidin-HRP
- biotinylated-mAb-N₁
- biotinylated-mAb-C₁
- c-AMH
- Capture Ab: anti-human-Fc-Ab

**D** AMH captured on AMHR2-Fc/Fc
- mAb-C₁
- mAb-N₁
- Absorbance (X 1000)

**E** % Intact AMH complex:
- mAb-N₂: Ratio C₁ to N₁

**F** Detection: streptavidin-HRP
- Capture Ab: mAb-N₁
- Capture Ab: mAb-C₁
- Capture Ab: anti-human-Fc-Ab

**G** AMHR2-Fc/Fc:
- mAb-N₁
- mAb-C₁
- Absorbance (X 1000)

**H** % Intact AMH complex:
- AMHR2-Fc/Fc: Ratio N₁ to C₁
- Ratio C₁ to N₁
0.5 nM AMH

mAb-C₁

200 nM AMH

mAb-N₁

20 µm 20 µm

20 µm 20 µm
A

Cleaved AMH

\[ \text{AMH}_{\text{RI}} \]

\[ K_1 \rightarrow RL \]

\[ K_2 \rightarrow RLR \]

mAb-C\(_1\) and 2\(^{nd}\) Ab added alone

\[
\frac{\text{bound mAb-C}\_1}{(\text{bound mAb-C}\_1)_{\text{max}}} = \frac{[RL + 2RLR]}{[R_0]}
\]

mAb-C\(_1\) and 2\(^{nd}\) Ab added as complex

\[
\frac{\text{bound mAb-C}\_1}{(\text{bound mAb-C}\_1)_{\text{max}}} = \frac{[RL + LRL]}{[R_0]}
\]

mAb-N\(_1\) and 2\(^{nd}\) Ab added alone

\[
\frac{\text{bound mAb-N}\_1}{(\text{bound mAb-N}\_1)_{\text{max}}} = \frac{[RL]}{[R_0]}
\]

mAb-N\(_1\) and 2\(^{nd}\) Ab added as complex

\[
\frac{\text{bound mAb-N}\_1}{(\text{bound mAb-N}\_1)_{\text{max}}} = \frac{[RL + 2RLR]}{[R_0]}
\]

B

HRP

Goat anti-mouse Fc-HRP

mAb-N\(_1\)

mAb-C\(_1\)

capture Ab. anti-human-Fc-Ab

HRP

mAb-secondary Ab complexes

HRP

mAb-C\(_1\) and 2\(^{nd}\) Ab added alone

\[ K_1 = 1.1 \text{ nM} \]

\[ [R_0]/K_2 = 3.7 \]

mAb-N\(_1\) and 2\(^{nd}\) Ab added as complex

\[ K_1 = 1.1 \text{ nM} \]

\[ [R_0]/K_2 = 4.9 \]

C

\[ [\text{Species}]/[R_0] \] or Fraction maximal signal

D

\[ [\text{Species}]/[R_0] \] or Fraction maximal signal

AMH bound monovalently (■)

\[ K_1 = 1.1 \text{ nM} \]

\[ [R_0]/K_2 = 4.9 \]

AMH bound bivalently (▲)
A

Species detected by mAb-C
Species detected by mAb-N

Monovalent interaction
Bivalent interaction

B

$K_1 = 1.0 \text{ nM}$

Width of bell shaped curve is proportional to $[R_0]/K_2$

Midpoint is equal to $K_1$

C

$K_T = K_{bi}$

$K_3 = 1.0 \text{ nM}$
$K_{mono} = 0.001 \text{ nM}$
$K_{bi} = 1.0 \text{ nM}$

$K_T$ between $K_{bi}$ and $K_{mono}$

D

Fraction intact complex

$K_3 = 1.0 \text{ nM}$
$K_{mono} = 0.001 \text{ nM}$
$K_{bi} = 1.0 \text{ nM}$
**Figure A**

Detection: streptavidin-HRP

Biotinylated-mAb-C

Capture Ab: mAb-N

**Figure B**

![Graph showing AMH complex concentration (ng/ml) over time](image)

- P value = 0.0016
- P value = 0.8890

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<th>u-AMH</th>
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**Figure C**

![Graph showing K₁ and Kᵣ concentration](image)

- Kₚ = 0.05 nM
- Kₚ = 0.5 nM
- Kₚ = 5.0 nM

Kₛᵢ = 1.1 nM

\(\frac{[R₀]}{Kₛ} = 7.2\)

Kₘ₀ₙ₀ = 0.0008 nM

**Figure D**

![Graph showing fraction intact complex](image)

- Kₛᵢ = 1.1 nM
- [R₀]/Kₛ = 9.1
- Kₘ₀ₙ₀ = 0.0008 nM