Structure of the Alk1 Extracellular Domain and Characterization of Its Bone Morphogenetic Protein (BMP) Binding Properties

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Supporting Information

ABSTRACT: Bone morphogenetic proteins (BMPs) are secreted signaling proteins — they transduce their signals by assembling complexes comprised of one of three known type II receptors and one of four known type I receptors. BMP-9 binds and signals through the type I receptor Alk1, but not other Alks, while BMP-2, -4, and -7 bind and signal through Alk3, and the close homologue Alk6, but not Alk1. The present results, which include the determination of the Alk1 structure using NMR and identification of residues important for binding using SPR, show that the β-strand framework of Alk1 is highly similar to Alk3, yet there are significant differences in loops shown previously to be important for binding. The most pronounced difference is in the N-terminal portion of the β4-β5 loop, which is structurally ordered and includes a similarly placed but shorter helix in Alk1 compared to Alk3. The altered conformation of the β4-β5 loop, and to lesser extent β1-β2 loop, cause clashes when Alk1 is positioned onto BMP-9 in the manner that Alk3 is positioned onto BMP-2. This necessitates an alternative manner of binding, which is supported by a model of the BMP-9/Alk1 complex constructed using the program RosettaDock. The model shows that Alk1 is positioned similar to Alk3 but is rotated by 40 deg. The alternate positioning allows Alk1 to bind BMP-9 through a large hydrophobic interface, consistent with mutational analysis that identified several residues in the central portion of the β4-β5 loop that contribute significantly to binding and are nonconservatively substituted relative to the corresponding residues in Alk3.

Bone morphogenetic proteins (BMPs) are small secreted signaling proteins that regulate embryonic patterning and organ development and maintain and regenerate tissues.1–3 They are present in both invertebrates and vertebrates and are the ancestors of an extended family of signaling proteins, known as the TGF-β superfamily.4 The other members of the superfamily include the closely related growth and differentiation factors (GDFs), which regulate cartilage and skeletal development, the activins and inhibins, which regulate cell growth and the release of pituitary hormones, and the TGF-βs, which regulate cellular growth and differentiation. The superfamily has expanded as eukaryotes have diversified, with three known ligands in Caenorhabditis elegans, seven in Drosophila melanogaster, and more than 30, including more than 15 known BMPs and GDFs, in humans.5 BMPs and other proteins of the TGF-β superfamily transduce their signals by binding and bringing together two serine/threonine kinase receptors, known as type I and type II receptors.5 This initiates a phosphorylation cascade in which the constitutively active type II receptor phosphorylates conserved serines within a negative regulatory domain of the type I receptor.6 Activated type I receptor in turn phosphorylates downstream signaling messengers called receptor-regulated Smads, or R-Smads, to relay the signal from cell membrane to the nucleus.7 There are seven type I and five type II receptors in humans and other higher vertebrates. The type I receptors, which include activin-like kinases 1–7 (or Alk1–7), can be further divided into subgroups based on the ligand they bind and the R-Smad they activate. The type I receptors Alk1, Alk2, Alk3, and Alk6 principally bind BMPs and GDFs and activate R-Smads 1, 5, and 8, whereas Alk4, Alk5, and Alk7 bind ligands such as activins, myostatin, and TGF-β and activate R-Smads 2 and 3. Activated R-Smads form a...
complex with a common partner, Smad4, and to translocate to the nucleus where they regulate transcription of target genes. R-Smads 2 and 3 and 1, 5, and 8 assemble distinct transcriptional complexes and target distinct genes, segregating the activities of BMPs and GDFs from activins, myostatin, and TGF-β.

Alk1 and endoglin, an unrelated cell surface proteoglycan with a large ectodomain and a short noncatalytic cytoplasmic domain, are expressed mainly by endothelial cells and have been linked to hereditary hemorrhagic telangiectasia (HHT), a disease caused by the fragility of blood vessels. Alk1 and endoglin lead to HHT. Alk1 and endoglin null mice have been further shown to have major defects in vasculogenesis and are embryonic lethal.8

Endoglin null mice have been further shown to have major defects in vasculogenesis and are embryonic lethal.8 Alk1 and endoglin, an unrelated cell surface proteoglycan including Alk3 and its close homologue, Alk6.14,18 Alk3, in contrast to Alk1 and endoglin, is expressed mainly by endothelial cells and has been linked to hereditary hemorrhagic telangiectasia (HHT) and to HHT.

The demonstrated importance of these residues for ligand binding, together with the demonstrated specificity differences between Alk1 and Alk3, suggests that the distinct specificity of Alk1 and Alk3 for different BMPs might arise from these amino acid differences.

To investigate the underlying specificity differences between Alk1 and Alk3, the structure of the unbound form of the Alk1 extracellular domain was determined using nuclear magnetic resonance (NMR) spectroscopy. The secondary structure and overall fold of Alk1 were found to be highly similar to Alk3; however, in contrast to Alk3, the helix in the binding region was shown to be both preformed and largely rigid on the nanosecond–picosecond time scale. The preformed helix and rigidity in this region of Alk1 could account for its restricted binding compared to Alk3, with its Glu-Leu motif serving as a critical specificity determinant. This was evaluated by generating chimeras in which the Glu-Leu motif from Alk3 was swapped with the homologous Phe-Gln motif from Alk3.

## EXPERIMENTAL PROCEDURES

### Chemicals and Other Reagents.

Tris(hydroxymethyl)-aminomethane (Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), sodium chloride, and oxidized and reduced glutathione were purchased from Sigma-Aldrich (St. Louis, MO). Tween 20, 1-ethyl-3-[3-dimethylaminopropyl]-carboimidium hydrochloride (EDC), N-hydroxysuccinimide (NHS), and CM-5 sensor chips were purchased from GE Healthcare (Piscataway, NJ). Human BMP-2 and human BMP-9 were purchased from Peprotech (Rocky Hill, NJ). All other chemicals were reagent grade or better and were purchased from ThermoFisher Scientific (Waltham, MA).

### Expression and Purification of Alk1-ED.

A DNA fragment corresponding to the full-length ectodomain of mature human Alk1 was inserted between the XhoI and NdeI cleavage sites in PET15b (Novagen, Madison, WI). This construct included in addition to residues 1–97 of the mature ectodomain an N-terminal histidine tag followed by a thrombin cleavage site. The construct was overexpressed in Escherichia coli BL21(DE3) cells cultured at 37 °C in LB medium containing 150 mg/L ampicillin. Protein expression was induced by adding 0.8 mM IPTG when the absorbance at 600 nm reached 0.6. Cells were harvested 6–8 h after induction. Cell pellets from 6 L of culture were resuspended in 200 mL of lysis buffer (100 mM Tris, 10 mM EDTA, pH 8.3

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Expression and Purification of Alk3-ED. A DNA fragment corresponding to residues 28–129 of the mature of human Alk3 ectodomain (Alk3-ED) was inserted downstream of an expression cassette that included thioredoxin, a hexahistidine tag, and thrombin cleavage site in plasmid pET32a (Novagen, Madison, WI). The fusion protein was overexpressed in E. coli BL21(DE3) cells at 37°C. The protein sample was diluted into 4 L of prechilled refolding buffer (50 mM Tris, 5% (v/v) glycerol, 0.5 M NaCl, pH 8.0) and oxidized glutathione was added to a final concentration of 30 mM. The refolding mixture was concentrated to 75 mL, incubated with thrombin (3 U/mg of protein) overnight at 4°C, dialyzed into 25 mM Tris-Cl, pH 8.0 and loaded onto a 25 mL Ni-NTA column that had been pre-equilibrated with Tris buffer. The column was washed with 100 mL of Tris buffer, and the flow-through and wash, which contained Alk3-ED, were pooled and dialyzed three times against 4 L of 25 mM Hepes, pH 7.0. The dialyzed protein was loaded onto a Source Q anion-exchange HPLC column (GE Healthcare, Piscataway, NJ) and eluted with a 0.0–0.5 M linear NaCl gradient (buffer A - 25 mM Hepes, pH 7.0; buffer B - 25 mM Hepes, 0.5 M NaCl, pH 7.0). Fractions were analyzed by nonreducing SDS–PAGE, and those found to contain Alk3-ED monomers were pooled and further fractionated on a C18 reverse phase column as for Alk1-ED.

Cell-Based Antagonist Assays. Mouse NIH-3T3 fibroblasts were cultured to near confluency in DMEM and transiently transfected with a construct encoding full-length Alk1 or Alk3 under the control of a CMV promoter. Twelve hours post-transfection, the transfected cells were transferred to serum free medium and cultured an additional six hours. The Alk1 transfected cells were then treated with 200 pM BMP-9 with increasing concentrations of the purified Alk1 and Alk3 extracellular domains (40 nM, 400 nM, or 2000 nM for Alk1 and 40 nM, 400 nM, or 4000 nM for Alk3). The Alk3 transfected cells were treated with 800 pM BMP-2 with increasing concentrations of the purified Alk1 and Alk3 extracellular domains (160 nM or 1600 nM for Alk1 and 160 nM, 1600 nM, or 16000 nM for Alk3). The cells were cultured for an additional hour, isolated by centrifugation, and lysed in buffer containing protease and phosphatase inhibitors. The signaling activity was then assessed by subjecting 30 μg of protein from the soluble fraction to SDS–PAGE followed by Western blotting with a phospho-Smad1/5/8 antibody (Cell Signaling, Boston, MA). The total protein loaded and Smad expression levels were controlled by stripping the blot and probing with Smad5 (Abcam, Cambridge, MA) and GAPDH (Calbiochem, San Diego, CA) antibodies.

NMR Samples and NMR Data Acquisition. Alk1-ED samples isotopically labeled with 15N or 13C for NMR were prepared by growing bacterial cells in M9 media containing 0.1% (w/v) 15NH4Cl or 0.1% (w/v) 13NH4Cl and 0.03% (w/v) 13C labeled glucose. All NMR samples were prepared in 25 mM sodium phosphate, 5% 2H2O, 0.02% w/v sodium azide at a protein concentration of 0.4–0.7 mM, pH 5.5. All NMR data were acquired at a sample temperature of 32°C.
The overall rotational correlation time was determined by first using the criterion described by Barbato to identify any residues undergoing large amplitude motion on the nanosecond–picosecond time scale or exchange. The trimmed data set (with these residues removed) was then analyzed using a conjugate gradient minimization procedure to identify the rotational correlation time by minimizing the difference between the calculated and experimental $T_1/T_2$ ratio. Internal dynamics were assessed by analyzing the experimental $^{15}$N relaxation parameters using the extended Model-Free formalism with the overall correlation time derived from the analysis described above. Internal motional parameters were derived using the program ModelFree4, which employs F-statistics for model selection. Five different models for internal motion were considered, $S^2$ (model 1), $S^2, \tau_e$ (model 2), $S^2, \tau_e, R_e$ (model 3), $S^2, \tau_e, R_e$ (model 4), and $S^2, S^0_b, \tau_e$ (model 5).

Alk1 and Alk3 Variants and Characterization of Their Binding Properties. Alk1-ED and Alk3-ED variants were generated by site directed mutagenesis of the corresponding expression plasmids using PCR (Quikchange, Stratagene, LaJolla, CA). All variants were expressed and purified as described for the corresponding wild type protein. Biacore 3000 biosensor instrument was used for quantitative measurement of the receptor–ligand interactions. BMP-9 and BMP-2 were diluted in 0.1 M acetic acid to a final concentration 2–6 µg/mL and coupled to the carboxymethylated dextran chip surface (CMS, GE Healthcare, Piscataway, NJ) using amine-coupling methods. The chip surface was first activated by injecting 35 µL of N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) followed by the introduction of a gap of 10–12 Å between Alk1 and BMP-9 to eliminate any bias in the initial steps of the docking. Alk1 was docked by performing an initial rigid body search, followed by optimization of side chain contacts. Alk3 was docked onto BMP-2 in a similar manner to evaluate the accuracy.

Data Deposition. Chemical shifts assignments for Alk1 ED were deposited under BMRB 17628. Ten lowest energy structures satisfying all the experimental distances, dihedral angles, and RDC restraints have been deposited under PDB 2LCR.

RESULTS

Isolation of Recombinant Alk1-ED and Alk3-ED and Initial Characterization by NMR. The seven type I receptors of the TGF-β superfamily include a conserved pattern of 10 cysteines (Figure 1). These cysteines form an identical pattern of five disulfide bonds in the three type I receptor structures.
Figure 3. SPR binding analysis of bacterial recombinant Alk1-ED and Alk3-ED. (A) SPR sensorgrams of serial 2-fold dilutions of Alk1-ED and Alk3-ED over a BMP-9 surface. Sensorgrams shown have been corrected for background binding to a surface with no coupled ligand. (B) SPR sensorgrams as in panel A, except over a BMP-2 surface. (C) Saturation plots obtained by fitting the maximal binding response (circular data points) as a function of injected receptor concentration, [R], to $R_{eq} = (R_{max}|R|)/(K_d + |R|)$. Data for Alk1 binding to BMP-9 and for Alk3 binding to BMP-2 are shown in the upper and lower panels, respectively.

which are known, Alk3,19,20 Alk5,39,40 and Alk6,41 suggesting that all type I receptors of superfamily share this common set of disulfides. The previously reported binding studies with the Alk1 extracellular domain were performed using protein produced in either CHO or HEK-293 cells.16–18,42 Though these recombinant forms of Alk1 bound BMP-9 with high affinity, they could not be used for the proposed NMR studies due to the difficulty of producing fully $^{15}N$, $^{13}C$ labeled samples for NMR. Thus, the bacterial expression and refolding procedure previously used to obtain the Alk5 extracellular domain43 was used.

This entailed expression of the mature human Alk1 extracellular domain (residues 1–97) in E. coli, folding in a nondenaturing buffer in the presence of a glutathione reductone couple, and purification by HPLC-based ion-exchange and reverse phase chromatography (Figure 2A–C). The identity of the isolated Alk1 extracellular domain (Alk1-ED) was confirmed by electrospray ionization mass spectrometry, which yielded a mass just 0.2 Da greater than that calculated for the fully oxidized (i.e., five disulfide) form of the protein (Supporting Information, Figure 1A).

The Alk3 extracellular domain (Alk3-ED) was obtained using the previously reported E. coli expression and purification procedure44 but modified to include a denaturation and renaturation step. This improved the recovery of native monomers and led to a highly homogeneous sample suitable for NMR and binding studies (n.b., following the prior procedure, residues 1–27 of the Alk3 extracellular domain were excluded from the expression construct since this region is structurally disordered and not required for BMP binding20). The identity of the isolated Alk3 extracellular domain was confirmed by electrospray ionization mass spectrometry, which yielded a mass just 0.1 Da greater than that calculated for the fully oxidized (i.e., five disulfide) form of the protein (Supporting Information, Figure 1B).

The Alk1 extracellular domain was subsequently analyzed by NMR to assess folding and homogeneity. This was accomplished by recording a two-dimensional $^1H$–$^{15}N$ heteronuclear single quantum shift correlation (HSQC) spectrum of a $^{15}N$-labeled sample in phosphate buffer at pH 5.5 and 32 °C. The spectrum recorded under these conditions included 90 of the 91 expected backbone amide signals dispersed over a broad range of $^1H$ chemical shifts (6.4–10.0 ppm) (Supporting Information, Figure 2). The Alk3 extracellular domain was analyzed in a similar manner, though in this case the spectrum was recorded at a somewhat higher pH (pH 6.3) and lower temperature (25 °C) to match the solution conditions under which it was previously studied.20 The spectrum recorded under these conditions had 94 of the 95 expected backbone amide signals (94/95) and closely matched that previously reported (Supporting Information, Figure 2). These data show that bacterial recombinant Alk1-ED and Alk3-ED are structurally ordered and homogeneous.

Characterization of the BMP-2 and BMP-9 Binding Properties of Alk1-ED and Alk3-ED. The prior SPR binding studies showed that the monomeric Alk1 extracellular domain, as well as Fc-Alk1, a constitutive Alk1 dimer in which the Alk1 extracellular was fused to the Fc region of antibody, bound BMP-9 with $K_d$’s of 20–45 nM and 2–3 nM, respectively16,18,42 (the higher affinity of the dimeric Alk1 is a result of multivalent binding, which has been previously observed with other receptors of the TGF-β superfamily45). This differs from other Alks, which appear to be incapable of binding BMP-9 either as Fc-Alk chimeras in SPR binding studies37 or as receptors expressed on the cell surface of cultured cells.14 Moreover, this pattern is the opposite of BMP-2 and BMP-4, which bound Alk3 and Alk6 with high affinity, but not Alk1.14

The functional activity of the bacterial recombinant Alk1-ED was evaluated by performing an equilibrium binding SPR experiment in which increasing concentrations of Alk1-ED were
injected over either a control surface with no coupled ligand or over surfaces with BMP-2 and BMP-9 at a surface density of roughly 500 resonance units (RU) each. This yielded a robust concentration-dependent SPR response relative to the control when the bacterial recombinant Alk1-ED was injected over the BMP-9 surface, but not the BMP-2 (Figure 3A,B). The bacterial recombinant Alk3-ED yielded the opposite result with a robust concentration-dependent response relative to the control when injected over the BMP-2 surface, but not the BMP-9 (Figure 3A,B). The maximal response could be fitted as a function of concentration to derive the dissociation constant, $K_d$, and maximal response, $R_{\text{max}}$, for Alk1-ED binding to BMP-9 and Alk3-ED binding to BMP-2, but not Alk1-ED binding to BMP-2 or Alk3-ED binding to BMP-9 due to the weak response (Figure 3C, Table 1). The $K_d$ values for Alk1-ED and Alk3-ED were evaluated in terms of their ability to compete against endogenously expressed Alk1 and Alk3 in cultured NIH-3T3 fibroblasts. The results showed that Alk1-ED diminished the BMP-9 stimulated activation of phosphoSmad1/5/8 in a dose-dependent manner, whereas Alk3-ED did not (Supporting Information, Figure 3). The Alk1-ED was further shown to fully attenuate the BMP-9 induced activation at a concentration of 2 $\mu$g/mL, which is roughly 5-fold lower than that reported by Alt et al.16 This confirms that the bacterially derived Alk1-ED and Alk3-ED are native and functionally active.

**NMR Assignments and Analysis of Secondary Shifts.** The backbone resonances of Alk1-ED were assigned by uniformly labeling it with $^{13}$C and $^{15}$N and by acquiring sensitivity-enhanced triple-resonance data sets with 0.4–0.7 mM samples in 25 mM sodium phosphate at pH 5.5 (Experimental Procedures). This enabled the sequence-specific assignment of all the backbone amide signals of Alk1-ED, except for the first five residues on the N-terminus (four of which correspond to a GSHM tetrapeptide derived from the vector) and Leu51 (Figure 4A). The side chain $^1$H and $^{13}$C side chain assignments were obtained by extending from the backbone using established methods (Experimental Procedures).

The secondary shifts of Alk1-ED were analyzed using the program PECAN, which provides secondary structure probabilities on a residue-by-residue basis47 (Figure 4B). This showed that the secondary structure of Alk1-ED is comprised of five $\beta$-strands. The PECAN analysis also identified a short region with helical propensity (residue 54–56), although this was with reduced probability compared to the regions of $\beta$-strand. The positioning and length of the predicted $\beta$-strands coincide closely with those based on unbound and bound Alk3-ED structures (Figure 4C). The positioning and length of the helical region also closely coincides with that observed in Alk3, though as already noted, this $\alpha$-helix was only observed in the BMP-2 bound form of Alk3-ED determined by crystallography,19 not the unbound form determined by NMR.20 The close correspondence of Alk1’s predicted secondary structure with that of Alk3 suggests that Alk1 shares the same disulfide-bonded three-finger toxin fold. The fact that the helical region can be detected based on the secondary shifts, though with reduced probability, suggests that this helix is at least partially formed in the unbound form of Alk1-ED.

**Solution Structure of Alk1-ED.** The solution structure of Alk1-ED was determined using simulated annealing with torsion angle dynamics, as implemented in the program Aria 2.3.48 The input data for the calculation consisted of 1816 experimental restraints, including 1612 distance restraints, 88 TALOS-derived $\phi$ and $\psi$ restraints,49 24 $^3$JHN-H$^2$O restraints, and 67 $^1$H–$^{13}$N residual dipolar coupling restraints (RDCs) (Table 2). The 10 lowest energy structures consistent with the experimental restraints are shown in Figure 5A. The regions of secondary structure — $\beta$1 (residues 12–14), $\beta$2 (residues 24–26), $\beta$3 (residues 30–36), $\beta$4 (residues 42–48), $\beta$5 (residues 54–56), and $\beta$6 (residues 66–69) — correspond closely to those predicted based on the secondary shifts and were well-defined, with an overall backbone root-mean-square deviation (rmsd) of 0.33 Å. The structurally ordered core, which extends from residues 10–82 and includes several loop regions, had an overall backbone rmsd of 0.73 Å (Table 2). The terminal regions, residues 1–9 and 83–97, had no medium and long-range NOEs and were disordered in the ensemble of calculated structures. The stereochemical quality, as assessed by the program PROCHECK,50 was typical of a well-refined structure, with 94% of the residues in the most favored or additionally allowed regions of the Ramachandran plot (Table 2). The residues in the generously allowed and disallowed

### Table 1. Binding of Alk1 and Alk3 Variants to BMP-2 and BMP-9

<table>
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<th>analyte</th>
<th>injected concentration range (μM)</th>
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<tr>
<td>Alk1-WT</td>
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*The SPR-derived $K_d$'s and associated errors are the mean and standard deviations of two to three independent measurements for all the variants, except N50A, R3SD, and L55Q, which were only measured once. Not determined due to weak binding.
regions of the Ramachandran plot were all in the terminal regions or loops.

The helical region of Alk3 bearing the Phe85-Gln86 motif important for binding to BMPs 2, 4, and 7 resides in the extended segment connecting \( \beta \)-strands 4 and 5. The Alk1 ensemble has increased disorder in this segment, yet analysis of the calculated structure using the program DSSP detects a 310 helix from residue 54 to 56 over all members of the ensemble (Figure 5A,B). Though the extended segment bearing this 310 helix is clearly disordered over its length, the N-terminal portion is evidently less so as backbone overlays of this segment up to Cys56 have a considerably lower rmsd (0.47 Å) than overlays of the C-terminal portion (1.14 Å, respectively) (Figure 5C). The structural basis for the apparent order in the N-terminal portion (hereafter designated L4) is probably a result of restrictions imposed by disulfide-bonded cysteines on the N- (Cys48) and C-terminal (Cys56) ends and the intrinsic propensity of residues 54 to 56 to form a 310 helix. The disorder in the C-terminal portion (hereafter designated L5) is probably the result of the absence of an anchoring cysteine on the C-terminal end coupled with the lack of any internal structure or interactions with the underlying structured core.

Table 2. Structural Statistics for Alk1-ED

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<tr>
<td>sequential ((</td>
<td>i - j</td>
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<td>(^{1}J_{HN, \alpha})</td>
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<tr>
<td>total restraints</td>
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Deviation Among Ensemble

- bonds (Å): 0.0054 ± 0.0003
- angles (degrees): 0.72 ± 0.04
- impropers (degrees): 2.1 ± 0.2
- dihedral restraints (degrees): 0.71 ± 0.22
- RDC \(^{1}D_{NH}\) (Hz): 0.58 ± 0.12
- \(^{1}J_{HN, \alpha}\) restraints (Hz): 0.71 ± 0.10

**Figure 4.** Assigned \(^{1}H-^{15}N\) HSQC spectrum and secondary structure probabilities of Alk1-ED. (A) \(^{1}H-^{15}N\) HSQC spectrum of 0.4 mM \(^{15}N\) Alk1-ED in 25 mM sodium phosphate, 0.02% sodium azide, 5% \(^2\)H\(_2\)O (pH 5.5) recorded at 32 °C at a magnetic field strength of 16.4 T (700 MHz \(^1\)H). Peaks are labeled according to their resonance assignments (residues are number as in Figure 1). Horizontal dashed bars designate the side-chain amide groups of asparagine and glutamine. (B) Secondary structure probabilities based on an analysis of the NMR secondary shifts using the program PECAN. (C) Secondary structure predictions for Alk1 based on the structures of the unbound and bound forms of Alk3 (PDB 2K3G and 1REW, respectively) and an alignment of the Alk1 and Alk3 amino acid sequences.
Internal Dynamics of Alk1. The internal flexibility of Alk1-ED was modeled based on the measured $^{15}$N $T_1$, $^{15}$N $T_2$, and $^{1}H-^{15}$N NOE parameters using the extended model free formalism as described in Experimental Procedures. The derived parameters show that the regions of regular secondary structure, including the $3_{10}$ helix in the extended segment between $\beta$-strand 4 and S, are rigid with a mean $S^2$ of 0.89 ± 0.06 (Figure 6). The N- and C-terminal regions from residues 1–10 and 82–97 are in contrast highly flexible, with $S^2$ of 0.18 ± 0.16, consistent with the lack of medium and long-range NOEs and high degree of disorder in these regions. The loops connecting $\beta$-strands 2 and 3 and $\beta$-strand 4 and the $3_{10}$ helix are highly rigid, with $S^2$ values of 0.89 ± 0.03. The loop connecting $\beta$-strands 1 and 2 is relatively rigid over most its length, except at the tip, which has an $S^2$ of 0.42. This is probably because this loop has an internal disulfide bond (Cys15–Cys20), which restricts mobility within the loop. The loops connecting $\beta$-strands 3 and 4 and the $3_{10}$ helix and $\beta$-strand 5 are flexible, with minimal $S^2$ values of 0.4–0.5. The significant differences in flexibility between the N- and C-terminal halves of the segment connecting $\beta$-strands 4 and 5 are consistent with the differences in the degree of order among these regions in the calculated structures (Figure 5C). Thus, the N-terminal half of the segment connecting $\beta$-strands 4 and S, including the $3_{10}$ helix from residues 54–56 is structurally ordered and rigid on the nanosecond–picosecond time scale, but the C-terminal half is not.

Structural Comparison of Alk1 and Alk3. The superposition of the structure of the unbound form of Alk1 with the unbound and bound forms of Alk3 shows that the $\beta$-strand core superimposes closely, with backbone RMSDs of 0.89 and 0.83 Å, respectively (Figure 7A,B). The largest difference is in the extended segment connecting $\beta$-strands 4 and 5 and is most pronounced for the unbound form of Alk3 where this region was shown to be flexible and structurally disordered (Figure 7A). The difference is less pronounced for the bound form of Alk3 where this region is ordered and includes a six residue helix, the third, fourth, and fifth residues of which, Phe85, Gln86 and Cys87, are positioned similarly to the three residues of Alk1’s $3_{10}$ helix, Glu54, Leu55, and Cys56 (Figure 7B). The similar spatial positioning of the helices in Alk1 and Alk3, coupled with the established importance of Alk3 Phe85 and...
Gln86 for binding of BMP-2 and other related ligands, suggests that residue differences in this region might be responsible for the restricted binding of these two type I receptors to their respective ligands. The other major structural difference between Alk1 and Alk3 is the conformation of loop 1. This loop includes an internal disulfide bond in both Alk1 and Alk3, though the two cysteines that form this disulfide are separated by four residues in Alk1 and three in Alk3 (Figure 1). This is probably responsible for the different conformations of the two loops as a disulfide cannot be accommodated if either of the cysteines are shifted toward one another in the Alk1 loop, or away from one another in the Alk3 loop.

Role of Alk1 REL and Alk3 DFQ Tripeptide Motifs on BMP Binding. To evaluate whether these residue differences might underlie the differences in specificity, Alk1 and Alk3 variants were generated in which Asp84, Phe85, and Gln86 in Alk3 and Arg53, Glu54, and Leu55 in Alk1 were swapped. These variants, designated Alk1-DFQ and Alk3-REL, were isolated as before and characterized in terms their binding using SPR. The SPR data showed that the affinity of Alk1-DFQ for its cognate ligand, BMP-9, was diminished approximately 200-fold compared to wild type Alk1 (Figure 8A,C, Table 1). Alk3-REL was similarly diminished in its affinity for its cognate ligand, BMP-2, compared to wild type Alk3, though the precise amount could not be determined due to weak binding (Figure 8E, Table 1). SPR further showed that the Alk1 and Alk3 variants with swapped tripeptides did not lead to a detectable increase in the binding of the receptors to their noncognate ligands, BMP-2 for Alk1 and BMP-9 for Alk3 (Figure 8B,D). Although the precise kinetic parameters were not determined, visual inspection of the sensorgrams indicates that the effects of the substitutions are to increase to disassociation rate, consistent with the disruption of interactions that normally stabilize the complex.

The contribution of individual residues within Alk1’s tripeptide motif were assessed by substituting them with the

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**Figure 7.** Structural comparison of Alk1 and Alk3 and BMP-2 and BMP-9. (A) An overlay of an ensemble of the unbound forms of Alk1 (blue) and Alk3 (light pink) are shown. The structured 3_10-helix in Alk1 is colored brown and the corresponding disordered α-helix region in Alk3 is colored red. (B) Cartoon representation of ligand bound Alk3 (pink/red) superimposed on Alk1 ensemble (blue). The extent of red coloring for Alk3 corresponds to fraction of total surface area buried in the Alk3/BMP-2 crystal structure. (C) Cartoon representation of Alk3-bound BMP-2 (pink/red) superimposed on unbound BMP-9 (blue). The extent of red coloring for Alk3-bound BMP-2 corresponds to fraction of total surface area buried in the Alk3/BMP-2 crystal structure.

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**Figure 8.** SPR binding analysis of Alk1 and Alk3 variants to BMP-9 and BMP-2. (A−C) SPR sensorgrams of serial 2-fold dilutions of Alk1-DFQ (A) and Alk3-REL (B) over a BMP-9 surface and corresponding saturation plots (C). (D−F) SPR sensorgrams of serial 2-fold dilutions of Alk1-DFQ (D) and Alk3-REL (E) over a BMP-2 surface and corresponding saturation plots (F). Sensorgrams shown have been corrected for background binding to a surface with no coupled ligand. Saturation plots were obtained by fitting the maximal binding response (circular data points) as a function of injected receptor concentration. Binding of Alk3 and Alk3-REL over BMP-9 and Alk1, Alk1-DFQ, and Alk3-REL over BMP-2 were not fitted due to weak responses.

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corresponding residues from Alk3, yielding the Alk1 variants R53D, E54F, and L55Q. The R53D and L55Q variants were diminished 15- and 13-fold compared to wild type, while the E54F variant was unaffected (Table 1). On the other hand, when these residues were substituted with alanine, E54A was diminished to the greatest extent (17-fold), L55A was diminished to an intermediate extent (7-fold), and R53A the least (3-fold). Four residues outside the tripeptide motif were also substituted with alanine; the first three, N50A, H52A, and R59A, are within extended segment connecting β-strands 4 and 5 and lie within the predicted binding interface (presuming that Alk1 binds BMP-9 in the same manner that Alk3 binds BMP-2), while the fourth, R26A, is in a loop on the opposite side of the protein and lies outside of the predicted binding interface. The N50A, H52A, and R59A substitutions diminished BMP-9 binding between 9- and 10-fold, while the R26A substitution had no detectable effect (Table 1). The precise kinetic parameters were again not determined, but visual inspection of the sensorgrams indicates that the substitutions that most strongly disrupted binding are characterized by increased disassociation rates, consistent with the disruption of interactions that normally stabilize the complex (Supporting Information, Figure 4). To determine whether any of the substitutions might have altered the folding or overall structure, the one-dimensional $^1$H NMR spectra of the variants were recorded using a WATERGATE solvent suppression scheme (Supporting Information, Figures 5 and 6). This showed that all of the variants had similar spectral patterns compared to their wild type counterparts in both the methyl and amide regions, indicating that they were unperturbed in terms of their folding. Taken together, these results show that the tripeptide motifs of these receptors are necessary for high affinity binding to their cognate ligands, but that other residues and structural features are required for high affinity binding to their noncognate ligands.

**Model of Alk1/BMP-9 Complex.** The results of the mutagenesis studies were interpreted by constructing a model of the Alk1/BMP-9 complex using the program RosettaDock. The criteria used to assess the docking were whether a consistent pattern of binding was observed among the lowest energy docked structures (designated as a “docking funnel”) and whether these were consistent with the mutagenesis data. The accuracy of the method was assessed by performing 1000 trial dockings to build the structure of the Alk3/BMP-2 complex from its two component structures; this yielded a pronounced docking funnel with the 10 lowest energy structures closely clustered to one another (rmsd 0.15 Å) and the Alk3/BMP-2 crystal structure (rmsd 0.29 Å). The subsequent docking runs were performed with the solution structure of Alk1 and the crystal structure of BMP-9; this yielded a defined, though less pronounced docking funnel, with 6 of the 10 lowest energy structures clustered around a common position (rmsd 0.76 Å) with Alk1 bound to BMP-9 through the extended segment bridging β-strands 4 and 5.

The overall positioning of Alk1 at the wrist epitope in the modeled complex is similar to that of Alk3 in the Alk3/BMP-2 complex, but differs in that the receptor is rotated by roughly 40 deg relative to the ligand (Figure 9A). This rotation is driven by steric clashes that prevent Alk1 from binding BMP-9 in an Alk3-like manner — this is illustrated in Figure 9B where the...
positioning of Alk1 in the modeled complex is shown to have few if any steric clashes (right panel), while Alk1 positioned onto BMP-9 in an Alk3-like manner leads to significant clashes (left panel). The first of these occur between residues in the putative ligand binding loop of Alk1, Asn50, His52, Leu55, and Arg57, and bulky residues in BMP-9, including Trp22, Phe43, and Leu45 (Figure 9C). The most severe of these is between the side chain guanidinium group of Arg57 and the indole ring of Trp22 and occurs because of differences in the N-terminal portion of the Alk1 β4-β5 loop, which is shifted toward the wrist region by 2 – 3 Å in BMP-9 compared to BMP-2 (Figure 7C). The clashes are also partly caused by differences in the N-terminal portion of the Alk1 β4-β5 loop, which is shifted toward β-strand 5 by about 3 Å in Alk1 compared to Alk3 (Figure 7B) and causes His52 and Leu55 to clash with Phe43 and Leu45 on BMP-9 (Figure 9C). The second of these occurs between His19 and Lys21 in loop 1 of Alk1 and between Gly5 and Leu45 in BMP-9. This clash occurs because of differences in the conformation of loop 1, which is less extended in Alk1 compared to Alk3 (Figure 7A). Similar, steric clashes are also observed when Alk1-ED is positioned onto BMP-2, or other ligands, such as BMP-7, GDF-5, and TGF-β1, in an Alk3-like manner (not shown), suggesting that the distinct conformation of the β1-β2 and β4-β5 loops that prevents Alk1 from binding BMP-9 in an Alk3-like manner also serves to prevent interactions with other ligands of the superfamily.

The interactions that stabilize Alk1 at the alternative interface are shown in Figure 9C. These are hydrophobic in nature and include interactions between Leu51, His52, and Leu55, and the aliphatic portion of the side chains of Glu54 and Arg59 in Alk3 with Trp22 and Trp25 (from the A-monomer) and Phe43, Pro44, and Leu63 (from the B-monomer) in BMP-9. The interactions are consistent with the mutational data — for example, Leu55 is surrounded by hydrophobic residues from the N-terminal portion of the TGF-β type I receptor Alk5 in contrast includes a five-residue PRDRP extension. The extension is structurally ordered in the unbound form and assumes a conformation similar to the bound form. This has been suggested to be important for Alk5’s restricted binding to the TGF-β/β3, not other superfamily ligands, such as BMPs and activins. The type I receptor Alk1, which was previously considered as an orphan receptor, has recently been shown to bind BMP-9 and BMP-10 with high specificity and affinity, yet the underlying mechanism responsible for its restricted binding is not known.

The results presented here have shown that the Alk1 extracellular domain binds with high affinity to BMP-9, but not to BMP-2 (Figure 3 and Table 1). The precise affinity difference could not be determined due to difficulty quantifying Alk1’s weak binding to BMP-2, but is estimated to be 1000-fold or higher based on the Kd of 29 ± 5 nM for binding BMP-9 and an estimated Kd of 50 µM or higher for binding BMP-2. This is the opposite of Alk3, which binds BMP-2 with an affinity estimated to be at least 500-fold greater than that of BMP-9. The analysis of the Alk1 structure showed that the overall fold and secondary structure elements are highly similar to Alk3, yet there are significant differences in the conformations of the β1-β2 and β4-β5 loops (Figure 7A,B). The differences of the β4-β5 loop are very pronounced for the unbound form of Alk3 where the loop was shown by NMR to be flexible on the nanosecond–picosecond time scale and disordered in the ensemble of calculated structures, but is also evident for the bound form of Alk3, where this region is ordered and assumes a longer, though similarly positioned, central helix (Figure 7A,B). The β1-β2 and β4-β5 loops have been shown previously as the specificity determining elements in Alk3 and Alk6, and therefore could be significant for Alk1 binding specificity as well.

This was tested by swapping the Arg53-Glu54-Leu55 tripeptide from Alk1 with the corresponding tripeptide from Alk3, Asp84-Phe85-Gln86. This reduced the binding affinity of both receptors for their respective ligands several hundred fold, but did not engender the receptors with high affinity binding to their noncognate ligands. This indicates that other residues and the conformation of the β1-β2 and β4-β5 loops might also be important for determining the affinity and specificity. This is supported by the mutational analysis that showed substitution of residues outside of the tripeptide motif but within the β4-β5 loop of Alk1 reduced its affinity for binding BMP-9 (Table 1). This is also supported by the model of the Alk1/BMP-9 complex which showed that Alk1 binds to the wrist in a manner similar to Alk3, but is rotated by roughly 40° due to steric clashes caused by differences in three different loops, including...
the β1-β2 and β4-β5 loops in Alk1 which were shown to be largely rigid and adopt a distinct conformation relative to the corresponding loops of Alk3 (Figure 7A,B) and the finger 1–2 loop of the ligands (Figure 7C). The alternate positioning allows Alk1 to bind BMP-9 through a large interface dominated by hydrophobic interactions. These interactions are consistent with the mutational analysis and place the aliphatic portion of Glu54 and the side chains of His52 and Leu55 in the hydrophobic pocket on the wrist region of BMP-9 and the side chain of Leu51 between the indole rings of Trp22 and Trp25. The aliphatic portion of Glu54 is constrained by a hydrogen-bonded ion pair formed between its side chain carboxylate group, and the guanidinium group of Arg57 and Arg53 protrudes into the solvent. The importance of Leu51, His52, Glu54, and Leu55 for binding is further supported by the finding that these residues are highly conserved among mammalian Alk1 sequences, whereas Arg53 is not (Supporting Information, Figure 7). The bound form of Alk3 is similarly incompatible with the Alk1 manner of binding — this is a result of the longer length of the β4-β5 loop in Alk3 as well as a displacement of the finger 3–4 loop of BMP-2 toward the wrist (Supporting Information, Figure 8). This positioning also places the side chain carboxylate of Glu81 between the indole rings of Trp28 and Trp31, which is expected to further disfavor this manner of binding.

Taken together, these results suggest that the specificity of Alk1 and Alk3 for their respective ligands arises as a consequence of relatively minor but important structural and residue changes in the binding regions of the receptors and ligands that reposition the receptor relative to the ligand. This provides distinct interfaces and stabilizing interactions and enables the high degree of specificity for these otherwise similar ligands and receptors. This type of repositioning and corresponding expansion of specificity has been previously observed for the TGF-β type I receptor, Alk5. This receptor includes a five residue extension in the N-terminal portion of the β4-β5 loop that is both rigid and forms a tight turn with an N-terminal cis proline. The extension precludes Alk5 from binding in an Alk3 manner due to clashes with underlying hydrophobic residues in the wrist — this causes the receptor to reposition so that it is shifted toward the fingertips where it interacts extensively with both TGF-β and TβR-II. The extension binds in the cleft between TGF-β and TβR-II and orchestrates a number of interactions that are specifically required for assembly of the TGF-β signaling complex.

Thus, changes in the length and composition of the N-terminal portion of the β4-β5 loop — anticipated to be relatively common mutational event — provides a mechanism for shifting the binding interface and in turn expanding the range of specificity between ligands and receptors that otherwise share the same structural scaffold.

The alternative manner of Alk1 binding identified through the docking calculations is dependent on the underlying assumption that the backbone conformation of the unbound form of Alk1 and unbound form of BMP-9 are representative of the bound forms. This is not known with certainty, though the fact that the structural elements involved — the β1-β2 loop and the N-terminal portion of the β4-β5 on the receptor are both rigid and reinforced by disulfide bonds — suggests that they might well be. The other important point is that the docking calculations yielded a consistent docking solution — this would not occur unless the backbone conformations are already close to those of the bound form as the backbone conformation of neither the receptor nor the ligand was adjusted in the docking calculations. The final point is that even if the β1-β2 and β4-β5 loops were able to adapt to bind BMP-9 in an Alk-3 manner — something that is not anticipated due to the demonstrated rigidity of the β1-β2 and β4-β5 loops — the interactions at the interface would be far from optimal. This includes not only the unfavorable interaction between Arg57 and Trp22 noted earlier, but also between Alk1 Arg 59 and BMP-9 Leu63 and between Alk1 Lys 21 and BMP-9 Gly5. Thus, it appears likely that Alk1 binds in an alternative manner relative to Alk3 and this underlies the high specificity of Alk1 and Alk3/6 for their respective ligands, though to be certain, this will have to await the determination of the structure of the Alk1/BMP-9 complex using crystallography, which is ideally suited for this system owing to the high affinity of Alk1 for BMP-9 and the relatively high molecular weight of the complex (50 kDa).

The prior binding studies have shown that endoglin and Alk1 noncompetitively bind BMP-9, while endoglin and ActRIIb competitively bind BMP-9. This suggests that endoglin binds BMP-9 at a site that overlaps with ActRIIb — this almost certainly includes the knuckle epitope as all BMP type II receptors studied to date have been shown to bind to the knuckle. The fact that endoglin noncompetitively binds with Alk1, together with our finding that Alk1 binds on the opposite side of the ligand at the wrist, indicates that the endoglin binding site does not extend into the wrist. The fact that both endoglin and Alk1 noncompetitively bind BMP-9 suggests that endoglin and Alk1 function together to bind and capture BMP-9 on the cell surface. The type II receptor BMPRII might then function to displace the bound endoglin to form a type I/type II receptor signaling complex. This is suggested by the fact that BMPRII has been shown to bind BMP-9 with the highest affinity of the type II receptors that bind BMP-9. These biochemical observations fit nicely with several lines of evidence that like Alk1 and endoglin, BMPRII also plays a role in normal vasculogenesis.

The mutations in Alk1 that have been linked to HHT, a disease caused by the fragility of the vasculature, are found throughout the protein. The most prominent ectodomain mutations involve either the addition or elimination of a cysteine residue. The cysteines are strictly conserved in all known type I receptors of the superfamily — addition or elimination of a cysteine would be expected to disrupt the formation of the disulfide bonds and interfere with the function of the receptor. There are 11 additional noncysteine HHT mutations in the ectodomain (R26P, G27R, G27E, A28P, T31A, H45P, R46W, R46Q, G58R, N75D, and N77S). The majority of these mutations are nonconservative — one falls in the predicted binding interface and would be expected to affect BMP-9 binding (G58R). The others fall outside the interface and may lead to disease by disrupting the folding of the receptor. This is suggested by the fact that many mutated residues lie at positions in the structure that would be expected to disrupt the fold — the R26P mutation for instance replaces an arginine residue located directly adjacent to one of the conserved cysteines with a constrained proline. This is further suggested by the finding that Alk1 ectodomain HHT mutants are either not expressed on the cell surface or are expressed poorly.
ASSOCIATED CONTENT

Supporting Information
Supplementary Figures 1–8. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

Alk, activin-like kinase; BMP, bone morphogenetic protein; BMPR-Ia, BMP type I receptor a; GDF, growth and differentiation factor; T/G/R-II, TGF-β type II receptor; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancements; RDC, residual dipolar coupling; TGF-β, transforming growth factor β; SPR, surface plasmon resonance; HHT, hereditary hemorrhagic telangiectasia

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