PROTEINS: Structure, Function, and Genetics 454:408–420 (2001)

Structural Determinants of Binding and Specificity in Transforming Growth Factor–Receptor Interactions

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ABSTRACT **Transforming growth factor (TGF-β) protein families are cytokines that occur as a large number of homologous proteins. Three major subgroups of these proteins with marked specificities for their receptors have been found—TGF-β, activin/inhibin, and bone morphogenic protein. Although structural information is available for some members of the TGF-β family of ligands and receptors, very little is known about the way these growth factors interact with the extracellular domains of their cell surface receptors, especially the type II receptor. In addition, the elements that are the determinants of binding and specificity of the ligands are poorly understood. The structure of the extracellular domain of the receptor is a three-finger fold similar to some toxin structures. Amino acid exchanges between multiply aligned homologous sequences of type II receptors point to a residue at the surface, specifically finger 1, as the determinant of ligand specificity and complex formation. The "knuckle" epitope of ligands was predicted to be the surface that interacts with the type II receptor. The residues on strands β2, β3, β7, β8 and the loop region joining β2 and β3 and joining β7 and β8 of the ligands were identified as determinants of binding and specificity. These results are supported by studies on the docking of the type II receptor to the ligand dimer–type I receptor complex. Proteins 2001;454:408–420.** © 2001 Wiley-Liss, Inc.

Key words: protein–protein interactions; evolutionary trace analysis; hydrophobic interface; charge distribution; sequence analysis and alignments

INTRODUCTION

The transforming growth factor β (TGF-β) family comprises a large number of structurally related polypeptide growth factors, each capable of regulating a fascinating array of cellular processes including cell proliferation, lineage determination, differentiation, motility, adhesion, and death. Expressed in complex temporal and tissue-specific patterns, TGF-β and related factors play a prominent role in development, homeostasis, and repair of virtually all tissues in organisms.[1] For example, the founding member of the TGF-β1 family was identified as a regulator of mesenchymal growth and, separately, as an antimitogen in epithelial cells.[2,3] Activins were identified as endocrine regulators of pituitary function and as inducers of mesoderm in frogs.[4,5] Bone morphogenic proteins (BMPs) were identified as bone repair factors and, independently, as dorsalizing agents in *Drosophila*.[6,7] Nearly 30 members of the TGF-β family have been described in human, and many TGF-β orthologs are known in mouse, *Xenopus* and other vertebrates.[1,6] Four are present in *Caenorhabditis elegans*[8] and seven in *Drosophila melanogaster*.[9] The family is divided into two general branches, the BMP/GDF (growth and differentiation factor) and the TGF-β/activin/nodal branches, whose members have diverse, albeit often complementary effects. Additional members, such as inhibin-α, act as ligand antagonists. Some family members are expressed in a few cell types or for limited periods of time during development, whereas others are widespread during embryogenesis and in adult tissues. AMH/MIS (antiMüllerian hormone or Müllerian-inhibiting substance) and GDF-8/myostatin are examples of the former; TGF-β1 and BMP-4, of the latter[10] (see the reviews[1,10–14] for the list of TGF-β family members, their activities, and a detailed description of their signaling mechanism). TGF-β1 to TGF-β3 are about 70% conserved among themselves, whereas BMPs are about 60% identical. About 30% of the sequences of TGF-βs and BMPs are identical, while inhibin-βB shares 30%, 40%, and ~27% of its sequence with BMP-7, BMP-2, and the TGF-βs, respectively. The decapentaplegic protein of *Drosophila melanogaster, dpp,* has 56%, 72%, 40%, and ~31% of its sequence identical to BMP-7, BMP-2, inhibin-βB, and TGF-β, respectively. Glial cell line–derived neurotrophic factor (GDNF) and its subfamily members undergo similar modes of dimerization as TGFs but have very little sequence similarity (~14%) with members of the TGF-β family. Therefore, the GDNF subfamily can be considered a member of the broader cystine-knot superfamily (whose structural details are explained below), including nerve growth factor

Abbreviations: TGF-β, transforming growth factor β; BMPs, bone morphogenic proteins; GDF, growth and differentiation factor; GDNF, glial cell line–derived neurotrophic factor; AtR2–ECD: extracellular domain of activin type 2A receptor; $BR1A_{ec}$, BMP receptor 1A extracellular domain; ET, evolutionary trace method; *dpp,* decapentaplegic protein of *Drosophila melanogaster;* ETC, evolutionary time cutoff.

Grant sponsor: Wellcome Trust, United Kingdom.

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Received 25 January 2001; Accepted 2 August 2001

TABLE I. Interactions of TGF and Other Superfamily Members With Their Receptors

Sequential binding

Ligand	Type II receptor	Type I receptor
TGF-β	TβR2	ALK-1, ALK-2?, TβR-I, ALK-7
Activins	AtR-II, AtR-II	AtR-I, AtR-I
BMP-7	AtR2, AtR-II	AtR-I
GDF-5	AtR-II, AtR-IIB	AtR-I, AtR-IB
MIS/AMH	AMHR	AtR-I

Co-operative binding

Ligand	Type II receptor	Type I receptor
BMPs	BR-2	BR-1A, BR-1B
dpp	Punt	Thick veins (tkv)
		Saxophone (sax)
GDF5		AtR-I, BR-1B

†Abbreviations include: TGF-β (transforming growth factor–β), BMP (bone morphogenic protein), *dpp* (decapentaplegic), GDF (growth and differentiation factor), ALK (Activin receptor-like kinases), MIS/AMH (Müllerian inhibiting substance/antiMüllerian hormone), TβR (transforming growth factor receptor), AtR (activin receptor), and BR (bone morphogenic protein receptor). This table was compiled according to Massague,[1] with a few corrections. Note that there is no species specificity observed in ligand–receptor interactions.

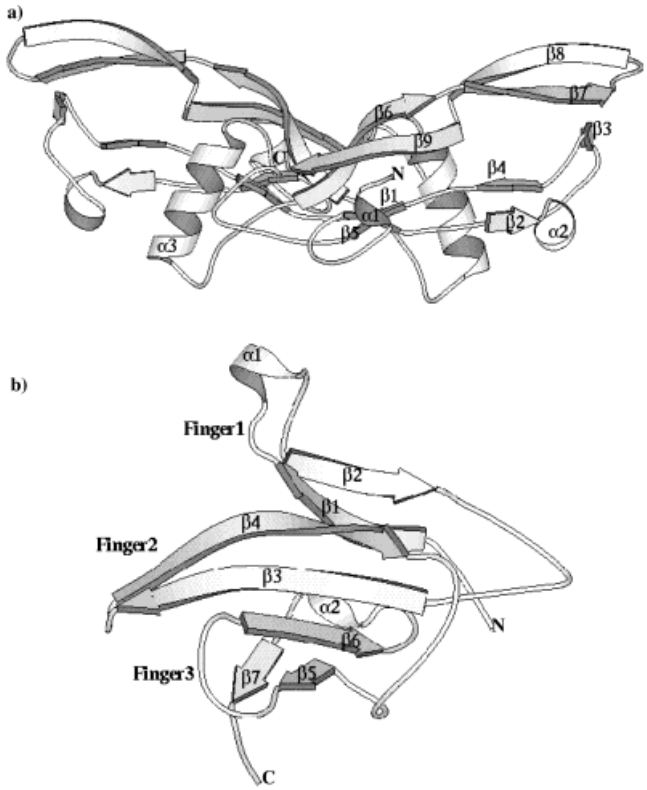

Fig. 1. Ribbon representation of **(a)** BMP-2 ligand (PDB code: 3bmp) and **(b)** ActR-II–ECD (PDB code: 1bte). Secondary structures are labeled. Fingers are marked on the receptor structure. Figure was prepared using MolScript.[60]

and platelet-derived growth factor, who members have similar protomer structures but display different modes of dimerization and share only ~15% of their sequence with the TGF-β family.[15,16]

Members of the TGF-β family of growth factors are synthesized as larger precursor molecules with an amino-terminal signal sequence and a prodomain of varying size. These precursor proteins are usually cleaved at a dibasic or RXXR site to release a mature carboxy-terminal segment of 110 to 140 amino acids[17] and are biologically active as dimers. Members of the TGF-β family regulate gene expression by bringing together two types of serine/threonine kinase receptors,[1,18] collectively known as the TGF-β receptor family. Unlike other members of the TGF-β family, GDNF family ligands activate intracellular signaling cascades via the receptor tyrosine kinase Ret.[19] The TGF-β receptor family is divided into two subfamilies, type I receptors and type II receptors, on the basis of their structural and functional properties. Type I receptors have a higher level of sequence similarity than type II receptors, particularly in the kinase domain.[1] Type I and type II receptors are glycoproteins of approximately 55 kDa and 70 kDa, respectively, with core polypeptides of 500 to 570 amino acids, including the signal sequence. Each receptor contains an extracellular, or ecto-, domain; a short membrane-spanning helix; and a cytoplasmic serine/threonine kinase domain.[20–24] Table I summarizes characteristics of various TGF-β family ligands and their receptors, which are identified biologically. Two general modes of ligand binding have been observed: One mode involves direct ligand binding (the biologically active form of TGF-β ligands is a dimer of two monomers; henceforth, when a ligand binding to a receptor is discussed, it is assumed that the receptor or receptors is interacting with a ligand dimer) to the ectodomain of the type II receptor and the subsequent interaction of this complex with the type I receptor. The type I receptor, in effect, becomes recruited to the complex, a quality that is characteristic of TGF-β and activin receptors. The second mode of binding is typical of BMP receptors and is cooperative, involving type I and type II receptor ectodomains that bind ligand with a high affinity when expressed together but with a low affinity when expressed separately.[1] In the mechanisms described above, type II receptors bind to ligand dimers, subsequently (or simultaneously) recruit type I receptors, and finally phosphorylate type I receptors at the GS domain (containing a characteristic TTSGSGSG motif) and thus activate them in transducing the signal to the nucleus.

The TGF-β isoforms show remarkable structural homology between themselves, including seven absolutely conserved cysteine residues that form three intrachain disulfide bonds and one interchain disulfide bond. The TGF-β and activins/inhibins contain an extra disulfide bridge at the N-terminus of the molecule. The structures of TGF-β2,[25–26] TGF-β3,[27] BMP-7/OP-1,[28] BMP-2,[29] and GDNF[30] were determined by X-ray crystallography, and a model of TGF-β1[31] was calculated from NMR restraints. The monomer is a thin, elongated, and slightly curved molecule resembling an open left hand. As shown in Figure

1(a), each monomer is folded into nine β-strands (β1–β9) and a long α-helix (discussed below as α3). The fold can be described as a hand with the "thumb" as the N-terminus and the extended sheets as "fingertips" representing the β2–β3 and β7–β8 loops. Accordingly, the convex surfaces of the fingers correspond to the "knuckles" and the helix region to the "wrist." The residues exposed on the convex surface involving β2, β3, β7, and β8 strands and loops joining them define the "knuckle" epitope.[33] All known ligand sequences contain seven invariant Cys residues, numbered as C2, C4, and C5 to C9; many of them contain an extra pair of Cys residues, numbered as C1 and C3. The structurally conserved region of the fold is described as a cystine "knot" because cysteines C4, C5, C8, and C9 participate in an eight-membered macrocycle wide enough for the last cystine bridge (formed by C3 and C7) to pass through. The cysteines that form the N-terminal disulfide bridge in the TGF-β are absent in other family members. Because the proteins of this family lack a hydrophobic core, the rigid cystine-knot scaffold is necessary for structural integrity. Further stabilization is achieved by dimerization, which creates a hydrophobic core between the protomers. In most cases such dimerization events are accompanied by the formation of a disulfide bridge connecting the two protomers at the C6 position.

The crystal structure of the extracellular domain of the activin type IIA receptor (ActR-II–ECD) has also been determined.[32] The fold of the ActR-II–ECD comprised of three antiparallel sheets formed by seven β-strands [Fig. 1(b)]. The molecule has both concave and convex surfaces, which result from a curvature in the first β-sheet (β1–β2). The ActR-II–ECD adopts a three-finger toxin fold, so named because it is also observed in several toxins, characterized by a common pattern of eight cysteines, forming a conserved scaffold of four disulfide bridges. The three fingers refer to three pairs of strands (β1–β2, β3–β4, and β5–β6), which all point roughly in the same direction [Fig. 1(b)]. ActR-II and cardiotoxin have the same disulfide pattern (C1–C3, C2–C4, C5–C8, and C9–C10), with the exception of an additional disulfide in ActR-II (C6–C7). Among the type II receptors there is some variability in the occurrence of cysteines. The majority of the extra cysteines in other receptors are clustered in finger 1, which constitutes the least conserved region, both sequence- and lengthwise. TβR-I has four additional cysteines in finger 1 but lacks the two cysteines that constitute the C5–C8 disulfide bond in ActR-II. Punt has two extra cysteines in finger 1.

The crystal structure of the human BMP-2 ligand in complex with two high-affinity receptor Ia extracellular domains (BR-Ia_{ec}) has been reported recently,[33] providing important information on TGF–receptor interactions at the molecular level. In this structure two molecules of the type I receptor are bound to the ligand dimer in the wrist epitope region of the ligand (additional details are described in the Results and Discussion sections) mainly by the hydrophobic surfaces of both molecules. This report also confirmed that both type I and type II receptor extracellular domains share the same fold, especially at the central β-sheet, despite poor sequence identity. Differences at loop regions and insertions of noncore secondary structures are evident; for example, a helix involved in primary interactions with the ligand in the type I receptor is absent in type II receptors.[33] Instead, an additional disulfide bridge, unique to type II receptors, links the equivalent loop region at the convex surface to the central β-sheet. This suggests that the two types of receptors have different modes of binding to the ligand at the atomic level.

Only a limited number of functionally important residues have been identified in TGF-βs and in related growth factors for binding the type II receptor. A study of the influence of segment deletions, residue replacements, and isoform chimeras on the binding affinity of TGF-βs for their type II receptor (TβR-II) highlighted the importance of C-terminal residues 83 to 112 of TGF-β1–3.[34] The structure–function analysis of activin-βA molecule is reported here, and two amino acids involved in the binding of the activin molecule to its type II receptor were identified[35] on the knuckle epitope as important for binding, Asp27 and Lys102.[36] Through alanine scanning mutagenesis experiments on ActR-II–ECD, Gray et al. were able to identify a cluster of hydrophobic residues (a hydrophobic triad)—Phe42, Trp60, and Phe83—as critical for binding to activins/inhibins.[36] It is known that type II receptors form a heteromeric complex with the ligand, but exactly how many receptor molecules interact with the ligand is not known.[1] It is apparent from Table I that TGF-β ligands can only bind to TβR-I and TβR-II, but no such specificity is observed in the case of BMPs and activins. ActR-I binds to activins/inhibins, BMP-7, and MIS/AMH; ActR-II binds to activins, BMP-7, and GDF-5.[1] This report suggested that the determinants of ligand binding to receptors may be conserved within the TGF-β subfamily and that the determinants of specificity are different for the TGF-β and activin/BMP subfamilies, whereas the residues that determine the specificity for activins and BMPs are similar. Activin receptors bind to activins/inhibins, BMPs, MIS, and GDF-5. Despite a remarkably similar structure, no such binding was observed for the TGF-β ligands.

To determine the functionally important residues, we compared the sequence distribution within the three fingers of the receptor and the charge distribution of the ligands, using the evolutionary trace (ET) method,[37] first applied by Litcharge et al. on SH2 and SH3 domains to identify potential binding-site residues as targets for mutagenesis in the TGF-β family of receptors. The five available structures of TGF-β ligands (three TGFs and two BMPs) and the three-dimensional models of *dpp* and inhibin-βB, which were derived by comparative modeling, were analyzed for differences in the distribution of polar and hydrophobic residues on the surfaces of the molecules, especially at the conserved residues[38] important for binding the type II receptor. The extracellular domain of the type II receptor was docked to the ligand-dimer type I receptor complex. From the results of both previous mutagenesis studies and of our analysis, the knuckle epitope was identified as a site of interaction with the type II receptor. Because the ligand molecules contain two sym-

metric knuckle epitopes, two receptors can bind to the ligand dimer, forming a tetrameric complex.

MATERIALS AND METHODS

Sequence Alignment and Clustering of Proteins

The 23 members of the TGF-β receptor[2] family were identified by a PSI-BLAST[39] search using the ActR-IIA ectodomain as a query sequence against the SWISS-PROT Protein Sequence Database[40] and used for evolutionary analysis. The ectodomains of the sequences were aligned using ClustalX[41] (Version 1.8) and manually edited, ensuring that gaps were not inserted into areas of known (or predicted) secondary structures. A PHYLIP (Version 3.5) distance matrix based on sequence dissimilarity indices was generated and input into a KITSCH clustering package to build a rooted phylogenetic tree.[42]

Evolutionary Trace Analysis of Receptor Sequences

An evolutionary trace is generated by comparing consensus sequences for a group of proteins that originate from a common node in a phylogenetic tree and are characterized by a common evolutionary time cutoff (ETC) and then classifying each residue as one of the three types: absolutely conserved, class specific, or neutral. Here *class specific* denotes residues occupying a strictly conserved location in the sequence alignment but differing in the nature of their conservation between various subgroups. When structural and functional residues of a protein family are not characterized, target residues can be chosen for mutagenesis. This can also be mapped to known protein structures to identify clusters of important amino acids on the surface of the protein.

The ET analysis[37] was performed using TraceSuite.[8] First, the phylogenetic tree was split along evolutionary time into five evenly distributed partitions: P01 to P05 in order of increasing ETC. For each partition a trace procedure was completed automatically in three steps: (1) proteins connected by a common node with evolutionary time greater than the given ETC were clustered together; (2) a consensus sequence was generated for each group to distinguish between conserved and nonconserved positions; and (3) a trace was generated by comparing the consensus sequences of receptors. Residues were classified into three types: absolutely conserved, class specific, or neutral. All the receptor sequences considered for the initial alignment were used for ET analysis. Punt sequence was not included because it is a lone element in the evolutionary tree and may bias the results.

Comparative Modeling and Visualization

Mature carboxy terminus peptides of *dpp* of *Drosophila melanogaster* and inhibin-βB of *Homo sapiens* were taken from the SWISS-PROT databank.[40] They were multiply aligned using ClustalX[41] to other members of the TGF-β family. MODELLER[43] (Version 4.0) was used to build three-dimensional models of both proteins. BMP-2 [Protein Data Bank (PDB) code: 3bmp] was used as a template for modeling *dpp*; BMP-2 (PDB code: 3bmp) and TGF-β3 (PDB code: 1tgj) were used as templates for modeling inhibin-βB. MODELLER constructs minimized 3-D model(s) of a protein by the satisfaction of spatial restraints extracted from the template PDB[44] files. In each case 20 models of the query sequence were generated. The final models were chosen on the basis of lowest energy and least violation of structural restraints. The stereochemistry and geometry of the models were assessed using PROCHECK[45] (Version 3.4.4), which ensured that the models had more than 85% residues in the core region of Ramachandran plot. The models were energy-minimized with the MAXIMIN2 option in SYBYL (Version 6.5, Tripos Association, Inc.) using the TRIPOS force field. For every run of energy minimization, 20 cycles of the Simplex method and an additional 50 cycles of the Powell algorithm were employed. The resulting models had no short contacts or bad geometry. The dimer coordinates were generated using a superposition program called SUPER (B. Neela, personal communication). The punt (type II receptor for the *dpp* molecule) receptor ectodomain was modeled using the same procedure, with the ActR-II–ECD crystal structure (PDB code: 1bte) as template. The resulting models and crystal structures were viewed by RASMOL, Version 2.6b2,[46] and solvent-accessible surfaces and electrostatic potentials were calculated and displayed using GRASP, Version 1.1.[47] Structure-based sequence alignment of TGF-β ligands was compiled using the program COMPARER, Version 2.0,[48] and structure-annotated using JOY, Version 4.0.[49,50]

Docking Studies on Ligand Type II Receptor Interactions

Global Range Molecular Matching GRAMM (Version 1.03) program[51–53] uses an empirical approach to predict the site of interaction between two molecules. Only the atomic coordinates of the two molecules are required (no information about the binding sites is needed). The program performs an exhaustive six-dimensional search through the relative translations and rotations of the molecules.

The X-ray structures of the activin type II receptor (PDB code: 1bte; solved at a resolution of 1.5 Å) and a complex of BMP ligand dimer with its type I receptors (PDB code: 1es7; solved at a resolution of 2.90 Å) were docked using GRAMM[51–53] with a generic, hydrophobic mode and a grid step of 2.1 Å. The program generated 1000 models to study possible ways of the ligand–receptor interactions. The models were examined for maximal hydrophobic interactions and total interactions between the 1es7 and 1bte structures using the distance cutoff value derived from known cytokine-receptor crystal structures.

RESULTS AND DISCUSSION

Analysis of Receptor Type II Sequences

The aligned nonredundant sequences of receptor type II, as shown in Figure 2, contain seven ActR-IIA sequences, seven ActR-IIB sequences, four BR2 sequences, four TβR-II sequences, and a punt receptor sequence from *Drosophila melanogaster*. Sequences of subfamilies show high conser-

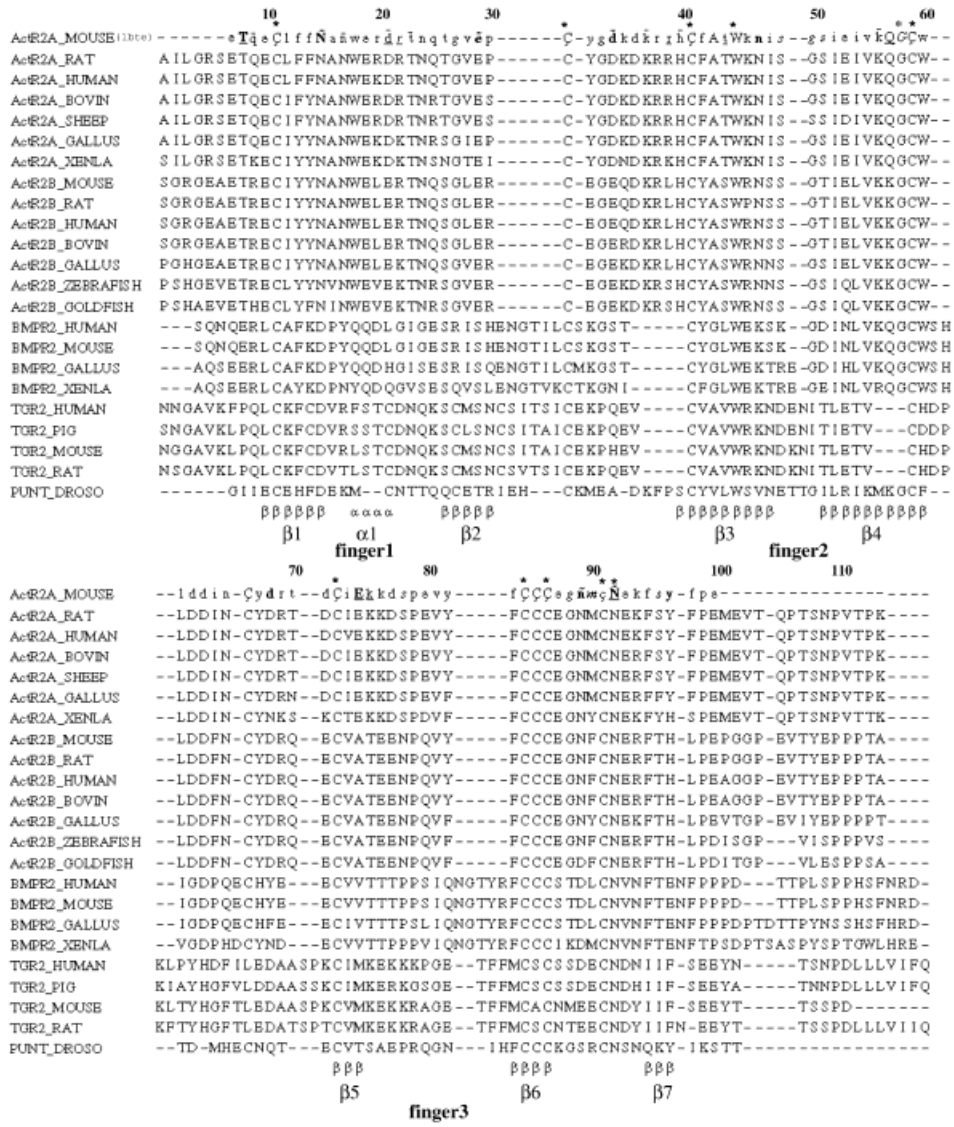

Fig. 2. Multiple alignment of 23 sequences of various type II receptor ectodomains with secondary structures and fingers marked. ActR2A_MOUSE,* ActR2A_RAT, ActR2A_HUMAN, ActR2A_BOVIN, ActR2A_SHEEP, ActR2A_GALLUS, and ActR2A_XENLA are activin type IIA receptors from mouse, rat, human, bovine, sheep, chicken, and *Xenopus*. ActR2B_MOUSE, ActR2B_RAT, ActR2B_HUMAN, ActR2B_BOVIN, ActR2B_GALLUS, ActR2B_ZEBRAFISH, and ActR2B_GOLDFISH are activin type IIB receptors from mouse, rat, human, bovine, chicken, zebrafish, and goldfish. BMPR2_HUMAN, BMPR2_MOUSE, BMPR2_GALLUS, and BMPR2_XENLA are bone morphogenic protein type II receptors from human, mouse, chicken, and *Xenopus*. TGR2_HUMAN, TGR2_PIG, TGR2_MOUSE, and TGR2_RAT are transforming growth factor type II receptors from human, pig, mouse, rat, and fruit fly. PUNT_DROSO is a homologue of activin type II receptor in the fruit fly. ActR2A_MOUSE (PDB code: 1bte) is an activin type II receptor ectodomain sequence from mouse with a known crystal structure.[32] The sequence is shown in structure-based annotation using JOY[49,50]. Please refer to the legend of Figure 6 for the JOY key.

vation among themselves, but across the subfamily there is hardly any conservation apart from the cysteines. Phe42, Trp45, Gly58, and Asn92 are characteristic of a three-finger toxin fold and are largely conserved. However, Phe42 is substituted by Tyr in ActR-IIB, BR2, and punt but replaced by Val in TβR-II; Gly58, which is conserved in ActR-IIB, BR2, and punt, is absent in TβR-II. In general, the average sequence identity is around 25%. The punt receptor shares 28% to 30% identity with BR2 and ActR-IIB, 22% to 23% identity with ActR-IIA, and about 15% identity with TβR-II. Trp45 and Asn92 are absolutely conserved among all the type II receptor subtypes considered. An evolutionary tree was generated using the PHYLIP package[42] (Fig. 3). As expected, AtR and BR sequences were found to be more similar and TβR sequence to stand on its own as a separate cluster.

Analysis of Residues in Fingers

Finger 1 contains loops of similar length that may be important for specificity in binding to the ligand because

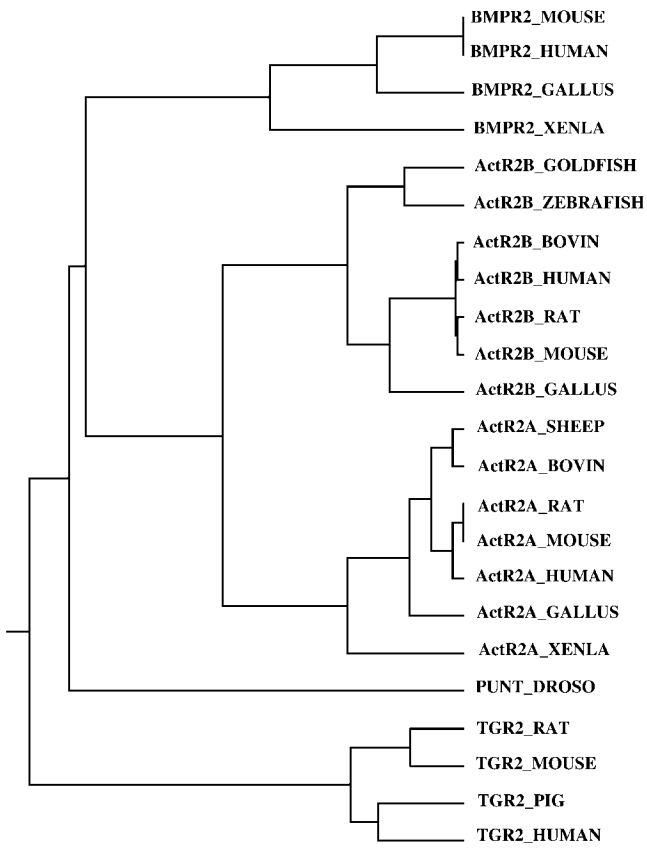

Fig. 3. Dendrogram containing 23 TGF-β families of type II receptor ectodomains, selected, using PHYLIP3.5,[42] on the basis of their sequence dissimilarity. The sequences are as described in the legend of Figure 2.

these loop regions display maximal sequence variation, which was also confirmed by evolutionary trace method (discussed later). Two negatively charged residues at the tip of finger 1, Glu19 and Asp21 (ActR-IIA numbering) are replaced by Asn and Leu in BR2 and by Ser and Cys in TβR-II. Finger 1 of punt contains an extra disulfide bridge, whereas that of TβR-II contains two extra disulfide bridges (Fig. 1). This confirms what was found in previous modeling and scanning-deletion mutagenesis studies,[54] that residues in finger 1 (residues 58–60 and 63–65 of TβR-II), facing the concave surface, are important for binding TGF. Finger 2 contains very few residues in each receptor sequence. However, punt, TβR-II, and BR2 receptors have relatively longer finger2 region: two-residue insertion in the case of TβR-II and punt and a one-residue insertion in BR2. Residues 74–79 of finger 3 are exposed on the concave surface; ActR-IIA has two positive and two negative charges in this loop, while ActR-IIB is predominately negative. BR2 is polar and TβR-II is predominantly positive in this region, whereas punt contains one positive and one negatively charged residue.

It has been reported that mutant receptors containing deletions corresponding to loop regions of finger 1, the β2–β3 loop, and finger 2 do not bind the ligand.[54] However, mutant receptors containing a deletion at finger 3, the loop region before β1, the β4–β5 loop, and after β7 do bind the ligand with affinities similar to those for wild-type receptors.[54] Because finger 2 is short, deletion of the loop region corresponding to finger 2 might cause structural changes to the receptor, rendering it unable to bind the ligand. Thus, finger 2 may or may not be important for binding. The highly variable finger 1 is not only a potential binding interface but is also the second most exposed conserved hydrophobic surface (as observed in the crystal structure of ActR-II), which is present at the convex side of the molecule. Finger 1 is a good candidate both to provide a hydrophobic docking surface and to act as a primary determinant of interaction and binding specificity.[32]

Evolutionary Trace of Receptor II Sequences

The output from the TraceSuite program (which uses the ET method[37] and is by Innis et al.[38]) on the extracellular domain of TGF type II receptors is shown in Figure 4. Analysis of the mapped traces for partitions P01 to P05 revealed clusters of potentially important residues on both the concave and convex surfaces of the receptor structures. The residues defined by the hydrophobic triad are at the concave surface.[36] In partition P01, apart from the structurally invariant cysteines, Trp45, Val55, and Asn92 are absolutely conserved among all receptor types considered in the ET analysis. The conserved Val55 lies on β4 and is in the vicinity of finger 2. Lys replaces Val55 in the punt sequence. Other residues identified in partition P01 are Thr8, Glu10, Asn15, Glu19, Glu29, Gly33, Ala43, Asn47, Asp62, Asp63, Val81, Glu93, and Phe95. Gly33 and Ala43 are buried in the core and may have a structural role, and Glu19 is on the β1–β2 loop (finger 1). Thr8, Glu10, Asn15, Asn47, Glu93, and Phe95 do not face the concave surface but are solvent accessible with no identified function. The trace residues facing the concave surface are Glu29 (on β2), Asp62 and Asp63 (on the β4–β5 loop), Val81 (on the β–β6 loop; finger 3), and Phe83 (on β6; finger 3). No class-specific residues were identified at P02.

Phe83 is in the hydrophobic triad, identified by alanine scanning mutagenesis to be important for ligand binding.[36] However, single mutations of Phe13, Phe14, Glu29, and Asp62 do not alter binding specificity for activins and inhibins.[36] The ET method does not identify Phe13 (exposed on the concave side) and Phe14 (exposed on the convex side) as trace residues, for which the implication is that these residues are probably involved in nonspecific binding. The method, however, identifies Glu29 (at the end of β1) and Asp62 (β4–β5 loop), which face away from the three fingers [Fig. 1(b)]. Glu29 is replaced by Ser in both TβR-II and BR2 and by Thr in punt; Asp62 is replaced by Gly in BR2 and by Tyr in TβR-II, whereas the corresponding residue in punt is deleted. This suggests that Glu29 and Asp62 might be playing a functional role in other subfamilies not tested so far by mutagenesis experiments.

In the crystal structure of ActR-IIA,[32] Thr44 (ActR-IIA numbering), identified as a conserved residue at partition P03 by ET analysis, is in the middle of a solvent-exposed hydrophobic surface (Table II), created by Ala16, Phe42,

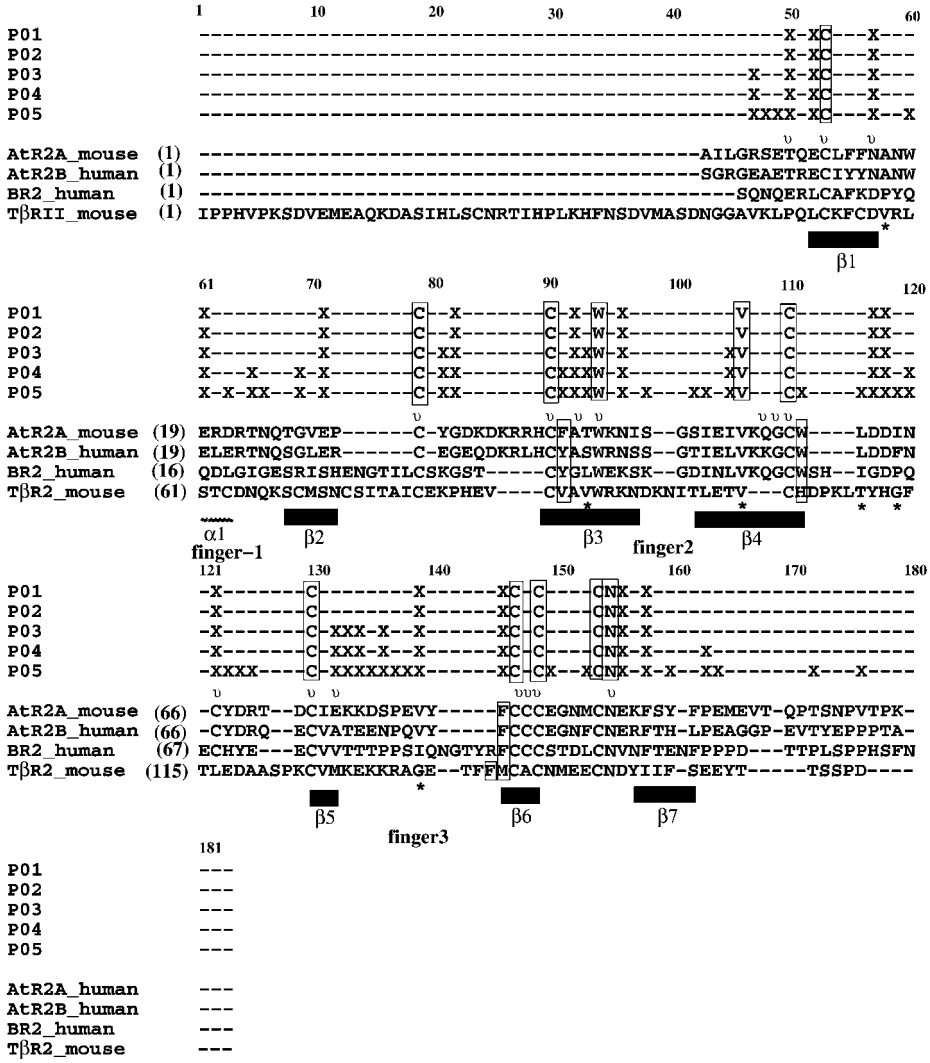

Fig. 4. Evolutionary Trace of type II receptor sequences (excluding punt) for partitions P01 to P05, aligned with the amino acid sequences of AtR2A_mouse (activin receptor IIA from mouse), AtR2B_human (activin receptor IIB from human), BR2_human (BMP receptor 2 from human), and TβR-II (TGFβ type II receptor from mouse). * indicates the residues are important for mutagenesis; n indicates solvent buried residues are as shown in the crystal structure of AtR-II–ECD.[32]

Val55, Trp60, Ile64, Val81, and Phe83 (recall that three of these define the hydrophobic triad, important in ligand binding[36]). Except for Ala16, all of these are trace residues. Ala16 is considered in the analysis as it is a solvent-exposed hydrophobic residue and lies in a loop region of finger 1. We refer to the hydrophobic triad as the residues defining the principal hydrophobic patch, which can be extended farther to include Ala16, Thr44, Val55, Leu61, Ile64, and Val81, termed the "surrounding" hydrophobic patch. Thr44 is replaced by Leu in BR2 and by a Val in TβR-II. Note that when there is a mutation of position Lys56—which is spatially proximate to this extended hydrophobic patch and is conserved in AtR, BR, and punt—Ala does not display a drastic change in binding.[36] Lys56 was not identified as a trace residue by our ET analysis.

Structure-Based Analysis of TGF-β Ligands and Identification of Determinants of Binding and Specificity

Large exposed hydrophobic patches on a protein surface often form part of the binding surface.[55] In the human growth hormone–receptor complex, a few hydrophobic residues at the interface contribute the most to the free energy of the interaction.[56] The recently solved crystal structure of the complex of the BMP-2–BRIA ectodomain,[33] exemplifying TGF–TGF type I receptor interactions, also demonstrates the same theme. Phe85 of $BRIA_{ec}$ helix α1 fits into a hydrophobic pocket of the ligand, where it interacts with BMP-2's Trp28 and Trp31, among other residues. In the crystal structure of free BMP-2, this pocket accommodates a 2-methylpentane-2,4-diol molecule from the buffer solution and, in the case of TGF-β3, a

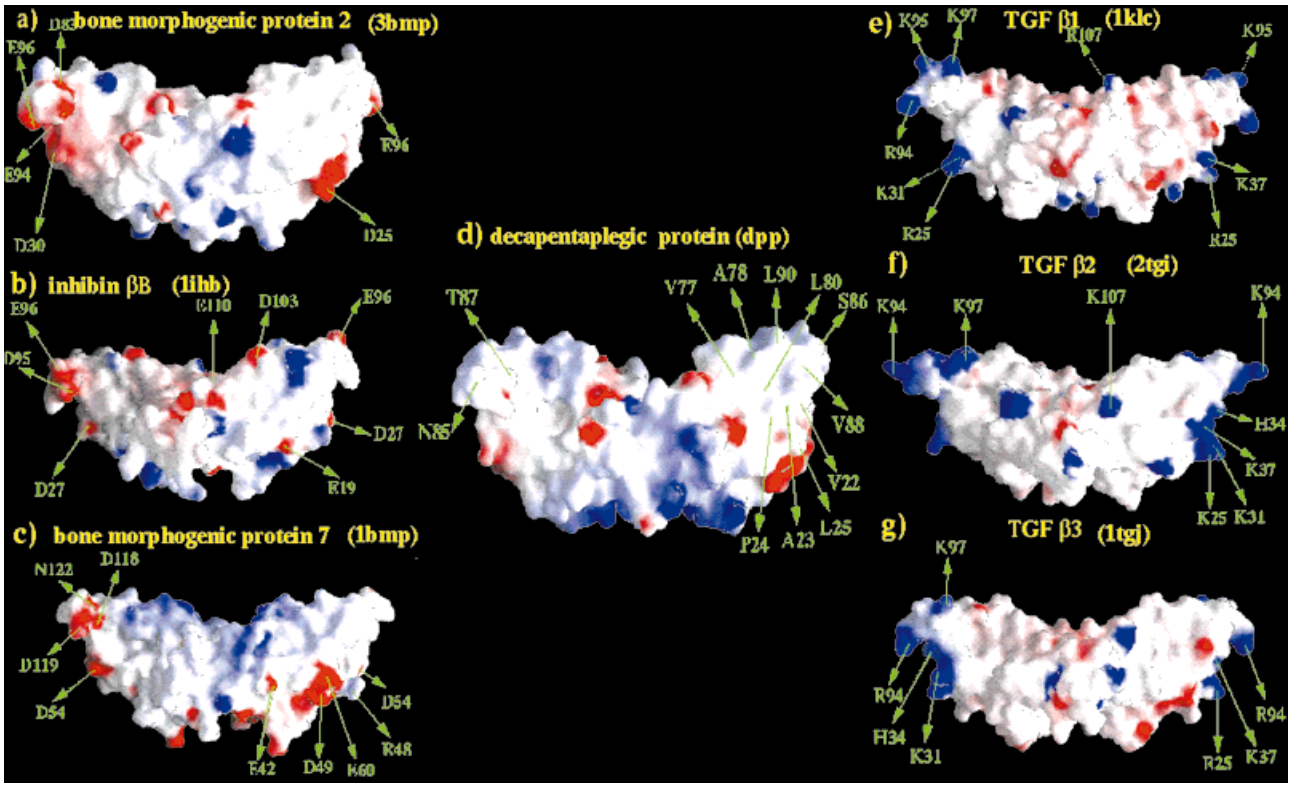

Fig. 5. Electrostatic potential representation of the known and modeled structures of the TGF-β family of ligands using GRASP.[47] Red surface patches indicate acidic residues, and blue patches indicate basic residues. The structures are **(a)** bone morphogenic protein 2, **(b)** inhibin-βB, **(c)** bone morphogenic protein 7, **(d)** decapentaplegic protein **(e)** transforming growth factor–β1, **(f)** transforming growth factor–β2, and **(g)** transforming growth factor–β3. The charged residues are marked, and the numbering is according to the structural positions given in PDB files. The hydrophobic residues are marked for *dpp*.

dioxane.[27,29,33] The Ile62, Val63, and Leu66 of BMP-2 provide an almost exclusively hydrophobic surface, which, together with Asn59, form the site of interaction with Phe85 of the receptor molecule.[30] In addition, Phe60, Met78, and Ile99 of BRIA are central to the ligand-binding interface.[33] The residues correspond to Asn59, Ile62, Val63, and Leu66 (BMP-2) in TGF-β ligands[38] and to Phe85, Phe60, Met78, and Ile99 (BRIA) in receptor 1 sequences, which were identified as trace residues (unpublished results of authors). To identify the determinants of binding and specificity for TGF–TGF type II receptors, the following approaches were taken.

Structure of TGF Growth Factors and Analysis of TGF-Like Sequences

The structures for TGF-β1–β3,[25–27,31] BMP-7,[28] and BMP-2[29] when superposed in the best fit display an overall root-mean-square deviation of less than 1.1 Å. However, there are clear differences in some structural elements between TGF-βs and BMPs; the N-terminus is not visible in the crystal structure of BMP-2[29] and BMP-7.[28] In contrast, TGF-β1–3 exhibits a short N-terminal α-helix (α1) that is anchored to the protein core by an additional disulfide bridge.[25–27,31] Moreover, BMP-2 and BMP-7 do not contain the short helix α2 observed after the second β-strand in TGF-βs and is replaced by a tighter nonhelical turn. This feature is conserved among known BMPs, GDFs, activins, and other subfamilies. However, the structures of BMP-2 and BMP-7 show a unique conformation at the loop preceding α3: a longer loop with a three-residue insertion (a short β-strand in BMP-2).

Analysis of surface residues of ligand molecules for difference in charge distribution

Figure 5 shows the GRASP surface representation[47] of structures of TGF-β1–3, BMP-2, and BMP-7 and models of inhibin-βB and *dpp*. Large hydrophobic areas are concentrated, especially on the wrist and knuckle epitope regions of the ligand dimers. It is clear from the figure that the charge distribution is different between TGF-β isoforms and the activin subfamily of proteins, especially in the knuckle epitope and in the loops of the β2–β3 and β7–β8 strands. These regions have a high negative charge in BMPs, inhibins, and *dpp*, whereas they are positively charged in TGF-βs (the conservation is confirmed using multiple sequence alignment of ligands). Unlike BMPs, *dpp* is in general polar at the β7–β8 loop, at the knuckle epitope, where Asp93, Glu95, and Lys96 of BMP-2 are replaced in *dpp* by Asn, Gln, and Thr, respectively. This difference in charge distribution together with the structural differences discussed before can be instrumental in giving rise to specificity while binding to receptors. In

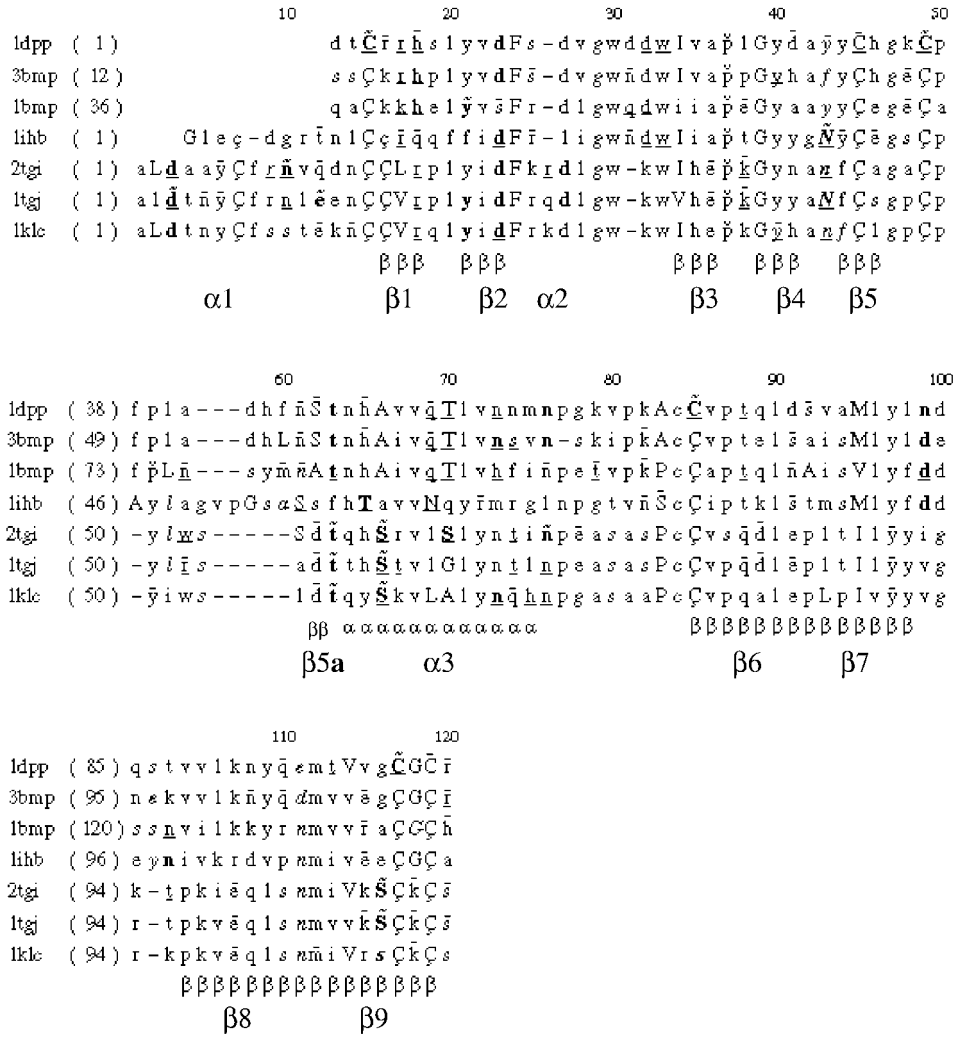

Fig. 6. Structure-based sequence alignment of the TGF-β family using COMPARER[48]; it was compiled using JOY.[49,50] Solvent-accessible and solvent-inaccessible residues are shown in upper case letters and lower case letters, respectively. Residues in positive phi are indicated in italics; residues with *cis* peptide in the backbone or that have disulfide bonds are indicated by the presence of a breve (š) or a cedilla (ç), respectively. Hydrogen bonds formed to the side chains, main chain amides, and main chain carbonyls of the other residues are indicated by the presence of a tilde (˜) on top, by boldface, and by underlining, respectively. The secondary structures are marked and numbered.

addition, all the structures have a positive charge at the N-terminus (conserved positively charged residue, two residues after C2), which accounts for their heparin binding.[57] However, in BMPs the N-terminus might fold back to shield this charge (as observed in the case of the inhibin-βB model), and TGF-βs are less positive than BMPs in this region.

Evolutionary Trace of Ligands and Identification of Residues Implicated in Binding and Specificity

Through applying the ET method to multiply aligned sequences of the TGF-β superfamily of ligands, Innis et al.[38] were able to identify trace residues. Trp28 and Trp31 of BMP-2, which have primary interactions with Phe85 of BRIAec (see above), were found to be absolutely conserved in the TGF-β family alignment and were identified as trace residues.[38] The mutation of Trp31 to alanine significantly decreases the stability of the BMP-2–$BRIA_{ec}$ complex.[33] Interestingly, neither Trp28 nor Trp31 is conserved in distant relatives like GDNF.

Here we will discuss those trace residues that occur on the knuckle epitope (important for receptor binding), are topologically equivalent (using COMPARER[48]), and display similar characteristics (identified by JOY[49,50]; Fig. 6). Two interesting clusters of residues were identified: first, at alignment positions 35, 36, 37, 92, 93, 94, 96, 98, 104, 105, and 106 (Fig. 6; residues forming the knuckle epitope). For BMP-2 (PDB code: 3bmp), these residues are Val33, Ala34, Pro35, Ala86, Ile87, Ser88, Leu90, Leu92, Val98, Val99, and Leu100. A second small cluster of residues, at alignment positions 17, 18, 43, and 115 (Arg16, His17, Phe41, and Glu109, according to BMP-2 numbering), is

TABLE II. Residues of Receptor Type II Important for Interaction with Ligands That Were Identified Using ET Method

	Principal patch			Surrounding patch					
Position	91	111	146	58	93	106	116	119	139
AtR-IIA	F42	W60	F83	A16	T44	V55	L61	I64	V81
AtR-IIB	Y42	W60	F84	A16	S44	V55	L61	F64	V81
BR-2	Y41	W59	F89	P13	L43	V54	I62	P65	I82
TβR-II	V85	H102	M135	V56	V87	V100	T108	G110	G130
Punt	Y37	F57	F81	E10	L39	K52	T58	M60	G77

TABLE III(A). Specific Residues Implicated for High-Affinity Binding

Position	21	23	25	37	43	94	96	98
Tgfβ-III	Y21	D23	R25	P36	N42	T87	L89	Y91
Tgfβ-II	Y21	D23	K25	P36	N42	T87	L89	Y91
Tgfβ-I	Y21	D23	R25	P36	N37	P87	V89	Y91
BMP-2	Y20	D22	S24	P35	F41	S88	L90	L92
BMP-7	Y44	S46	R48	P59	Y65	S113	L115	F117
dpp	Y9	D11	S13	P24	Y30	A78	L80	L82
IHB B	F17	D19	R21	P32	N39	S89	L91	F93

TABLE III(B). Subfamily-Specific Residues Implicated for Receptor Specificity and Binding

Position	17	18	32	35	36	92	93	104
Tgfβ-III	V17	R18	K31	H34	E35	P85	L86	P96
Tgfβ-II	L17	R18	K31	H34	E35	P85	L86	P96
Tgfβ-I	V17	R18	K31	H34	E35	P85	L86	P96
BMP-2	R16	H17	D30	V33	A34	A86	I87	V98
BMP-7	K40	H41	D54	I57	A58	A111	I112	V123
dpp	R5	H6	D19	V22	A23	S76	V77	V88
IHB B	R13	Q14	D27	I30	A31	T87	M88	I99

Position	105	106	107	108	115
TGF-β3	K97	V98	E99	Q100	K107
TGF-β2	K97	I98	E99	Q100	K107
TGF-β1	K97	V98	E99	Q100	R107
BMP-2	V99	L100	K101	N102	E109
BMP-7	I124	L125	K126	K127	R134
dpp	V89	L90	K91	N92	V99
IHB B	V100	K101	R102	D103	E110

rather surprising. However, note that the N-terminus of the BMP family of ligands, not seen in the crystal structure, could fold back in this region, attributing a structural rather than a functional role to these residues. The trace residues at the knuckle epitope are divided into two classes [Table III(a,b)]: (1) the residues implicated for ligand binding and (2) the residues implicated for binding as well as specificity, most of which are subfamily-specific and class-specific residues. The first cluster could be the preferred site of interaction with hydrophobic clusters on a type II receptor, as identified by mutagenesis studies and in this study. Residue Pro35 is absolutely conserved in all the ligand sequences considered for our analysis but is absent in GDNF. Val33 and Ala34 are replaced by charged residues in TGF-β isoforms, whereas Leu100 is replaced by a charged residue in inhibins; other residues in cluster 1 are conserved substitutions. In cluster 2 in TGF-βs Arg16 is replaced by a hydrophobic residue. Alignment position 18 (Fig. 6; His17 of BMP-2) is occupied by a positively charged residue in all the known sequences. Phe41, adopting unusual ϕ/ψ angles (67°, 178°) in the Ramachandran plot[58–59] although not in the dimer interface, forms interchain contact at the backbone carbonyl with the neighboring subunit in BMP-2, and its side chain is solvent-exposed[29] on the knuckle epitope. This residue is present where the β-strands β2, β5, β 6, and β9 are arranged close enough to form a short segment of the four-stranded antiparallel β-sheet, as evident in the crystal structure.[29] This arrangement is also observed in all known proteins in the TGF-β superfamily. As shown in Table III, Glu109 (BMP-2 numbering) is specific for BMP-2 and inhibin subfamilies, which is a positively charged residue (Arg/Lys) in TGF-βs and BMP-7 and a valine in *dpp*. Such differences of trace residues point to class-specific electrostatic distribution and receptor specificity.

Given the above results and the crystal structure of BMP-2 with its type I receptor, it is plausible that finger 1 (with loop region of β2–β3) of the type II receptor interacts

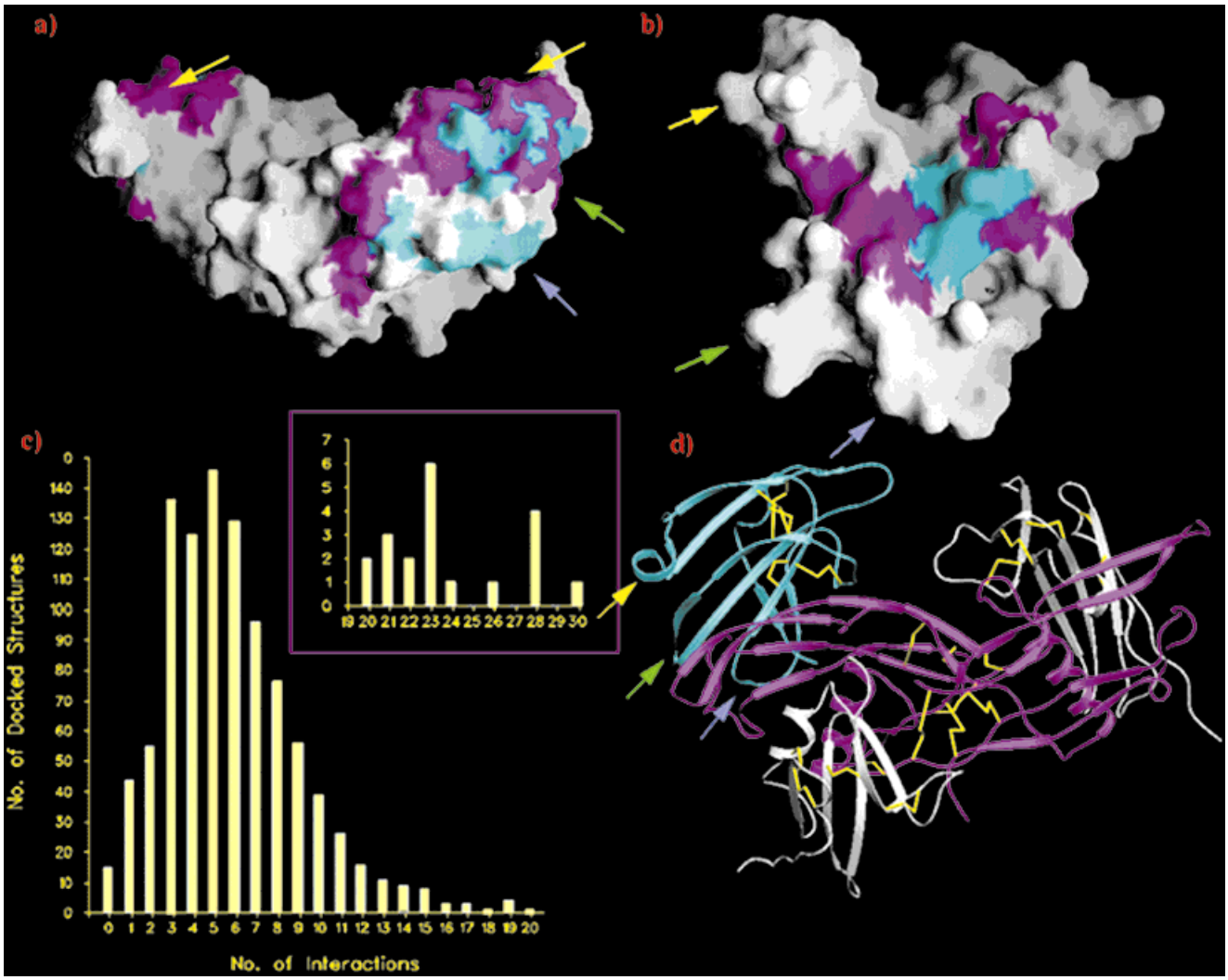

Fig. 7. Predicted mode of interaction between transforming growth factor and type II receptor. **(a)** Structure of BMP dimer in GRASP surface representation.[47] Key residues were identified by the evolutionary trace (ET) method[37]; those that are probably important for binding (principal patch) are denoted in cyan. Additional residues (surrounding patch), also identified by the ET method, are shown in magenta. **(b)** Same as (a) except for the extracellular domain of the activin type II receptor. Regions corresponding to finger 1, finger 2, and finger 3 (also see Fig. 1) are denoted with yellow, green, and violet arrows. The proposed complimentary areas of interaction in the ligand dimer are marked in similar colors in (a). **(c)** Distribution of the total number of hydrophobic contacts for 1000 models of the interaction between the type II receptor (PDB code: 1bte) and ligand dimer complexed with two type I receptor molecules (PDB code: 1es7). Hydrophobic contacts on the type II receptor were measured between the two molecules as the number of C^{α}—C^{α} distances of hydrophobic residues of 1es7 within 12 Å. Inset to Figure 7(c) models with high hydrophobic contacts (seven or more) at the principal patch of the type II receptor were examined for additional hydrophobic interactions, including the surrounding hydrophobic patch. (d) Ribbon representation of one of the models of the interaction between a type II receptor and a type I receptor-bound ligand dimer. This model, suggested by GRAMM, has a high number of hydrophobic residues at the predicted binding site [Fig. 7(c)]. Activin type II receptors are shown in cyan, BMP dimer in magenta, and the two type I receptors in gray. The knuckle epitope of the ligand dimer and finger 1 and finger 3 of the type II receptor (denoted by yellow and violet arrows) were predicted to be the key regions for forming the binding interface. This picture was prepared using SETOR.[61]

with the knuckle epitope of the ligand if the C-termini of both type I and type II receptors need to point roughly in the same direction and the type II receptor interacts with the ligand at its concave surface[32,33,54] with the hydrophobic triad residues. The finger 3 loop region of the type II receptor does not play an important role in binding. Some residues identified by the ET method in the surrounding patch of receptor type II are also reported in the deletion studies of the core region (53–55, 83–85, 98–100, and 143–145, TβR-II numbering; see Table II) of TβR-II.[54] Thus, the ET method can be used both to rationalize the result of the mutagenesis studies and to predict the targets for the mutagenesis.

Docking Studies of the Type II Receptor to the Ligand–Dimer Type I Complex

The nature of interactions at the ligand type I receptor binding site was primarily hydrophobic.[33] The above analyses of the trace residues of solvent-exposed hydrophobics and the overall similarities between type I and type II receptors suggest similar hydrophobic interactions in TGF ligand type II receptor binding [Fig. 7(a,b)]. One thousand

GRAMM[51–53] models of the type II receptor interacting with the ligand dimer complexed with two type I receptors were generated. The models were examined for maximal hydrophobic interactions (defined as interaction between the hydrophobic residues of both structures) and total interactions (defined as interaction between all the residues of both structures) using the C^{α} distance cutoff of 12 Å to define interacting pairs. Figure 7(c) shows the distribution of the number of models with the number of hydrophobic interactions between the ligand type I receptor complex and receptor type II. Models with an appreciable number of hydrophobic interactions at the principal patch (data not shown) were specifically examined after including the surrounding hydrophobic patch residues [Fig. 7(c), inset]. Interestingly, models with the highest number of hydrophobic interactions [Fig. 7(c)] and of total interactions [as shown in Fig. 7(d)] with key residues of the type II receptor closely correspond to the ET results, suggesting a theme involving the knuckle epitope at the ligand as the receptor-binding site.

CONCLUSIONS

We have identified the clusters of residues that lie on the knuckle epitope of ligand molecules and the concave surface of type II receptor molecules, which may play an important role in complex formation. These clusters are hydrophobic patches surrounded by charged residues on the surface of molecules. We propose that finger 1 and a part of finger 2 of the type II receptor, with the central hydrophobic patch, interact with the knuckle epitope of the ligand (mainly the convex side on β2–β 3, β7–β8, and the loop regions joining them) as they provide the large conserved hydrophobic surface for docking. The β2–β3 loop region may be interacting with the smaller cluster identified using the ET method, which shows good agreement with GRAMM docking studies. Although each type I receptor interacts simultaneously with both ligand protomers at the wrist epitope,[33] we predict that the type II receptor interactions are with one protomer each at the knuckle epitope. These predictions are supported by deletion studies on ligands,[31,34,35] deletion and mutagenesis studies on receptor type II sequences,[36,54] and the orientation of receptor type I in the crystal structure in complex with BMP-2.[33] We have discussed the important amino acids that can be targets for future mutagenesis studies. It will also be interesting to prepare TGF chimeras of the loop region between the β2–β3 loop and the β7–β8 loop because the difference in charge distribution in this region may contribute to specificity in the identification of receptors. Various tools, such as the study of evolutionary trees, conserved residues of the aligned sequences, spatial positions of interesting residues, charge distribution on their three-dimensional fold, and docking studies, have been used to provide structural explanations for ligand–receptor specificity, which have general value for analyzing protein–protein interactions.

ACKNOWLEDGEMENTS

Author C.M.B was a visiting scientist at the National Centre for Biological Sciences (NCBS), and her stay was supported by NCBS. We thank Professor K. VijayRaghavan for initiating the idea for this article and for useful discussions. We thank Professor Tom Blundell, Jiye Shi, and Axel Innis for providing the program TraceSuite for our use and for useful discussions.

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