Structural and Biophysical Characterization of the EphB4-EphrinB2 Protein-Protein Interaction and Receptor Specificity*

Jill E. Chrencik †, Alexei Brooun †, Michelle L. Kraus †, Michael I. Recht §, Anand R. Kolatkar †, Gye Won Han §, Jan Marcus Seifert †, Hans Widmer †, Manfred Auer †, and Peter Kuhn †

From the †Department of Cellular Biology, The Scripps Research Institute, La Jolla, California 92037, ‡Scripps-PARC Institute for Advanced Biomedical Sciences, Palo Alto Research Center, Palo Alto, California 94304, §Discovery Technologies-Innovative Screening Technologies, Novartis Institutes for BioMedical Research GmbH & Co. KG, A-1235 Vienna, Austria, ¶Novartis Institutes for BioMedical Research, CH-4002 Basel, Switzerland

Increasing evidence implicates the interaction of the EphB4 receptor with its preferred ligand, ephrinB2, in pathological forms of angiogenesis and in tumorigenesis. To identify the molecular determinants of the unique specificity of EphB4 for ephrinB2, we determined the crystal structure of the ligand binding domain of EphB4 in complex with the extracellular domain of ephrinB2. This structural analysis suggested that one amino acid, Leu-95, plays a particularly important role in defining the structural features that confer the ligand selectivity of EphB4. Indeed, all other Eph receptors, which promiscuously bind many ephrins, have a conserved arginine at the position corresponding to Leu-95 of EphB4. We have also found that amino acid changes in the EphB4 ligand binding cavity, designed based on comparison with the crystal structure of the more promiscuous EphB2 receptor, yield EphB4 variants with altered binding affinity for ephrinB2 and an antagonistic peptide. Isothermal titration calorimetry experiments with an EphB4 Leu-95 to arginine mutant confirmed the importance of this amino acid in conferring high affinity binding to both ephrinB2 and the antagonistic peptide ligand. Isothermal titration calorimetry measurements also revealed an interesting thermodynamic discrepancy between ephrinB2 binding, which is an entropically driven process, and peptide binding, which is an enthalpically driven process. These results provide critical information on the EphB4-ephrinB2 protein interfaces and their mode of interaction, which will facilitate development of small molecule compounds inhibiting the EphB4-ephrinB2 interaction as novel cancer therapeutics.

The protein-protein interaction between the membrane-bound Eph receptor tyrosine kinases with the membrane-bound ephrin ligands have now been reported in the overexpression/dysregulation in numerous tumor cell lines (1). First reported for their role in axonal guidance, this group of proteins now has defined roles in regulating several cellular processes including developmental patterning, cell attachment, and motility (2–4). The importance of these proteins in development is underscored by the fact that deletion of either the EphB4 receptor or the ephrinB2 ligand results in lethality by embryonic day 11 as a result of arrested angiogenesis but not vasculogenesis (5). Understanding the EphB4-ephrinB2 interaction and exploring the determinants for the unique specificity of this receptor-ligand complex is at the core of modulating this activity and will allow for a deeper understanding into the basic biology behind this interaction and for the development of novel anti-angiogenesis and anti-tumorigenesis therapeutic approaches.

The EphB4 receptor and the ephrinB2 ligand are capable of transducing a signal bi-directionally into either the EphB4-expressing cell (forward signaling) or the ephrinB2-expressing cell (reverse signaling) (12, 13). Therefore, a cellular response is conducted only upon cell-cell contact. The Eph receptors are divided into two subclasses, A and B, based on both sequence conservation and binding preferences with the ephrins (6). The receptors have a modular domain architecture, extracellularly characterized by an N-terminal ligand binding domain, a cysteine-rich domain, and two fibronectin type III-like repeats. Intracellularly, the receptors consist of a juxtamembrane domain, a conserved tyrosine kinase domain, a C-terminal sterile α-domain, and a PDZ binding motif. The ephrin ligands are also divided into two subclasses, A and B; ephrinA ligands are anchored to the membrane through a glycosylphosphatidylinositol linker, whereas members of the B-subclass are tethered to the membrane by a transmembrane region and contain a cytoplasmic PDZ domain binding motif. In general, EphA receptors (EphA1-EphA10) are promiscuously activated by ephrinA ligands (ephrinA1-ephrinA6), whereas EphB receptors (EphB1-EphB6) are promiscuously activated by ephrinB ligands (ephrinB1-ephrinB3) (7). Cross-subclass interactions have been identified specifically between EphB2 and ephrinA5 (8) and between EphA4 and ephrinB2/B3 (9); however, these interactions are rare and appear to be exceptions to the general rule. Unlike the other promiscuous receptors in this family, however, the EphB4 receptor has a distinctive specificity.
for a single ligand, ephrinB2 while binding only weakly to both ephrinB1 and ephrinB3. EphrinB2, on the other hand, binds to several receptors within the B-subclass (10). The ligand specificity of the EphB4 receptor is somewhat surprising, since the receptor has the characteristics of a promiscuous receptor, including a large and hydrophobic binding cavity and a flexible “lid” capable of accommodating a range of ligands (11). Here we provide a structural perspective describing the molecular determinants of EphB4 specificity.

Crystal structures have been previously determined for ephrin ligands (ephrinB2, ephrinB1) and Eph receptors (EphB2, EphB4) as well as receptor-ligand complexes (EphB2-ephrinB2, EphB2-ephrinA5, EphB4-ephrinB2) (8, 10, 19). The crystal structure of the homodimeric ephrinB2 ligand reveals a β-barrel structure arranged in a deviation of the common Greek Key topology (17). Comparison of the crystal structures of both apo and receptor-bound ephrinB2 revealed that the solvent-exposed and high affinity G-H loop is conserved in position between the two structures. This critical loop shares high sequence homology in both A and B subclasses, respectively, and is involved in receptor-ligand homodimerization as well as in ephrinB2 homodimerization (10, 17). In addition, the crystal structures of the ligand binding domains of EphB2 and EphB4, which share only 44% sequence identity, show a similar jellyroll folding topology, with 13 antiparallel β-sheets connected by loops of varying length (18, 19). The crystal structure of the EphB4 receptor in complex with the phage display derived TNYL-RAW peptide revealed that the peptide binds to the ephrin binding cavity of the receptor, effectively inhibiting interaction with the ligand (19). Furthermore, the structure of the EphB2-ephrinB2 complex showed that the ligand binding channel of the receptor is located at the upper convex surface of EphB2 and is formed by the flexible J-K, G-H, and D-E loops, which become ordered to accommodate the solvent-exposed ephrin G-H loop (10). A low affinity tetramerization interface was also identified at the concave surface of the receptor H-I loop, which interacts with the C-D loop of the ephrin.

Given that the EphB4-ephrinB2 interaction is important in angiogenesis and that EphB4 is overexpressed in several tumor types (1, 20–22), modulating this protein-protein interaction is a potential approach to slowing tumor angiogenesis and tumor growth. In mouse models of breast cancer, high EphB4 expression correlates with increased malignancy and tumor aggressiveness (23–25). EphB4 expression is also increased in human primary infiltrating ductal breast carcinoma and is correlated to increased malignancy (26). There is evidence that the EphB4 ectodomain stimulates endothelial cell migration and proliferation, suggesting that ephrinB2-expressing endothelial cells interact with the EphB4 ectodomain to promote angiogenesis and tumor progression. Furthermore, a kinase-deficient EphB4 mutant has been shown to increase breast cancer cell growth, indicating that downstream forward kinase signaling is not an absolute requirement for tumorigenesis, at least in breast cancer cells (27). Several groups have more recently demonstrated that the full extracellular domain of EphB4 is indeed a viable therapeutic target. First, the soluble extracellular domain of EphB4 was described to block both forward and reverse signaling, resulting in an inhibition of tumor growth in vivo (28, 29). Second, phage display studies have identified a peptide (TNYL-RAW) that antagonizes the EphB4-ephrinB2 interaction in the high nanomolar range (30). However, a more complete understanding of the biological role of EphB4-ephrinB2 signaling in tumorigenesis and in forms of pathological angiogenesis is now required.

To enable anti-EphB4-ephrinB2 therapeutic development and probe EphB4 specificity, we determined the three-dimensional crystal structure of the EphB4-ephrinB2 complex to high resolution. In addition, we conducted site-directed mutagenesis and biophysical analyses to investigate the role of several residues within the ligand binding cavity of EphB4 in contributing to the binding of both ephrinB2 and the antagonistic TNYL-RAW peptide. These results will enable the development of predictive models for structure-based drug design of small molecule compounds for use as therapeutics and to probe the biology of EphB4-ephrinB2 bi-directional signaling.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The human EphB4 receptor (17–196) was expressed and purified in insect cells as described elsewhere (19). The wild type EphB4 construct was used as a template for the generation of site-specific mutants. The human ephrinB2 (extracellular domain, residues 25–187) was designed based on the previously published EphB2-ephrinB2 structure and cloned into a modified pFastBac1 vector containing a GP67 leader peptide. Recombinant baculovirus was generated using the Bac-to-Bac system (Invitrogen). Briefly, large scale expression of ephrinB2 was carried out using Wave Bioreactors on a 5-liter scale at a multiplicity of infection of 5 for 48 h, resulting in ~10 mg of ephrinB2/liter of Hi-5 insect cells (Invitrogen). Media containing secreted ephrinB2 protein was concentrated and buffer was exchanged using a Hydrostart Crossflow Filter (Sartorius Edgewood, NY). The ligand was purified by immobilized metal affinity chromatography and cleaved overnight with tobacco etch virus protease. Material was further re-purified by immobilized metal affinity chromatography (S2000) to remove the protease and an N-terminal fragment containing the histidine tag. The EphB4-ephrinB2 complex was formed with a 1.5-fold molar excess of ephrinB2 overnight at 4 °C in buffer containing 50 mM Tris, pH 7.8, 100 mM NaCl, and 10 mM Imidazole. The complex was purified by immobilized metal affinity chromatography followed by size exclusion chromatography to remove trace aggregates (Phenomenex, Torrance, CA). The final purity of the complex was greater than 95%.

**Crystallization, Data Collection, and Structure Solution**—The EphB4-ephrinB2 complex was concentrated to 20 mg/ml in a buffer containing 25 mM Tris, pH 7.8, 150 mM NaCl, and 5 mM CaCl₂ and crystallized by sitting drop vapor diffusion at 20 °C against a precipitant of 2.2 M ammonium sulfate and 100 mM Tris, pH 7.8. EphB4-ephrinB2 crystals were cryoprotected in 25% glycerol and flash-cooled. Crystals formed in the P41 space group and contained one monomer of receptor and one monomer of ligand in the asymmetric unit. Data were collected at the Advance Photon Source (Argonne, IL) on beamline GM/CA-CAT. Images were processed and reduced using HKL2000 (31). The structure was solved by molecular replacement with MolRep (CCP4i), using the EphB2-ephrinB2 struc-
ture (Protein Data Bank code 1KGY) as a search model (10, 32). The structure was refined by a rigid body refinement followed by model building in O and iterative refinements with Refmac (32, 33). The structure exhibits good geometry with no Ramachandran outliers.

**Isothermal Titration Calorimetry**—All mutants and ligands were dialyzed into buffer containing 50 mM Tris-Cl, pH 7.8, 150 mM NaCl, and 1 mM CaCl$_2$ before use in isothermal titration calorimetry (ITC) experiments. All experiments were performed with a Microcal MCS ITC at 25 °C. Titrations were completed as described (19). EphB4 (wild type or mutant) was present in the sample cell at a concentration of 12–15 μM, and the injection syringe contained either 127 μM ephrinB2 or 200 μM TNYL-RAW. Titrations of TNYL-RAW with the L95R mutant of EphB4 were performed with 2 mM TNYL-RAW in the injection syringe and 15 μM EphB4 (L95R) in the sample cell. Data for these titrations were fit assuming a stoichiometry of 1, and at least 60% saturation at the final peptide concentration as described (19, 34).

**Fluorescent Polarization Assay**—Alexa-532 labeled TNYL-RAW peptide was synthesized by Biopetide (San Diego, CA). A serial dilution of EphB4 was prepared in assay buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1 mM CaCl$_2$, 0.1% Pluronic 124). TNYL-RAW-Alexa-532 labeled peptide was prepared as a 100 μM stock solution in the assay buffer, and a 300 nM working solution was made fresh before the measurements by dilution in the assay buffer. 5 μl of serially diluted EphB4 (9–2362 μM concentration range) was combined with 5 μl of labeled peptide (final concentration 75 nM) in the final volume of 20 μl in the absence and presence of 200 μM TNYL-RAW as a control for nonspecific binding. The mixture was allowed to equilibrate for 30 min at room temperature, and measurements were performed with a Tecan Genios Pro (Tecan Instruments) using 535-nm excitation and 580-nm emission wavelength. All experimental data were analyzed using Prism 4.0 software (GraphPad Software Inc., San Diego, CA), and $K_d$ values were generated by fitting the experimental data using a one-site binding hyperbola nonlinear regression model. The calculated Z-factor for 108 samples, each at 2 different protein concentrations representing upper and lower plateaus of the dose response curve, is 0.715.

**RESULTS**

**Overall Structure**—EphB4 and ephrinB2 were co-concentrated to 20 mg/ml and crystallized by sitting drop vapor diffusion against a precipitant of 2.2 M ammonium sulfate and 100 mM Tris, pH 7.8, at 20 °C. The co-crystal structure was refined to 2.0-Å resolution with an $R$-factor of 23% and a free $R$ factor of 29% (Table 1; Fig. 1). Unlike crystals of the of the EphB2-ephrinB2 complex, which consisted of a heterotetramer, crystals of the EphB4-ephrinB2 complex consist of a heterodimer. Previously, formation of EphB2-ephrinB2 heterotetramers was observed for a concentration range around 1 mM using size exclusion chromatography analysis, whereas analytical ultracentrifugation demonstrated that the EphB2-ephrinB2 complex was a heterodimer at concentrations in the low micro-

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**TABLE 1**

| Crystallographic statistics for the EphB4-ephrinB2 complex |  |
|---|---|
| Resolution (Å) | 20-2.0 (2.1-2.0)* |
| Wavelength (Å) | 0.98 |
| Space group | P$_4_1_2_1_2_1$ |
| Unit cell dimensions (Å) | $a = b = 81.09$, $c = 50.95$ |
| Completeness (%) | 99.6 (99.9) |
| $R_{	ext{sym}}$ (%) | 3.9 (20.8) |
| $I/σ$ | 4.8 |
| Mean redundancy | 4.7 |
| No. of reflections | 19,785 |
| $R_{\text{cryst}}$ (%) | 22.6 (26.5) |
| $R_{\text{free}}$ (%) | 29.5 (30.0) |
| Root mean square deviations |  |
| Bond length (Å) | 0.02 |
| Bond angle (°) | 1.7 |
| Average B factor (Å$^2$) | 56.6 |
| Number of atoms |  |
| Protein | 4,992 |
| Solvent | 78 |

*The number in parentheses is for the highest shell.

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FIGURE 1. The ephrin binding domain of the EphB4 receptor in complex with the ephrinB2 extracellular domain. The EphB4 receptor (red) consists of a jellyroll folding topology with 13 anti-parallel B-sheets connected by loops of varying lengths, whereas the ephrin ligand (blue) is similar to the Greek key folding topology. The interface is formed by insertion of the ligand G-H loop into the hydrophobic binding cleft of EphB4.

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2 The abbreviations used are: ITC, isothermal titration calorimetry; L, ligand; R, receptor.

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between previously described apo and receptor-bound structures, shifting only 0.91 and 0.90 Å, respectively (10, 17).

**EphB4-EphrinB2 Interface**—The high affinity EphB4-ephrinB2 heterodimer is formed by insertion of the solvent-exposed ligand G-H loop into the upper convex and hydrophobic surface of the EphB4 receptor, positioned above receptor strands E and M. Hydrophobic contacts drive receptor-ligand binding in this region. Ligand (L) residues Phe-120L, Pro-122L, Trp-125L, and Leu-127L participate in van der Waals interactions with receptor residues lining the receptor binding cavity in the D-E, G-H, and J-K loops (Fig. 2). Phe-120L forms hydrophobic interactions with Leu-95R (R, receptor; see below), Leu-
EphB4-EphrinB2 Complex Structure

FIGURE 3. Stereoview of αA weight F_{obs} - F_{calc} electron density at 2.0 Å resolution, contoured at 1σ for the EphB4-ephrinB2 interface. EphB4 is in green, and ephrinB2 is in yellow. Clear density of the interface shows Phe-120 in a novel position with respect to previously described structures in order to interact with Leu-95.

100R, and Pro-101R, whereas Leu-124L interacts with Thr-147R from the receptor J-K loop. Meanwhile, Trp-125L extends to the surface of the receptor in between the J-K and G-H loops, participating in hydrophobic interactions with residues Leu-48R, Glu-50R, Val-159R, Leu-188R, and Ala-186R. In addition, Pro-122L, similar to all previous crystal structures, maintains its position by participating in a direct interaction with the receptor Cys-61R-Cys-184R disulfide bridge. Few polar contacts are formed at the receptor-ligand dimer interface. Ser-121L forms a side chain-side chain hydrogen bond with Glu-59R as well as a main chain-side chain hydrogen bond with Lys-149R, whereas Asn-123L forms a hydrogen bond with the main chain oxygen of Leu-48R. Additionally, Lys-149R extends to the body of the ephrinB2 G-H loop, forming side chain-side chain hydrogen bonds with Glu-128L, and side chain-main chain hydrogen bonds with Ser-121L and Asn-123L, which are both part of the high affinity ligand FSPN sequence (Fig. 3). The introduction of this new interaction at the EphB4-ephrinB2 interface is certain to contribute to the high affinity of this receptor-ligand complex.

Similar to the EphB2-ephrinB2 structure, a second portion of the high affinity heterodimerization interface exists immediately adjacent to the ligand binding cavity, formed by ligand strands C, G, and F, and receptor strands B-C, E, and D. This region of the complex deviates minimally from the corresponding EphB2-ephrinB2 complex, with a maximum deviation of 2.1 Å from furthest atoms, and is predominantly characterized by backbone-backbone, backbone-side chain, and side chain-side chain hydrogen bonds. In particular, side chain-side chain interactions between Glu-59R (Glu-68 in EphB2)-Glu-118L and Ser-121L, Asp-29R (Glu-40 in EphB2)-Lys-112L, and Glu-43 (Glu-52, EphB2)-Lys-116L provide the binding potential characteristic of this low nanomolar interaction. Side chain-main chain interactions between Ser-55 and Lys-116L, and between Glu-44R and Lys-60R complete the binding network in this region.

Although the overall shape of the EphB4-ephrinB2 interaction interface is in good agreement with that previously described in the EphB2-ephrinB2 structure, marked differences exist within the receptor loops that frame the ligand binding channel. The EphB4 J-K loop assumes a distinct position compared with previously described crystal structures and is situated directly above the ligand G-H loop and 15 Å from the D-E loop (Fig. 4). The corresponding J-K loop from the EphB2-ephrinB2 structure, on the other hand, is positioned only 6.4 Å from the D-E loop and, therefore, maintains a more compact binding cavity. In fact, the J-K loops differ in position by up to 10 Å from furthest positions between the two ephrinB2-bound complex structures. Not surprisingly, the J-K loop shows remarkable flexibility in each structure described, also shifting in position by up to 20 Å from furthest positions between the EphB4-TNYL-RAW...
avoid steric interference with residues lining the ephrinB2 G-H loop. The smaller Leu-95R together with Phe-120L allows the J-K loop of EphB4 to adopt a novel position directly above the ligand G-H loop.

**Biophysical Characterization of EphB4 Specificity; Enthalpic Versus Entropic Contributions**—A series of site-specific mutations was generated by changing residues lining the EphB4 G-H and J-K loops to the corresponding residues found in EphB2 (Table 2). The EphB4 mutants were analyzed based on their binding to fluorescently labeled Alexa-532-TNYL-RAW peptide. Fluorescence polarization (FP) analysis corroborated the prediction that Leu-95 is a critical determinant for binding of the TNYL-RAW peptide because the L95R mutant did not exhibit significant binding of the peptide in our assay. EphB4 mutants T147F, A186S, and K149Q showed an ~4-5-fold reduction in binding affinity of the fluorescently labeled peptide.

FIGURE 4. Stereoview of the superposition of the Eph receptor ligand binding domains from the EphB4-ephrinB2 (red), EphB2-ephrinB2 (green), and EphB4-TNYL-RAW complex structures (blue). Clear deviation is seen in the J-K loop, whereas more minor changes are seen in the receptor D-E and G-H loops (Ref. 10; Protein Data Bank code 1KGY). The overall root mean square deviation between the EphB4-ephrinB2 and the EphB2-ephrinB2 and EphB4-TNYL-RAW structures is 5.0 and 2.5 Å, respectively.

FIGURE 5. EphrinB2 specificity region in the EphB2/EphB4-ephrinB2 complexes. Left, the region near the EphB4 Leu-95R of the EphB4-ephrinB2 complex structure is shown in schematic representation. The van der Waals interaction between the ephrinB2 Phe-120L and the EphB4 Leu-95R is depicted as a dotted line. Right, the region near the EphB2 Arg-103R of the EphB2-ephrinB2 complex structure is shown in the same orientation as that on the left. The EphB2 Arg-103R, Ser-156R, and Ser-107R side chains are shown as colored sticks. Hydrogen bonds between Arg-103R and the two serines are shown as dotted lines. The J-K loops of EphB2 and EphB4 are shown in yellow highlighting the change in loop position between the two complexes.

Based on the structural information and preliminary binding characterization by fluorescence polarization analysis, two EphB4 mutants, L95R and K149Q, were chosen for a more detailed thermodynamic analysis of their binding to both ephrinB2 and the TNYL-RAW peptide ligand using ITC. As reported previously, EphB4 binds to ephrinB2 with an affinity of 40 nm and a $\Delta H_{\text{obs}}$ of 3.3 kcal mol$^{-1}$ (19). Mutation of EphB4 Lys-149 to Gln has no effect on the binding affinity or enthalpy of ephrinB2 binding (Table 3). In contrast, mutation of EphB4 Leu-95 to Arg reduces the binding affinity of ephrinB2 by nearly 2 orders of magnitude. Binding of ephrinB2 to all forms of EphB4 is endothermic, and the binding of ephrinB2 is more endothermic with the L95R mutation in EphB4 (5.2 versus 3.3 kcal mol$^{-1}$ for wild type EphB4). Preliminary experiments carried out in a buffer with different enthalpy of ionization showed a similar enthalpy change to that reported here. For example, binding of ephrinB2 to EphB4 (K149Q) resulted in a $\Delta H_{\text{obs}}$ of 3.9 ($\pm$0.1) kcal mol$^{-1}$ in phosphate ( $\Delta H_{\text{ion}} = 0.8$ kcal mol$^{-1}$) compared with the $\Delta H_{\text{obs}}$ of 3.7 ($\pm$0.2) kcal mol$^{-1}$ value obtained in Tris ( $\Delta H_{\text{ion}} = 11.34$ kcal mol$^{-1}$) (Table 3). Thus, it appears that protonation/deprotonation is not coupled to ephrinB2 binding under the conditions of the ITC experiments.

Binding of the TNYL-RAW peptide to the wild type, K149Q, and L95R forms of EphB4 was also monitored by ITC. TNYL-RAW binds to EphB4 with an affinity of 70 nm and a $\Delta H_{\text{obs}}$ of $-14.7$ kcal mol$^{-1}$ (19). In contrast to the different effects of mutations in EphB4 on the interaction of EphB4 with ephrinB2, mutation of EphB4 of either Lys-149 to Gln or Leu-95 to Arg reduces the affinity of the EphB4-TNYL-RAW interaction (3- and 500-fold, respectively; Table 3). Binding of the TNYL-RAW peptide to all three forms of EphB4 is characterized by an exothermic enthalpy.

Thus, thermodynamic analysis reveals that TNYL-RAW binding to the EphB4 ligand binding domain is an enthalpically driven process, whereas ephrinB2 binding to EphB4 is an entropically driven process. The differences in the binding thermodynamics are consistent with the available structural information. Burial of the hydrophobic ligand G-H loop within the hydrophobic receptor binding cleft should entropically drive the interaction through the release of water,
increasing the solvent entropy. In addition, the ephrinB2 ligand
G-H loop is quite rigid, both in apo and receptor-bound struc-
tures, minimizing massive conformational entropy losses. The
small loss of conformational entropy counteracts the het-
erodimerization process by ordering the otherwise flexible
receptor J-K, D-E, and G-H loops. Unlike ephrinB2, however,
the free peptide ligand will lose significant conformational
degrees of freedom upon EphB4 binding, resulting in an overall
entropy loss. This is compensated by an enthalpy gain due to
the formation of favorable interactions, both polar and nonpo-
lar, at the receptor-peptide interface. A complete structure-
based thermodynamic analysis is precluded, however, due to
our inability to crystallize apo EphB4.

It should be noted that we produced the ephrinB2 extracel-
lular domain in insect cells in a glycosylated form, whereas the
ephrins used for previous crystal structure determinations were
produced in Escherichia coli and, therefore, not glycosylated. A
conserved glycosylation site exists in ephrinB2 at residue Asn-
39, in proximity to the low affinity tetramerization interface.
Consistent with its possible glycosylation, Asn-39 is located
near the surface of the protein, and its side chain extends
toward the surface of the complex. Although the carbohydrate
was not observed in our electron density map, likely because it
was disordered, it is conceivable that a sugar at this location
could interfere with the formation of receptor-ligand tetramers
in the crystal lattice. However, previous reports have suggested
that carbohydrate moieties would play more a favorable than an
unfavorable role in tetramerization (36).

**DISCUSSION**

We have determined the three-dimensional crystal structure of
the EphB4 receptor ligand binding domain in complex with the
extracellular domain of its ligand, ephrinB2, and identified the
determinants for EphB4 specificity. A multiple sequence align-
ment with members of the EphB subclass reveals that the EphB4
receptor lacks a conserved arginine and instead contains a leucine
at position 95. A L95R mutation was previously predicted to result
in steric interference with the antagonistic TNYL-RAW peptide
ligand (19). Here we report that this mutation would also result in
steric clashes with Phe-120L in the G-H loop of ephrinB2 due to
the unique positioning of the J-K loop of EphB4. A leucine instead
of an arginine at position 95 of the EphB4 receptor indeed appears
to be sufficient to cause a substantial structural rearrangement of
the receptor J-K loop. Of particular interest is the novel position of
the conserved Phe-120L in the high affinity FSPN sequence of the
ephrinB2 G-H loop. Although ephrinB2 is conserved in structure
in both receptor-bound and apo structures, there appears to be
variability within the rigid G-H loop to conform to a specific
receptor.

EphrinB2 shares 46 and 40% sequence identity with ephrinB1
and ephrinB3, respectively. EphB4 binds only weakly to both of
these ligands while exhibiting high affinity for ephrinB2. Consid-
ering the B-subclass ephrin G-H loop (ephrinB1-B3), it is interest-
ing to speculate on why EphB4 preferentially binds ephrinB2 over
other B-subclass ligands. EphrinB1 shares significant sequence
identity with the high affinity ephrinB2 G-H loop, except at posi-
tion 124, which is a Tyr in ephrinB1 and a Leu in ephrinB2. Although Leu-124L does not form substantial interactions with
EphB4, the small size of the leucine allows tight packing within the
receptor binding cavity and maintains the hydrophobic nature of
the binding cleft. Superposition of a tyrosine on the ephrinB2
structure would require the rearrangement of the EphB4 J-K loop
to accommodate the bulky tyrosine, likely accounting for the
reduced affinity of EphB4 for ephrinB1. The ephrinB3 G-H loop is
also very similar to the ephrinB2 G-H loop but deviates in the
FSPN sequence, which contains a tyrosine instead of the pheny-
lalanine (YSPN). Phe-120L forms critical interactions with resi-
dues lining the EphB receptor-ephrinB2 binding cavity in the three
complex crystal structures thus far described (8, 10, 19). In the
previous crystal structures Phe-120L extends to the surface of the
binding cavity, adjacent to the receptor G-H loop. Thus, superpo-
position of a tyrosine on the EphB2-ephrinB2 structure would not
affect the dynamics of the ligand binding cavity, and this residue
would likely interact with several water molecules on the surface of
the complex. However, in our structure the Phe-120L of ephrinB2 is
observed in a novel position, buried within the hydrophobic
binding cleft and forming interactions with Leu-95R and the Cys-
61R–Cys-184R disulfide bridge. Insertion of a tyrosine at this posi-
tion would, therefore, result in both steric clashes within the recep-
tor binding cavity and a polar redistribution of the active site,
consistent with the weak affinity of EphB4 for ephrin-B3.

**Insights into the Modulation of EphB4-EphrinB2 Interaction**—
Unlike the heterotetrameric EphB2ephrinB2 crystal structure, EphB4 and ephrinB2 only form heterodimers, both in the cry-

**TABLE 2**

| EphB4 mutant | $K_d$ (μM) |
|--------------|-----------|
| Wild type    | 5 ± 0.9   |
| L95R         | ND*       |
| T147F        | 25 ± 5.6  |
| R148S        | 4.5 ± 0.7 |
| K149Q        | 23 ± 8    |
| R150V        | 6.1 ± 0.8 |
| R148S/K149Q/R150V | 21 ± 6 |
| A186S        | 16 ± 4    |

* Fluorescence polarization (FP) window is not significant to accurately determine $K_d$.
EphB4-EphrinB2 Complex Structure

...stals and in solution. Interestingly, this is most similar to the EphB2-ephrinA5 co-crystal structure than the more related EphB2-ephrinB2 structure. Our studies suggest that higher order oligomerization may require regions outside of the ligand binding domain, which is consistent with previous reports (14, 37). Mutagenesis studies have demonstrated that biologically active Eph-ephrin multimeric complexes are dependent on three interfaces; the high affinity dimerization interface, the tetramerization interface, and a third interface in the cysteine-rich region, which is immediately adjacent to the ligand binding domain and previously reported to mediate receptor-ligand low affinity interaction.

The thermodynamic discrepancies between peptide and ephrin binding should be considered in the design of therapeutics to treat disease related to the Eph receptor family. Iterative rounds of structure-based drug design will require an understanding of the enthalpic and entropic contributions of small molecule compounds. The ephrin ligand, with entropically driven binding, interacts with multiple members of the EphB family. In contrast, the TNYL-RAW peptide, with enthalpically driven binding, is a specific inhibitor of the EphB4-ephrinB2 interaction. The specific nature of the enthalpically driven peptide binding is consistent with the observation made by the group of Ernst Freire concerning the selectivity of binding of human immunodeficiency virus-1 protease inhibitors (15). Further studies are needed to determine whether the correlation between the specificity of the interaction and the contribution of the enthalpic component to the total binding energy is a coincidence or a more general property of the interaction of inhibitors.

We have characterized the interaction between the EphB4 ligand binding domain and the extracellular domain of ephrinB2 both structurally and biophysically. We now better understand the structural determinants that confer the high affinity and specificity of EphB4 for ephrinB2 and the role of the critical EphB4 residue, Leu-95. Structure-based design of EphB4 and ephrinB2 mutants will provide more precise tools for understanding the mechanisms of tumorigenesis stimulated by their interaction. The characterization of enthalpic versus entropic contributions in EphB4 ligand binding using ITC complements the structure-based approach to compound and ligand design. Our results combined with the information from other related Eph receptor-ephrin complex structures offer the possibility to rationally design small molecule compounds and biotherapeutics with improved pharmacokinetic properties that would antagonize the EphB4-ephrinB2 interaction.

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