Three-dimensional Structure of the EphB2 Receptor in Complex with an Antagonistic Peptide Reveals a Novel Mode of Inhibition$^{[5]}$

Received for publication, August 1, 2007, and in revised form, September 25, 2007 Published, JBC Papers in Press, September 26, 2007, DOI 10.1074/jbc.M706340200

Jill E. Chrencik$, Alexei Brooun$†, Michael I. Recht$, George Nicola$, Leila K. Davis$, Ruben Abagyan$, Hans Widmer$, Elena B. Pasquale**, and Peter Kuhn‡

From the Departments of $^{4}$Cellular Biology and $^{4}$Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, the $^{5}$Scripps-PARC Institute for Advanced Biomedical Sciences, Palo Alto Research Center, Palo Alto, California 94304, the $^{1}$Novartis Institutes of BioMedical Research, CH-4002 Basel, Switzerland, and $^{**}$The Burnham Institute for Medical Research, La Jolla, California 92037

The Eph family of receptor tyrosine kinases has been implicated in tumorigenesis as well as pathological forms of angiogenesis. Understanding how to modulate the interaction of Eph receptors with their ephrin ligands is therefore of critical interest for the development of therapeutics to treat cancer. Previous work identified a set of 12-mer peptides that displayed moderate binding affinity but high selectivity for the EphB2 receptor. The SNEW antagonistic peptide inhibited the interaction of EphB2 with ephrinB2, with an IC_{50} of ~15 μM. To gain a better molecular understanding of how to inhibit Eph/ephrin binding, we determined the crystal structure of the EphB2 receptor in complex with the SNEW peptide to 2.3-Å resolution. The peptide binds in the hydrophobic ligand-binding cleft of the EphB2 receptor, thus competing with the ephrin ligand for receptor binding. However, the binding interactions of the SNEW peptide are markedly different from those described for the TNYLRAW peptide, which binds to the ligand-binding cleft of EphB4, indicating a novel mode of antagonism. Nevertheless, we identified a preserved structural motif present in all known receptor/ligand interfaces, which may serve as a scaffold for the development of therapeutic leads. The EphB2-SNEW complex crystallized as a homodimer, and the residues involved in the dimerization interface are similar to those implicated in mediating tetramerization of EphB2-ephrinB2 complexes. The structure of EphB2 in complex with the SNEW peptide reveals novel binding determinants that could serve as starting points in the development of compounds that modulate Eph receptor/ephrin interactions and biological activities.

$^{[5]}$ This work was supported by Novartis Institutes for BioMedical Research Grant SFP-1543, National Institutes of Health Protein Structure Initiative Specialized Centers Grant GM074961 (to P. K.) and training Grant ST32/A107354–16 (to J. C.). This is The Scripps Research Institute manuscript number 18926. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

$^{†}$ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

The atomic coordinates and structure factors (code 2QBX) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

$^{1}$ Present address: Pfizer Inc., 10770 Science Center Dr., CB2/2213, San Diego, CA 92121.

$^{2}$ To whom correspondence should be addressed: 10550 N. Torrey Pines Rd., CB265, La Jolla, CA 92037. Fax: 858-784-8996; E-mail: pkuhn@scripps.edu.

The erythropoietin-producing hepatocellular carcinoma (Eph) family is the largest family of receptor tyrosine kinases identified to date, with 16 structurally similar family members (1). The Eph family plays important roles in both developing and adult tissues, and regulates biological processes such as tissue patterning, development of the vascular system, axonal guidance, and neuronal development (2–6). The EphB2 receptor plays a role in the development of several tissues. The loss of EphB2 and the related EphB3 receptor, which has some redundant functions, results in cleft palate, the failure to remodel the vascular plexus, impaired heart development, defects in urogenital development, and abnormal development of dendritic spines in the hippocampus (7–10). Furthermore, some strains of EphB2 knock-out mice have defects in the inner ear (11).

The EphB2 receptor has also been recently found to be over-expressed in many types of cancer, including gastric (12, 13), colorectal (14–18), ovarian (19), breast (20), and prostate cancers (21, 22), and glioblastoma (23, 24). In some tumor types EphB2 appears to have tumor promoting effects, whereas in others it has tumor suppressor effects (17, 18). In addition, similar to EphB4, EphB2 on the surface of tumor cells may promote tumor angiogenesis by interacting with ephrinB2 in tumor blood vessels (25). Therefore, targeting EphB2 represents a promising avenue for therapeutic intervention. Peptides and chemical compounds that bind to EphB2 may be used to target anti-cancer agents to EphB2-expressing tumors (26) and, in certain tumor types, interfering with EphB2 ligand binding should inhibit tumor progression and tumor angiogenesis.

The Eph receptors and the ephrins ligands are classified into two major classes, A or B, based on their sequence identities and ligand binding preferences. Class A receptors (EphA1–EphA10) bind preferentially to ephrinA ligands (ephrinA1–ephrinA6), whereas class B receptors (EphB1–EphB6) bind preferentially to ephrinB ligands (ephrinB1–ephrinB3) (27). Although the Eph receptors bind promiscuously the ephrins of the same A or B class, interactions across classes are rare (28). An interesting feature of the Eph/ephrin interaction is the fact that both the receptor and the ligand are membrane bound, and are capable of transducing signals bidirectionally either in the...
EphB2-SNEW Complex Structure

forward direction through the Eph receptor cytoplasmic domain, or in the reverse direction through the ephrin, to elicit a series of biological responses in both receptor- and ephrin-expressing cells.

The domain architecture of the Eph receptors is highly conserved. The cytoplasmic region of the receptors consists of a juxtamembrane region, a kinase domain, a SAM domain (sterile α-domain), and a PDZ binding motif (PSD-95 post-synaptic density protein, Discs large, and Zona occludens tight junction protein). Extracellularly, the receptors contain a ligand-binding domain at the N terminus, a cysteine-rich region, and two fibronectin type III repeats. The ligand-binding domain of the Eph receptor has been characterized as the minimal region required for high affinity interaction with the ephrins (29). The ephrin ligands also have a structurally conserved extracellular domain characterized by a Greek-key topology. The ligands deviate, however, in their attachment to the cell membrane. The ephrinB ligands contain a transmembrane region, whereas the ephrinA ligands are anchored to the membrane by a glycosphatidylinositol linkage.

Previously reported crystal structures of Eph receptors in complex with a ligand revealed a heterodimerization interface on the surface of the Eph receptor ligand-binding domain that mediates a high affinity receptor/ligand interaction. In addition, a lower affinity tetramerization interface was identified in EphB2-ephrinB2 complex crystals that mediated the multimerization of EphB2 receptor/ephrinB2 dimers (29). In the high affinity interface, the G–H loop of the ephrin inserts into the hydrophobic ligand-binding cavity formed by the D–E, G–H, and J–K loops of the Eph receptor, resulting in a nanomolar binding affinity (29, 30). Previously, the crystal structure of the EphB4 receptor in complex with the high affinity (40 nM) and antagonistic TNYL-RAW (TNYLFSQPNPILARRAW) peptide was elucidated (31). The structure revealed that the antagonistic peptide binds within the EphB4 ligand binding cavity, precluding interaction with the G–H loop of ephrinB2.

Phage display screens have identified a series of 12-mer peptides that target the ligand-binding domains of several Eph receptors and antagonize their interactions with ephrins, whereas maintaining exceptional specificity for a particular Eph receptor (32). One peptide, SNEW (SNEWIQPRLPQH, Biopeptide, Inc.), and J–K loops of the Eph receptor, resulting in a nanomolar binding affinity (29, 30). Previously, the crystal structure of the EphB4 receptor in complex with the high affinity (40 nM) and antagonistic TNYL-RAW (TNYLFSQPNPILARRAW) peptide was elucidated (31). The structure revealed that the antagonistic peptide binds within the EphB4 ligand binding cavity, precluding interaction with the G–H loop of ephrinB2.

Isothermal Titration Calorimetry—EphB2 was dialyzed into 50 mM Hepes, pH 7.2 (at 25 °C), 150 mM NaCl, and 1 mM CaCl2, prior to use in calorimetry experiments. The SNEW peptide and all mutant peptides were dissolved in the dialysis buffer. All ITC experiments were performed with a Microcal MCS ITC at 25 °C. ITC experiments were performed as described previously (30, 31).

In Silico Combinatorial Mutagenesis—All in silico mutagenesis analyses were conducted with the ICM program (42) and incorporated the SNEW structure as a template. First, the
structure was regularized with a multistep procedure. This consists of creating full-atom geometrical approximation model, rotational positioning of methyl groups, iterative optimization of geometry, and energy of the whole structure, and adjustment of polar hydrogen positions. Finally, free minimization was performed to relieve any bad contacts and check the consistency of the resulting structure.

Next, residues 1 through 9 of the peptide were each mutated to all 20 amino acids individually, resulting in 120 new peptides. After each mutation, a full energy optimization of the new side chain was then performed using the Monte Carlo procedure. The associated energy includes van der Waals terms, a hydrophobicity term based on the solvent accessible surface buried upon binding, a solvation electrostatic term using a boundary-element solution of the Poisson equation, a hydrogen-bond interaction term, and the entropic contribution (43). For the double and triple mutants, three peptide mutation positions, 3, 6, and 8, were kept constant based on the highest scoring candidates from the previous round. The combinatorial approach was then repeated with the remaining positions in a similar manner as the first round. Finally, an empirical scoring function between the receptor and each peptide was calculated and stored in a table. Six single mutants, two double mutants, and a triple mutant peptide were then selected based on high scores and rational feasibility, to be synthesized and tested by ITC.

RESULTS

The overall structure of the human EphB2 ligand-binding domain in complex with the antagonistic SNEW peptide was refined to a 2.3-Å resolution and a free R factor of 27%. The structure of EphB2 in complex with SNEW is similar to that of the apo-EphB2 receptor (38), and consists of a jellyroll folding topology with 13 β-strands arranged into 2 antiparallel β-sheets forming a compact β-sandwich (Fig. 1). The β-sheets are connected by loops that vary in amino acid number and are characterized by a high degree of flexibility. For example, the D–E and J–K loops are disordered in the apo-EphB2 structure due to their inherent flexibility. However, binding of the antagonistic SNEW peptide promotes the ordering of these loops. The EphB2 structure is further stabilized by two conserved disulfide bridges, one in the G–H loop, and the other in the E–F/L–M loops (Cys105/Cys115 and Cys70/Cys192, respectively).

The peptide-bound and apo-EphB2 structures superpose well, with an overall root mean square deviation of 1.7 Å over 178 respective Ca atoms. Furthermore, the EphB2-SNEW complex superposes well with respect to the ephrinB2- and ephrinA5-bound EphB2 structures, with root mean square deviations of 2.7 and 1.9 Å, respectively. Although the core of the EphB2 ligand-binding domain remains unchanged in the apo and peptide-bound structures, as well as the peptide-bound EphB4 structure, there are notable differences in the surface-exposed loop regions, particularly the D–E, G–H, and J–K loops (Fig. 2) (29–31, 38). Interestingly, the J–K loops from the EphB2-bound structures (PDB code 1NUK and 1KGY) are generally positioned toward the D–E loop, whereas the J–K loops from the EphB4-bound structures (PDB codes 2BBA and 2HLE) are positioned toward the G–H loop (29–31, 38). The J–K loop from the EphB2-SNEW complex is positioned between these two formations, which is a result of the N-terminal residues of the SNEW peptide, which sterically precludes the J–K loop from occupying the area closer the D–E loop, as in the EphB2/ephrinB2 structure (29). The structural flexibility of these loops in several ligand-bound EphB forms has been well documented.
and it allows the receptors to use an induced fit mechanism to recognize and accommodate cognate ligands (29, 30, 44).

**SNEW Binding**—The SNEW peptide was readily placed into the electron density after one round of refinement using \(|F_{\text{obs}}| - |F_{\text{calc}}|\), \(F_{\text{calc}}\) maps (Fig. 3). Similar to the antagonistic TNYL-RAW peptide that binds to the EphB4 receptor, the antagonistic SNEW peptide resides in the ephrin binding cavity otherwise occupied by the long and hydrophobic G–H loop of the ephrin ligand. The SNEW peptide adopts an extended structure with no regular secondary structure elements. The N-terminal end of the peptide binds at the top of the receptor binding cleft, between the J–K and G–H loops. The position of the peptide at the junction of the J–K loop likely results in its ordering. The 12-mer peptide binds across the \(\beta\)-sheet floor formed by strands D and E of the receptor, forming a small network of interactions with residues lining the receptor binding cavity. The C-terminal end of the peptide emerges at the tip of the C–D and E–F loops, forming side chain/main chain interactions with residues in these loop regions. The last amino acid of the peptide, His\(^{12}\), could not be readily modeled into the electron density presumably due to its position outside of the ligand binding cavity, and is, therefore, is not part of the final model of the complex.

The interaction network between the EphB2 receptor and the SNEW peptide relies on just a few hydrogen bonds as well as hydrophobic interactions (Fig. 4). The first three residues of the SNEW peptide (SNEWIQPRLPQH) interact with residues lining the receptor binding cavity. Ser\(^{1}\), Asn\(^{2}\), and Glu\(^{3}\) form polar interactions between side chain residues of the peptide and main chain atoms of amino acids lining the receptor J–K and G–H loops. The fourth amino acid in the peptide, the bulky Trp\(^{4}\), forms a hydrogen bond with Asn\(^{2}\) of the SNEW peptide, and likely stabilizes the overall structure of the peptide at the N-terminal end. Trp\(^{4}\) occupies a small portion of the large ligand binding cavity and interacts favorably with hydrophobic residues lining the cavity. Both Ile\(^{8}\) and Pro\(^{9}\) of the SNEW peptide interact with a highly conserved disulfide bridge between the E–F and L–M loops of EphB2 (Cys\(^{70}/\text{Cys}^{192}\)). A proline residue is conserved in the ephrin-B2 ligand G–H loop (FSPN sequence), and has been described to stabilize the Cys\(^{70}/\text{Cys}^{192}\) disulfide bridge in all known Eph crystal structures thus far described, whereas an Ile residue is present at the corresponding position in the TNYLFSNPNGPAR (TNYL-RAW) peptide and plays a similar stabilizing role as the proline (29, 30, 44). These isoleucine and proline residues are critical for a high affinity receptor/ligand interaction to stabilize the critical disulfide bridge in all known Eph receptors (31). The side chains of the Ile/Pro residues may therefore serve as a scaffold for the development of therapeutic leads, as these residues appear to be absolutely required for effective binding and stabilization of the disulfide bridge. The next three residues in the SNEW peptide, Glu\(^{6}\), Pro\(^{7}\), and Arg\(^{8}\), form hydrogen bonds and hydrophobic interactions with residues of the E–F loop lining the EphB2 binding cavity. Finally, Pro\(^{10}\) and Glu\(^{11}\) form a hydrophobic interaction and a side chain/main chain hydrogen bond with residues in the E–F and C–D loops, respectively, contributing to the interaction network between receptor and peptide.

**FIGURE 2.** Superposition of the J–K and D–E loops of the EphB4 (EphB4/TNYL-RAW complex; PDB code 2HLE) and EphB2 receptors (EphB2-ephrinB2 complex; PDB code 1KGY) on the EphB2 receptor from the EphB2-SNEW structure. The EphB4 receptor is shown in cyan, the EphB2 (complex with ephrinB2) is shown in magenta, and the EphB2 (complex with SNEW) is shown in green. The structures are superimposed with an overall root mean square deviation of 7.8 and 2.7 Å between 180 eq Cu positions, respectively. The J–K loop is displaced by as much as 20 Å between structures.

**FIGURE 3.** Stereoview of \(\sigma-A\) weighted \(2|F_{\text{obs}}| - |F_{\text{calc}}|\) electron density at 2.3-Å resolution, contoured at 1\(\sigma\) for the antagonistic SNEW peptide.
FIGURE 4. Detailed ligplot diagram of critical EphB2-SNEW interactions. All interactions that are less than 4 Å are indicated by dashed green lines. The ligand is depicted with all bonds shown, whereas receptor residues (orange) are drawn schematically. The protein is identified by chain B, whereas the peptide is identified by chain D.
A large stacking network is observed at this interface, wherein the Asp127 residues are capped by the Arg179 residues, and a sandwich is formed with the stacking of Tyr124 with Arg residues. This highly polar interface is capped by Phe128 residues from each monomer.

Comparison with TNYL-RAW Binding to EphB4—Numerous differences exist between binding of the SNEW antagonistic peptide to the EphB2 receptor and binding of the TNYL-RAW antagonistic peptide to the EphB4 receptor (31). The overall directionality of the peptides at the N-terminal ends is similar, with the exception that the N terminus of the EphB2-SNEW peptide binds between the receptor G–H and J–K loops of EphB2, whereas the N-terminal end of the EphB4 TNYL-RAW peptide binds between the G–H and J–K loops of EphB4 (Fig. 5, left). Secondary structural elements do not exist at the N-terminal end of either peptide. The TNYL-RAW peptide binds along the b-sheet floor formed by b-sheets D and E of the EphB4 receptor, whereas the SNEW peptide binds across this region in the EphB2 receptor. Furthermore, whereas the TNYL-RAW peptide forms a 90° turn at the center of the peptide due to the presence of a G–P motif, the SNEW peptide remains highly linear. As a result of the kink in the TNYL-RAW peptide, the C-terminal end of the TNYL-RAW peptide is angled back at the tip of the G–H and J–K loops of EphB4, and plays a critical role in stabilizing the loops through an extensive interaction network, particularly with respect to the C-terminal RAW sequence. Interestingly, the N-terminal end of the SNEW peptide resides in the same region of EphB2 that is occupied by the C-terminal end of the TNYL-RAW peptide, albeit in a deeper groove between the G–H and J–K loops, resulting in an overall 8-Å displacement of the J–K loop between the two receptor structures. The SNEW peptide, on the other hand, emerges near the C–D and E–F loops of the EphB2 receptor. Although the two peptides occupy distinct surfaces within the receptor binding cavity, there is a single point of intersection at a conserved Ile residue (Fig. 5, right). This residue, corresponding to SNEW Ile5 as described above, promotes the stability of a critical disulfide bridge that is conserved across EphA and EphB classes. The conservation of a residue in the ligand or peptide that stabilizes this disulfide bridge suggests that this interaction is critical for high affinity ligand binding. The overall interaction network of the SNEW peptide with EphB2 is less extensive than that described for the TNYL-RAW peptide binding to the EphB4 receptor, consistent with the observed lower binding affinity of SNEW for EphB2.

EphB2 Homodimeric Crystals—Unlike crystals of the apo-EphB2 receptor, which contained one monomer of the EphB2 receptor in the asymmetric unit (38), EphB2 in complex with the SNEW peptide exists as a homodimer in the crystallographic asymmetric unit. The two EphB2 monomers are related by a mirror image, and interact extensively through the H–I, K–L, and F–G loops. At the center of the interface is the stacking of both Arg179 and Asp127 from each monomer of EphB2 (Fig. 6). The side chain of Asp127 forms a hydrogen bond with the side chain amine of Arg179 from the same EphB2 monomer, stabilizing the position of these residues at the homodimer interface. In addition, Arg179 forms a side chain–main chain hydrogen bond with a Glu125 residue from the opposing EphB2 monomer. Tyr124 residues from each EphB2 monomer stack on the top and bottom of the equivalent Arg179 residue, forming a pseudo-sandwich at this interface. Tyr124 forms a hydrogen bond with the side chain amine of Arg179 from the opposing receptor and a polar interaction with Asp127, also from the opposing receptor. Finally, the top of the interface is capped off by the cumulative stacking of Phe128 residues from both monomers. The hydrophobic stacking of these residues fills out the binding network at the homodimer interface. Aside from this hydrophobic interaction, the remainder of the interface consists of just a few hydrogen bonds. Size exclusion chromatography studies with the EphB2 receptor at concentrations up to 10
mg/ml demonstrated the presence of both monomer and dimer in solution (data not shown). However, the apo-EphB2 receptor crystallized as a monomer, which is suggestive that ligand binding may be responsible for the discrepancy between monomer and dimer forms in the crystal. Conversely, the EphB4 receptor consistently eluted as a monomer, even at concentrations as high as 20 mg/ml. The functional relevance of the EphB2 homodimer has yet to be elucidated.

**Thermodynamic Characterization**—Isothermal titration calorimetry studies determined a $K_d$ value of 6 $\mu M$ for the interaction of the SNEW peptide with human EphB2, which is consistent with the I$_{50}$ of ~15 $\mu M$ previously reported in enzyme-linked immunosorbent assays using mouse EphB2 (supplementary data Fig. S1) (31). In addition, a $\Delta H$ of $-9.8$ kcal/mol was determined. For comparison, binding of the TNYL-RAW peptide to the EphB2 receptor was also tested and found to be undetectable. This is consistent with previous reports that the SNEW and TNYL-RAW peptides are highly specific for the EphB2 and EphB4 receptors, respectively.

**In Silico Combinatorial Mutagenesis**—A series of mutations were made within the SNEW peptide in an effort to optimize the binding potential of the peptide. The mutations were based on an *in silico* combinatorial mutagenesis approach using the ICM program package (42). The program involves regularization of the peptide, a substitution mutation of each peptide residue to every amino acid, full side chain optimization after conformational analysis on these peptides are shown in Table 2. The highest affinity peptide was SNEW-3, containing a single Glu$^6$ → Leu mutation, which resulted in a $K_d$ of 3 $\mu M$, or a 2-fold increase in the binding affinity from the original peptide. Mutation of the same position to serine decreased the affinity to 11 $\mu M$. Interestingly, the Glu$^3$ to Gln mutation led to a dramatically decreased affinity. It was expected that the polar environment generated by this substitution would assist in stabilization with main chain atoms of residues in the K $\beta$-strand of the receptor.

Instead, this perturbation adversely affected the existing hydrogen bonding network. This indicates the interaction between Glu$^3$ of the peptide and Val$^{164}$ of the receptor is more important than expected.

**DISCUSSION**

We have determined the crystal structure of the EphB2 receptor in complex with an antagonistic peptide to 2.3-Å resolution, and described the molecular determinants of binding by both structural and biophysical methods. Two crystals structures of Eph receptors in complex with antagonistic peptides have now been described: EphB4/TNYL-RAW and EphB2-SNEW. Surprisingly, the binding interfaces for each peptide with the receptor are uniquely distinct, despite the fact the ligand-binding domains of the EphB2 and EphB4 receptors share 45% sequence identity. Nevertheless, both peptides sterically inhibit the interaction of the Eph receptor with the ephrin G–H loop, and therefore antagonize ephrin binding. It appears that a general requirement for effective peptide binding is the formation of an interaction network capable of mediating the conformational stability of the flexible loops of the Eph receptor, and particularly the J–K loop. The ordering of this loop has previously been implicated in the recognition and binding of a target ligand, likely through an induced fit mechanism whereby the loop adopts a suitable conformation to accommodate an ephrin or a peptide ligand. In fact, this loop has been found to deviate up to 20 Å in position depending on the bound ligand (31). Whereas the ordering of this loop would certainly result in an entropic penalty, the enthalpic gain of peptide/compound binding would drive the reaction, as observed in the EphB4/TNYL-RAW complex (30). It also appears that the large ephrin binding cavity of an Eph receptor can accommodate diverse ligands in a highly variable manner. This implies that chemical compounds identified in high-throughput screening efforts may be found to bind to a variety of surfaces in the ephrin binding pocket to modulate the Eph/ephrin interactions.

A second notable feature of the Eph/ephrin interaction is the conservation of an Ile or Pro residue in an identical location in each Eph/ligand structure thus far described. For example, the Pro residue from the conserved FSPN sequence of the ephrinB2 G–H loop is situated so that it stabilizes a critically important disulfide bridge. The nature of this interaction is similarly con-

### Table 2

| Name    | Sequence         | $K_d$ $\mu M$ | $\Delta G$ kcal mol$^{-1}$ | $\Delta H$ kcal mol$^{-1}$ | $\Delta S$ kcal mol$^{-1}$ |
|---------|------------------|---------------|-----------------------------|-----------------------------|-----------------------------|
| SNEW$^a$| SNEWIQPRLPQH     | 6             | $-7.1 \pm 0.1$             | $-9.8 \pm 0.3$             | $-2.6 \pm 0.3$             |
| SNEW_1  | SNEWIQPRLPQH     | >50           |                             |                             |                             |
| SNEW_2  | SNEWIQPRLPQH     | 11            | $-6.8 \pm 0.1$             | $-9.2 \pm 0.6$             | $-3.1 \pm 0.6$             |
| SNEW_3  | SNEWIQPRLPQH     | 3             | $-7.5 \pm 0.1$             | $-13.9 \pm 0.2$            | $-6.3 \pm 0.2$             |
| SNEW_4  | SNEWIQPRLPQH     | 6             | $-7.1 \pm 0.1$             | $-11.7 \pm 0.1$            | $-4.6 \pm 0.1$             |
| SNEW_5  | SNEWIQPFLPQH     | >50           |                             |                             |                             |
| SNEW_6  | SNEWIQPRLPQH     | >>50          |                             |                             |                             |
| SNEW_7  | SNEWIQPRLPQH     | >50           |                             |                             |                             |
| SNEW_8  | SNEWIQPFLPQH     | >50           |                             |                             |                             |
| SNEW_9  | SNEWIQPFLPQH     | >50           |                             |                             |                             |
| TNYL-RAW| TNYLFSPNGPILARAW  | >50           |                             |                             |                             |

$^a$ Reported $K_d$ is an average of 2 measurements.
served in the Eph-peptide complexes, where an Ile residue plays a similar role in stabilizing the disulfide bridge. Modification of this residue in the antagonistic TNYL-RAW peptide led to a dramatic reduction in affinity from 70 nM to 60 μM for the EphB4 receptor (31). It therefore appears that the backbone architecture of either an Ile or a Pro provides a key molecular determinant for ligand recognition and stability. This suggests that chemical compounds that target this disulfide bridge of the receptor may be particularly effective at inhibiting ligand binding.

The EphB2-SNEW complex crystallized as a dimer, whereas the EphB4/TNYL-RAW complex crystallized as a monomer, and whereas one has to be cautious interpreting the physiologic relevance of different crystal forms, it nevertheless provides data worthwhile considering. Interestingly, the loops involved in the homodimer interface have also been implicated in the tetramerization interface of the EphB2-ephrinB2 complexes (29). The EphB2/ephrinB2 tetramer interface is also characterized by a polar nature, with numerous weak polar interactions and few main chain–side chain hydrogen bonds. In addition, a critical hydrophobic stacking interaction between Phe128 (receptor) and Tyr124 (ephrinB2 ligand) was proposed to impart rigidity to the tetramer interface. The similar stacking of n-plexylalanines at the dimer interface in the EphB2-SNEW structure is predicted to play a similar role in imparting stability to this complex. Superposition of the EphB4 receptor on the EphB2 dimers provides clues as to the inability of EphB4 to homodimerize. Although the sequence is highly conserved at the dimer interface, there are also some important differences. Similar to the EphB2 receptor, EphB4 has an Asp at position 127 (EphB2 numbering) and a Tyr at position 124, which would form interactions at the homodimer interface similar to those described for EphB2. In addition, EphB4 also has a long basic side chain at position 179, although it is from a lysine rather than an arginine. Thus, the overall polar interface would be predicted to remain unchanged. However, the hydrophobic stacking interactions of Phe128 residues that cap the homodimer interface are absent due to the presence of Ala at the corresponding position in EphB4. Interestingly the lack of the Phe127 was also predicted to disrupt the tetramerization interface between EphB4 and ephrinB2 (31). Whether or not the lack of a bulky residue at this position is required for proper multimerization has yet to be elucidated. Although a Phe is not conserved across the Eph subclasses, all other Eph receptors have a polar or aromatic residue at this position, whereas Ala is only found in EphB4. In addition, unlike the EphB2/ephrinB2 size exclusion chromatography profile, which indicated the presence of both a dimer and a tetramer (45), the EphB4-ephrinB2 complex was only detected as a dimer, even at concentrations as high as 20 mg/ml. The EphB2 monomer is functional in an isotothermal titration calorimetry binding assay, and the possible functional relevance of the EphB2 homodimer for receptor activation and signaling remains to be elucidated.

We attempted to systematically optimize the SNEW peptide to increase its affinity for the EphB2 receptor. Our studies show that three of the modified peptides we synthesized have binding affinities within an order of magnitude of the affinity of the original SNEW peptide. In particular, a Gln → Leu substitution resulted in a 2-fold increase in affinity. In the EphB2-SNEW crystal structure, the glutamine at position 6 of the peptide does not point to the active site cavity and thus the advantages of its replacement by a leucine are not immediately apparent. However, the strongly hydrophobic side chain of leucine is near the long side chains of receptor residues Arg103 and Gln148 in the docked pose, and may provide a slightly better stabilization of these residues. Alternatively, the effects of the hydrophobic interaction mediated by Leu5 may assist in the initial recognition of the peptide by the receptor. In this scenario, the peptide adjusts its conformation to a final resting state after binding, whereby the leucine becomes oriented in an outward position. We have initiated further studies of in silico combinatorial mutagenesis using SNEW_3 as the template structure in an attempt to identify peptides with even higher binding affinities.

We have characterized both structurally and biophysically the interaction between the EphB2 ligand-binding domain and the antagonistic SNEW peptide. The detailed molecular understanding of the SNEW peptide binding determinants with the EphB2 receptor should accelerate structure-based drug design efforts utilizing the peptide as a starting scaffold. Similar to the fluorescence polarization assay developed to measure EphB4/TNYL-RAW interactions (30), the SNEW peptide could also be used as a probe in a fluorescence polarization assay to screen for EphB2 antagonists in high-throughput format. It is important to note that the binding affinity of the SNEW peptide for the EphB2 receptor is 6 μM, much unlike the high affinity of the TNYL-RAW peptide for the EphB4 receptor, which is 70 nM. The moderate binding affinity of the SNEW peptide will result in a low stringency for the assay that will impact the hit rate and affinity of compounds identified in a HTS assay. Identification of protein-protein interaction inhibitors is often a daunting task, and structural and thermodynamic characterization of prototype peptide antagonists provides a strong foundation for the development of small molecule modulators of Eph/ephrin interactions, which are dysregulated in various cancers. Comparative analysis of hits identified in both EphB2 and EphB4 HTS screens will also allow the ability to probe fundamental questions with regards to the molecular determinants that are required for lead compounds to inhibit other protein-protein interacting pairs.

Acknowledgments—We acknowledge Raymond C. Stevens and Michelle Kraus for insightful discussions and guidance. We also acknowledge the helpful support of the staff scientists at the Advanced Photon Source (BL GM-CA CAT) for help in data collection. The General Medicine and Cancer Institutes Collaborative Access Team (GM/CA CAT) is supported by NCI National Institutes of Health Grant Y1-CO-1020 and NIGMS National Institutes of Health Grant Y1-GM-1104.

REFERENCES
1. Eph Nomenclature Committee (1997) Cell 90, 403–404
2. Brantley-Sieders, D. M., and Chen, J. (2004) Angiogenesis 7, 17–28
3. Carmeliet, P., and Collen, D. (1999) Curr. Top Microbiol. Immunol. 237, 133–158
4. Kullander, K., and Klein, R. (2002) Nat. Rev. Mol. Cell. Biol. 3, 475–486
