Engineering nanomolar peptide ligands that differentially modulate EphA2 receptor signaling

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Running title: Nanomolar peptides that activate or inhibit EphA2 signaling

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Keywords: Eph receptor, ephrin, receptor tyrosine kinase, peptide, agonist, antagonist, dimerization, cancer, inflammation, crystal structure

ABSTRACT

The EPH receptor A2 (EphA2) receptor tyrosine kinase plays an important role in a plethora of biological and disease processes, ranging from angiogenesis and cancer to inflammation and parasitic infections. EphA2 is therefore considered an important drug target. Two short peptides previously identified by phage display, named YSA and SWL, are widely used as EphA2-targeting agents owing to their high specificity for this receptor. However, these peptides have only modest (micromolar) potency. Lack of structural information on the binding interactions of YSA and SWL with the extracellular EphA2 ligand-binding domain (LBD) has for many years precluded structure-guided improvements. We now report the high-resolution (1.53–2.20 Å) crystal structures of the YSA peptide and several of its improved derivatives in complex with the EphA2 LBD, disclosing that YSA targets the ephrin-binding pocket of EphA2 and mimics binding features of the ephrin-A ligands. The structural information obtained enabled iterative peptide modifications conferring low nanomolar potency. Furthermore, contacts observed in the crystal structures shed light on how C-terminal features can convert YSA derivatives from antagonists to agonists that likely bivalently interact with two EphA2 molecules to promote receptor oligomerization, autophosphorylation, and downstream signaling. Consistent with this model, quantitative FRET measurements in live cells revealed that the peptide agonists promote the formation of EphA2 oligomeric assemblies. Our findings now enable rational strategies to differentially modify EphA2 signaling toward desired outcomes by using appropriately engineered peptides. Such peptides could be used as research tools to interrogate EphA2 function and develop pharmacological leads.

EphA2 has been implicated in many disease processes. It is overexpressed in many cancer types where, however, ligand-induced EphA2 kinase-dependent signaling is often low (1–3). This apparent paradox can be explained by the fact that
the receptor has pro-oncogenic activities in the absence of ligand. In contrast, EphA2 activation by ephrin-A ligands can inhibit oncogenic signaling networks (such as AKT-mTORC1 and RAS-ERK) and the pro-oncogenic EphA2 phosphorylation on S897 as well as induce EphA2 internalization and degradation. Thus, agents promoting EphA2 activation can be useful to suppress cancer cell malignancy as well as to deliver drugs, toxins and imaging agents to tumor cells (3-5). On the other hand, inhibiting EphA2 activation could be useful against pathological forms of angiogenesis, inflammation and parasitic infections (3,6,7). Thus, there are exciting opportunities to target EphA2 for multiple therapeutic applications, but their potential has not yet been realized.

EphA2 belongs to the Eph family of receptor tyrosine kinases, which signal via lateral association in the plasma membrane (2,3,6). The extracellular region of EphA2 includes an N-terminal ligand-binding domain (LBD), a cysteine-rich region and two fibronectin type III domains. The ligands, ephrin-A1 through ephrin-A5, bind to a pocket in the LBD. A hydrophobic transmembrane helix connects the EphA2 extracellular region to the cytoplasmic region, which contains the kinase domain. Upon EphA2 oligomerization (dimerization and clustering), typically induced by ligand binding, the EphA2 molecules cross-phosphorylate each other on tyrosine residues, triggering downstream signaling cascades.

Efforts to target EphA2 and modulate its activation and downstream signaling have included different strategies. The ATP binding site in the kinase domain is suitable for targeting with small molecule inhibitors, but it is difficult to achieve specific targeting given the high conservation of this site in Eph receptors and other kinases (3,6,8). The ephrin-binding pocket in the LBD can also be targeted with engineered forms of the ephrin-A ligands, but these ligands bind promiscuously to all nine EphA receptors and are therefore not well-suited as selective EphA2 modulators (2,3,6). In addition, the ephrin-binding pocket has proven too large for selective high-affinity binding of small molecules (3,8). In contrast, two related 12 amino acid-long peptide ligands (YSA and SWL) that we identified by using phage display, and their modified derivatives, are exquisitely selective because they bind only to EphA2 among the Eph receptors (5,9). Some of these peptides function as ephrin mimics that promote EphA2 tyrosine phosphorylation, downstream signaling and endocytosis (9-11). They have therefore been widely used to target cancer cells and other cell types with high EphA2 expression. The peptides have been conjugated to different chemotherapeutic drugs and many types of nanoparticles containing drugs, imaging agents and/or siRNAs (3,5). However, they have modest EphA2 binding affinity, which severely limits their potential applications.

Here we report structure-guided approaches that have greatly improved the EphA2 binding affinity of YSA derivative peptides from micromolar to low nanomolar. We also identify features conferring agonistic or antagonistic properties, which can be useful for different applications, and show that the peptide agonists promote EphA2 oligomerization through an unexpected bivalent binding mode.

Results

The YSA peptide binds to the ephrin-binding pocket of EphA2

In our initial characterization of YSA (peptide (1) in Table 1), we used a modified version of the peptide including a C-terminal GSGSK linker with a biotin tag attached to the side chain of the lysine (YSA-GSGSK-bio (2), Table 1) (9). We have now solved the crystal structure of this peptide in complex with the EphA2 LBD at a resolution of 1.9 Å (Fig. 1, Table S1). The structure contains two peptide–EphA2 complexes in the asymmetric unit and in both complexes the electron density is well defined for the first 10 amino acids of the peptide (Fig. S1A), indicating that this part of YSA (1) and YSA-GSGSK-bio (2) is mainly responsible for interaction with EphA2. The peptide binds to the ephrin-binding pocket of EphA2, which is the region that interacts with the G-H loop of ephrin-A1 (12) (Fig. 1, panels A,C,E versus B,D). The first 4 amino acids of YSA-GSGSK-bio (2) bound to EphA2 closely overlap with residues Phe111 to Phe114 in the G-H loop of ephrin-A1 bound to the EphA2 LBD (Fig. 1F). In fact, the first 4 amino acids of YSA (1) (YSAY) conform to a ΩXXΩ motif (where Ω is an aromatic residue and X can be any residue) that is also present in the SWL (6) peptide and the G-H loop of all the ephrin-A ligands
weak replacement of Ser did, was, d group of the biotin from one of the two peptide asymmetric unit, which we interpret as the head group of the biotin from one of the two peptide molecules (Figs. 1A, C, E versus B, D).

The YSA-GSGSK-bio (2) peptide forms an extensive network of hydrophobic and polar interactions with EphA2 (Fig. 1H, Fig. S2A). Key interactions involve peptide Tyr1 (which binds to a hydrophobic pocket in EphA2 formed by Val72, Met73, Phe108, Pro109, and the Cys70-Cys188 disulfide bond) and Tyr4 (which is deeply buried in a hydrophobic pocket formed by EphA2 Ile64, Met66, Thr101, Val161, Ala190, and Leu192). These interactions of the peptide are similar to those observed for Phe111 and Phe114 of ephrin-A1 (12).

Additional hydrophobic interactions are formed by peptide Pro5 with EphA2 Phe156 and Val161, peptide Pro9 with EphA2 Met55 and peptide Met10 with EphA2 Leu54 and Tyr65. Key polar interactions include a salt-bridge between peptide Asp6 and EphA2 Arg159 as well as hydrogen bonds between the backbone of peptide Ser2 and the side-chain of EphA2 Arg103, the backbone of peptide Pro5 and EphA2 Asn57, the backbone of peptide Val8 and the backbone of EphA2 Gln56, and the backbone of peptide Met10 with the backbone of EphA2 Leu54 (Fig. 1H and S2A). We did not build peptide Met11 and Ser12 and the GSGSK linker in the structure because of their weak or absent electron density (Fig. S1A).

Consistent with the crystal structure data, the YSA1-10am (5) peptide lacking the residues not clearly visible in the crystal structure, and with an amidated C-terminus (am) to avoid the negative charge that is not present in Met10 of YSA (1), is as potent as YSA (1) (Fig. 2A and Table 1). This suggests that Met11 and Ser12 are not important for the interaction with EphA2, in agreement with data from a previous alanine scan (13).

Although the crystal structure shows that only the first 10 amino acids of YSA-GSGSK-bio (2) stably interact with the EphA2 LBD, we did observe additional electron density in the interface between the two EphA2 molecules of the asymmetric unit, which we interpret as the head group of the biotin from one of the two peptide molecules (Figs. 1A, E and S1A). The biotin interacts mainly with residues Leu44, Thr45 and Tyr48 of the other EphA2 molecule in the asymmetric unit (Fig. 1I, J and S1B, C).

Discrepancies between IC_{50} values measured by ELISA and K_d values measured by isothermal titration calorimetry (ITC) support a model where the biotin moiety of YSA-GSGSK-bio (2) interacts weakly with a second EphA2 molecule. In fact, YSA-GSGSK-bio (2) appears to be ≈5 fold more potent than YSA-GSGSK (3), which contains the linker but not the biotin, in ELISAs measuring inhibition of ephrin-A5 binding to the immobilized EphA2 extracellular region fused to the Fc portion of an antibody (Table 1 and Fig. 2A). This suggests that the GSGSK linker enables the biotin moiety of a peptide bound to the ephrin-binding pocket to reach a second EphA2 LBD when the receptor extracellular regions are immobilized in the wells in close proximity to each other. In contrast, weak biotin binding is not sufficient to affect the interaction between the peptide and the soluble monomeric EphA2 LBD measured in ITC experiments (Table 1). The biotin moiety of the YSA-K-bio (4) peptide lacking a linker may instead not effectively reach a second EphA2 molecule, resulting in a higher IC_{50} value in ELISAs (Table 1 and Fig. 2A).

**Modifications increasing the potency of YSA derivatives**

The other peptide previously identified by phage display, SWL (6) (9), has some residues in common with YSA (1) (Fig. 1G), suggesting that it may be possible to incorporate SWL (6) residues in YSA (1) to combine favorable features of both peptides. We found that replacement of Tyr1 and Ser2 of YSA (1) with SWL (6) residues Trp2 and Leu3, respectively, improves peptide potency by ~2-fold (Table 1 and Fig. 2A, compare WLAam (7) with YSA1-10am (5)), consistent with previous data (13). Since the previous alanine scan showed a favorable effect of replacing Ser1 in SWL (6) with Ala (14), we further modified WLAam (7) by adding an N-terminal βAla. This unnatural amino acid is similar to Ala, but should improve peptide resistance to proteolytic degradation by plasma aminopeptidases (4,15). This replacement indeed further increased potency by ~2-fold (Table 1 and Fig. 2A, compare βA-WLAam (8) with WLAam (7)). Replacement of Met10 with Tyr (the corresponding residue in SWL (6)), which based on
the crystal structure was predicted to increase hydrophobic interactions with EphA2, improved potency by another ~2 fold (Table 1 and Fig. 2A, compare βA-WLA-Yam (9) with βA-WLAam (8)).

The crystal structure of the βA-WLA-Yam (9) peptide in complex with the EphA2 LBD, which we solved at a resolution of 1.53 Å, confirms additional interactions with EphA2 that account for the increased potency (Figs. 3A,B, S1D and S2B). Most important among these are the extended hydrophobic interactions mediated by Trp2 and Tyr11. βAla1 does not significantly interact with EphA2, suggesting that the observed ~2-fold increase in potency due to addition of βAla1 might be caused by the elimination of the N-terminal positive charge of the Trp residue.

To investigate whether the addition of biotin might improve the potency of βA-WLA-Yam (9) in ELISAs, as it does for YSA-GSGSK (3), we extended the peptide by adding K-biotin to the peptide C-terminus directly or through linkers of different lengths in βA-WLA-YK-bio (10), βA-WLA-YSK-bio (11), βA-WLA-YGSK-bio (13) and βA-WLA-YGSGK-bio (14) (Table 1). The resulting peptides showed similar potency as βA-WLA-Yam (9) or βA-WLA-YSK (12), except for βA-WLA-YK-bio (10), which has no linker and is less potent (Table 1). To investigate whether the linker residues might not be optimal, we crystallized the βA-WLA-YSK-bio (11) in complex with EphA2 to gain information about the linker. However, the 2.03Å resolution structure we solved did not contain electron density for Lys13 and the stem of the biotin (Figs. 3C,D and S1). This is consistent with a linker that is not optimal and does not provide information useful for optimization. We therefore tried an additional peptide modification based on SWL (6).

Addition of Arg12, the residue at the corresponding position of SWL (6), did not further increase potency but improved peptide solubility in aqueous solutions (Table 1 and Fig. 2A, compare βA-WLA-YR (15) with βA-WLA-Yam (9)). Since Arg12 could introduce sensitivity to proteolytic degradation of C-terminal peptide extensions (16), we included a proline at position 13 because arginine followed by a proline is resistant to cleavage by trypsin-like proteases (16). We also included a lysine at position 14 to allow attachment of biotin or other tags (Table 1, βA-WLA-YRPK (16)). Remarkably, the addition of both Pro13 and Lys14 increased potency by ~7 fold (Table 1 and Fig. 2A, compare βA-WLA-YRPK (16) with βA-WLA-YR (15)). The binding affinity of βA-WLA-YRPK (16) for the EphA2 LBD measured by ITC is ~200 nM, a 50-fold improvement compared to YSA-GSGSK-bio (2) (Tables 1 and S2). The corresponding biotinylated peptide also exhibits much higher potency in ELISAs and much higher binding affinity measured by ITC (Tables 1 and S2; Fig. 2A, βA-WLA-YRPK-bio (17)). Replacement of Arg12 with Ser, to eliminate possible residual cleavage by trypsin-like proteases (16), yielded a peptide with only slightly decreased potency but with the disadvantage of being poorly soluble in aqueous solutions (Table 1, βA-WLA-YSPK-bio (18)). Thus, Arg12 does not seem to be critical for high potency (Fig. S3E,F), unlike what has been proposed for the corresponding Arg11 in another YSA derivative peptide named 135E2 (17).

The crystal structures of the βA-WLA-YRPK-bio (17) peptide in complex with the EphA2 LBD, solved in two different space groups at resolutions of 1.55 Å and 2.20 Å (Figs. 3E,F, S1F, S2D, S3 and Table S1), explain the increased potency of this peptide. In one of the four complexes observed in the two structures, Arg12 interacts with EphA2 residues Asp53 and Tyr48. Peptide Pro13 packs against peptide Tyr11 and helps fill the hydrophobic pocket lined by EphA2 Leu54. In addition, the structures suggest that C-terminal amidation of βA-WLA-YRPK (16) could further improve potency by eliminating the C-terminal negative charge positioned near the negatively charged Glu40 of EphA2 (inset in Fig. S5D). Indeed, the amidated βA-WLA-YRPKam (19) and βA-WLA-YRPKam-bio (20) peptides show a ~2-fold higher potency than the peptides with an unmodified C-terminus (Table 1 and Fig. 2A).

Importantly, YSA derivatives with greatly increased potency, such as βA-WLA-YRPK-bio (17), retain high specificity for EphA2 because even at concentrations 100-fold higher than the IC₅₀ value for inhibition of ephrin-A5-EphA2 binding they do not inhibit ephrin binding to any other Eph receptor (Fig. 2B and not shown).

**C-terminal biotin and negative charge potentiate the agonistic properties of YSA derivatives**

The YSA-GSGSK-bio (2) peptide has been previously shown to be an agonist that induces EphA2 tyrosine phosphorylation and downstream
signaling (9). The conserved tyrosine 588 (Y588) autophosphorylation site in the EphA2 juxtamembrane segment can be used as a marker indicative of EphA2 activation (18). Dose-response curves measuring Y588 phosphorylation of endogenous EphA2 expressed in PC3 prostate cancer cells stimulated with YSA-GSGSK-bio (2) yield an EC50 value in the low micromolar range (Fig. 4A). Surprisingly, the non-biotinylated version of the peptide induces only a very small increase in Y588 phosphorylation, which is only detectable at high peptide concentration (Fig. 4B).

The two most potent biotinylated peptides, βA-WLA-YRPK-bio (17) and βA-WLA-YRPKam-bio (20), are also agonists that induce high levels of EphA2 phosphorylation comparable to YSA-GSGSK-bio (2) (Fig. 4C,D and not shown). However, as expected given their much higher potency, these two peptides are active at nanomolar concentrations. These data suggest that the C-terminal biotin promotes the agonistic activity of YSA derivative peptides. The general role of the biotin in promoting EphA2 activation was confirmed by analysis of other biotinylated and non-biotinylated peptides, including the βA-WLA-YSK-bio (11) and βA-WLA-YSK (12) pair and biotinylated peptides with C-terminal linkers of different lengths (Table 1 and Fig. S4). We found that all nine peptides with biotin near the C-terminus strongly activate EphA2, and that the precise position of the biotin (relative to the N-terminal peptide residues interacting with the ephrin-binding pocket) does not have a strong effect on EphA2 activation in cells. This is in agreement with the crystal structures, in which the linker regions including the stem of the biotin are poorly defined (Figs. 1A,E,I and S1A,E), suggesting that these regions do not interact with EphA2. Thus, peptides with 1 to 7 residues between Pro10, which is present in all YSA derivatives, and the Lys-biotin residue can all efficiently activate EphA2 (Fig. S4A-F). In contrast, most non-biotinylated peptides either do not detectably activate EphA2 or are very weak activators that induce barely detectable EphA2 Y588 phosphorylation only when they are present at high concentrations (Table 1 and Fig. S4G-L).

The observation that C-terminal amidation of βA-WLA-YRPK-bio (17) increases its binding affinity and potency in ELISAs (Table 1) but decreases its agonistic potency in cells (Fig. 4C,D), suggests that the negative charge of the unmodified peptide C-terminus may play a role in EphA2 activation. Indeed, we found that the non-amidated βA-WLA-YRPK (16) has substantial ability to activate EphA2 in cells, even though the concentrations needed are about 10-fold higher than for the biotinylated peptide and the maximal Y588 phosphorylation induced by saturating peptide concentrations is about 40% lower (Fig. 4C,E). Interestingly, the C-terminally amidated version of the peptide essentially loses the ability to activate EphA2 (Fig. 4F), consistent with a role of the C-terminal negative charge for EphA2 activation even in the absence of biotin.

To determine whether the loss of the positive charge in the side chain of Lys14 may contribute to the agonistic properties of the biotinylated peptides, we examined a version of βA-WLA-YRPK (16) with acetylation of the Lys14 side chain (Table 1, βA-WLA-YRPacK (21)). We found that the acetylated peptide has only slightly increased agonistic ability compared to βA-WLA-YRPK (16) (Fig. 4E,G), suggesting that the Lys14 positive charge has only minor detrimental effects on EphA2 activation. This is consistent with a direct effect of the biotin in promoting EphA2 activation in cells.

The crystal structures of the peptides in complex with the EphA2 LBD provide further insights into the mechanisms underlying the agonistic properties of the peptides. In the structures of two of the three biotinylated peptides, we observed electron density for the biotin moiety of one of the two peptides in the asymmetric unit (Figs. 1A,E,I, 3C and S1A,E). In both structures, the biotin binds at the interface between two EphA2 LBD molecules and makes similar contact with EphA2 residues (Figs. 1I, S5A,C and not shown). This raises the possibility that in cells two biotinylated peptides may bridge two EphA2 molecules, with each peptide binding to the ephrin-binding pocket of an EphA2 molecule and the biotin-binding site of another EphA2 molecule (Fig. 1I). In addition, the C-terminus of βA-WLA-YRPK-bio (17) forms a salt bridge with Arg137 of the other EphA2 molecule in the asymmetric unit (Fig. S5D inset). The bivalent binding of biotinylated peptides could thus promote oligomerization, leading to reciprocal phosphorylation of EphA2 molecules. In βA-WLA-YRPK-bio (17) this could be further enhanced by
the C-terminal negative charge. Interestingly, the four different structures with the three biotinylated peptides show EphA2 dimers that interact through the “heterodimerization” or “dimerization” interface observed in crystal structures of the EphA2 extracellular region, which were also documented in the cellular context (19,20) (Fig. S5A,C,D,E,F). In contrast, in the structure with the non-biotinylated βA-WLA-Yam (9) peptide, the EphA2 molecules in the asymmetric unit interact differently, through an interface that is incompatible with the orientation of the receptors on the cell surface (Fig. S5B).

According to our model, a YSA derivative with biotin near the N-terminus should not efficiently activate EphA2 because such peptide would not simultaneously interact in the correct orientation with the ephrin-binding pocket of an EphA2 molecule and the biotin-binding site of another EphA2 molecule. Indeed, we found that the biotinylated K-bioA-WLA-YPKam (22) does not efficiently activate EphA2 in cells, despite its low nanomolar potency in ELISAs (Table 1 and Fig. 4H). Interestingly, ITC measurements revealed that this peptide has by far the highest EphA2 binding affinity among the YSA derivatives we engineered (Tables 1 and S2). Nevertheless, K-bioA-WLA-YPKam (22) remains highly selective for EphA2 (Fig. 2B).

We also monitored the effects of YSA derivative peptides on AKT S473 phosphorylation, since EphA2 activation induced by ephrin-A ligands is known to inhibit AKT phosphorylation and activation (1,21). This confirmed that the peptide agonists promote not only EphA2 activation but also downstream signaling (Figs. 4 and S4).

**The peptide agonists promote EphA2 oligomerization**

Using a quantitative FRET approach in live cells, we have previously shown that in HEK293 cells transiently transfected with EphA2, the receptor weakly dimerizes in the absence of a bound ligand (22) through an extracellular interface known as the “clustering” interface (19,20). Furthermore, we found that the YSA-GSGSK (3) peptide increases the formation of these EphA2 unliganded dimers (23,24). In contrast, the monomeric soluble form of ephrin-A1 induces the formation of EphA2 dimers that assemble through another extracellular interface known as the “dimerization” interface (19,20).

To understand the effects of the YSA derivatives with agonistic properties on the assembly of EphA2 oligomers (dimers and higher order clusters), we performed quantitative FRET experiments with HEK293 cells expressing EphA2 tagged at the C-terminus with a donor (mTURQ) or acceptor (EYFP) fluorescent protein and treated with the YSA-GSGSK-bio (2) peptide.

In these experiments, FRET was measured in 300-400 individual cells with different EphA2 expression levels. FRET efficiency, EphA2-mTURQ (donor) concentration, and EphA2-EYFP (acceptor) concentration in the plasma membrane were measured in micron-sized regions of the plasma membrane as described (23), and the data derived from the individual cells were combined to yield binding curves. A least square error analysis of the FRET data, performed as described previously (25), showed that these data are best described by a monomer-dimer association model rather than a higher order association model. Thus, the binding curves were fitted according to a monomer-dimer association model to determine the two-dimensional dissociation constants (25) (Fig. 5).

The FRET measurements revealed that YSA-GSGSK-bio (2) substantially increases the dimeric fraction of EphA2 wild-type (WT) on the cell surface, as expected (Fig. 5A,F). The peptide also promotes substantial dimerization of the EphA2 L223R/L254R/V255R triple mutant, which has impaired ability to assemble through the clustering interface (26) (Fig. 5B,F). In contrast, YSA-GSGSK-bio (2) does not increase dimerization of the EphA2 G131Y mutant, which has impaired ability to assemble through the dimerization interface (Fig. 5C,F). Comparison of the curves for EphA2 WT and the two mutants in the absence of YSA-GSGSK-bio (2) shows that only the L223R/L254R/V255R mutations impair dimerization (26) (Fig. 5D,F). In contrast, in the presence of the peptide the G131Y mutation strongly impairs dimerization while the triple mutation has a smaller effect.

The simple dimerization model cannot explain the observation that mutations in both interfaces decrease EphA2 association propensity. One possibility is that the biotinylated peptide mainly induces EphA2 oligomerization through the
dimerization interface (Fig. 5E,F), with additional low affinity interactions occurring through the clustering interface. Such dimer-of-dimers model is consistent with the FRET data.

Therefore, the FRET data are in agreement with the crystal structures, where the two EphA2 LBDs in the asymmetric unit interact through the dimerization interface when bound to one of the three biotinylated peptides (Fig. S5A,C,D,E) but not when bound to the non-biotinylated βA-WLA-Yam (9) (Fig. S5B). Thus, our FRET and X-ray crystallography data show that the peptide agonists induce EphA2 activation and downstream signaling by promoting the oligomeric assembly of receptor molecules on the cell surface through the dimerization interface.

**YSA derivatives lacking agonistic properties inhibit ephrin-induced EphA2 activation and signaling**

A number of the YSA derivatives appear to be essentially inactive in the assays measuring EphA2 activation in cells (Figs. 4 and S4). However, these peptides inhibit ephrin binding to EphA2 in ELISAs, some with low nanomolar potency (Table 1). To determine whether they can also inhibit EphA2 activation by ephrin-A ligands in cells, we examined the effects of the two most potent: βA-WLA-YRPKam (19) and K-bioA-WLA-YRPKam (22). This revealed that both peptides inhibit EphA2 Y588 phosphorylation induced by ephrin-A1 Fc and thus can serve as antagonists (Fig. 6). The two peptides also prevent the inhibitory effects of EphA2 activation on AKT. K-bioA-WLA-YRPKam (22) is more effective, consistent with its low nanomolar EphA2 binding affinity (Tables 1 and S2).

**Discussion**

We have engineered nanomolar peptide agonists as well as antagonists that target the ephrin-binding pocket of the EphA2 receptor tyrosine kinase by using as the starting points two peptides with high specificity for EphA2 but modest (micromolar) binding affinity. Iterative improvements guided by structural information obtained from four different peptides crystallized in complex with the EphA2 LBD have resulted in up to 350-fold increase in binding affinity. This was achieved with only small changes in the size of the optimized peptide agonists (from 1.99 kDa for YSA-GSGSK-bio (2) to 1.89 kDa for βA-WLA-YRPK-bio (17)) and antagonists (from 1.35 kDa for YSA (1) to 2.0 kDa for K-bioA-WLA-YRPKam (22)). We have preserved the AYPDSVP core of the YSA (1) peptide and replaced other residues to increase contacts with EphA2.

The extensive network of interactions with EphA2 involving almost all the residues of βA-WLA-YRPK-bio (17), which is documented in the crystal structure of the peptide in complex with the EphA2 LBD, is consistent with the incremental potency improvements we observed with each additional amino acid modification in the series of peptides we engineered. Interestingly, the binding of the YSA derivatives we have analyzed by ITC is characterized by unusually large decreases of both entropy and enthalpy, which are most pronounced for the high affinity βA-WLA-YRPK (16), βA-WLA-YRPK-bio (17) and βA-WLA-YRPKam (19) peptides (Table S2). This might be expected for linear peptides that are unstructured and highly flexible in solution (resulting in an unfavorable decrease in entropy upon binding EphA2) but in which many of the residues contribute to the binding interaction with the receptor (resulting in a favorable decrease in enthalpy) (27). The enthalpy component predominates in the best peptides we have developed, which exhibit low nanomolar affinity for EphA2. They therefore represent a marked improvement over the original YSA (1) and SWL (6) peptides and their derivatives of similarly low potency that have been used by many groups over the years (5).

Our most potent peptide agonists and antagonists retain the high selectivity for EphA2 over other Eph receptors that characterizes the YSA (1) and SWL (6) peptides. This would be expected because a number of the EphA2 residues that interact with βA-WLA-YRPK-bio (17), such as Asn57, Met66, Ser68 and Phe156, are only present in EphA2 among the Eph receptors (Figs. 3F and S2D). In addition the small Ser68 residue, which allows enough room for the peptide in the ephrin-binding pocket, is a much bigger Gln or Glu in all other Eph receptors.

It has been puzzling that monomeric peptides can function as EphA2 agonists, since Eph receptor activation is known to require oligomerization (2,3,5). Surprisingly, we found that a C-terminal biotin confers the ability to efficiently promote EphA2 activation and downstream signaling in...
cells. Several pieces of evidence presented here suggest that a likely explanation for the agonistic activity of the biotinylated YSA derivatives is that they function as bivalent ligands capable of bridging two EphA2 molecules to induce or stabilize EphA2 oligomeric assemblies. The X-ray crystal structures show distinct binding sites in the EphA2 LBD for the peptide N-terminal residues and the biotin, but do not conclusively show whether a peptide binds to two different EphA2 LBD molecules or to two binding sites within the same molecule, because of the lack of definition of the connecting residues. Nevertheless, the orientation of the biotin suggested by the shape of its electron density strongly suggests its interaction with a second EphA2 LBD molecule. Reciprocal binding of the N-terminal and biotin moiety of two peptides to two EphA2 molecules anchored on the cell surface could induce/stabilize receptor oligomers even if the biotin binds weakly to EphA2. According to the crystal structures, the EphA2 molecules would be brought together/stabilized by the biotinylated peptides through the extracellular “dimerization” interface (Fig. S5). Supporting this model, our FRET data show that the G131Y mutation, which weakens this interface (23,28), severely disrupts EphA2 oligomers induced by YSA-GSGSK-bio (2).

Further supporting the bivalent binding of the peptide agonists to two EphA2 molecules is our observation that the negative charge of the βA-WLA-YRPK-bio (17) C-terminus interacts with a neighboring EphA2 molecule in the crystal structure. We found that this negative charge is required for EphA2 activation in cells in the absence of the C-terminal biotin (Fig. 4E,F) as well as potentiates the effects of the biotin on EphA2 activation (Fig. 4C,D). Further evidence shows that the localization of the biotin near the peptide C-terminus is critical, since an N-terminal biotin does not confer agonistic properties (Fig. 4H). The bivalent binding involving biotin may be a distinctive feature of peptides targeting EphA2 because the three main EphA2 residues mediating biotin binding (Leu44, Thr45 and Tyr48), or homologous residues, are not all present in any other Eph receptor. In addition, biotinylated peptides binding to the ephrin-binding pocket of other Eph receptors do not function as agonists ((29,30) and unpublished data).

The bivalent binding model we propose for the biotinylated YSA-derived peptide agonists is analogous to that observed for the monomeric forms of the ephrin-A ligands. Although the ephrin-A5s are typically anchored on the cell surface through a glycosylphosphatidylinositol linkage (2,3), they can be released by metalloproteases as soluble monomeric proteins that also activate EphA2 signaling (31). Interestingly, monomeric ephrin-A1 has been previously shown to induce EphA2 dimers that use the dimerization interface (23), similar to what we observe with the biotinylated peptide agonist YSA-GSGSK-bio (2). In these dimers, each ephrin-A molecule uses two different interfaces to bind to two EphA2 molecules, inducing the assembly of “heterotetramers” containing two EphA2 and two ephrin-A molecules (19,28), similar to what we propose for the peptide agonists (Fig. 1J).

FRET measurements have also previously shown that EphA2 can form some dimers in cells even in the absence of a bound ligand, for example when it is highly expressed in transiently transfected HEK293 cells (26). Furthermore, FRET analysis of the EphA2 L223R/L254R/V255R clustering interface mutant implicated this interface in the assembly of the EphA2 unliganded dimers. We found that destabilization of the clustering interface slightly decreases EphA2 oligomers induced by YSA-GSGSK-bio (2), but to a much lesser extent than the G131Y mutation. We hypothesize that the binding of peptide agonists such as YSA-GSGSK-bio (2) can induce dimerization of EphA2 monomers through the dimerization interface as well as the assembly of larger EphA2 oligomers (perhaps tetramers) derived from pre-existing unliganded dimers, and that these oligomers would use both interfaces. In contrast, oligomers induced by monomeric ephrin-A1 are not significantly affected by the EphA2 clustering interface triple mutation (23), suggesting that the binding of monomeric ephrin-A1 disrupts the unliganded dimers whereas the binding of the peptides does not.

While non-biotinylated peptides can induce weak EphA2 tyrosine phosphorylation when present at very high concentrations, or when the receptor is highly expressed by transient transfection (24), at lower concentrations these peptides mainly function as antagonists that inhibit EphA2 signaling by an activating ligand such as
ephrin-A1 Fc. Interestingly, recent FRET studies have revealed that the non-biotinylated YSA-GSGSK (3) increases the proportion of EphA2 dimers assembled through the clustering interface. A possible explanation of this effect is that the peptide disrupts the recently described lateral interaction between the ephrin-binding pocket of an EphA2 molecule and the second fibronectin type III domain of a neighboring EphA2 molecule on the same cell surface (32). This “head-to-tail” interaction may not be detectable by our FRET approach due to excessive distance of the C-terminally attached fluorescent tags in this configuration of two EphA2 molecules. By disrupting the “head-to-tail” EphA2 complexes, a monovalent peptide targeting the ephrin-binding pocket of EphA2 would increase the proportion of receptor molecules available to dimerize through the clustering interface according to the law of mass action, through the same mechanism used by unliganded EphA2 (26).

While this work was in progress, Gambini et al. reported a series of peptide derivatives obtained through replacement of various YSA (1) residues with unnatural amino acids or chemical moieties (17). Although the monomeric YSA derivatives of Gambini et al. were presumed to be agonists, our experimental findings and mechanistic insights from high resolution structures strongly suggest that they might be antagonists instead. Further investigation of their biological function and structural characteristics can yield additional insights into the design principles for EphA2 modulators.

YSA derivatives represent a valuable resource to study EphA2 receptor signaling functions, by enabling potent and selective modulation of EphA2 activation. Many basic questions about EphA2 function remain unanswered, and the new peptides offer a unique opportunity to selectively induce or suppress EphA2 kinase-dependent signaling and investigate the biological outcomes of EphA2 modulation.

The YSA derivatives can also serve as starting points towards pharmacological leads that modulate EphA2 activity for different therapeutic applications. Given the complex signaling roles of EphA2, agents that either promote or inhibit EphA2 signaling can both have utility in the clinic, since both gain and loss of EphA2 kinase activity have been linked to human pathologies. The novel peptides we have developed are made from common building blocks (mostly natural amino acids) and are, therefore, straightforward to synthesize. The most potent derivatives have good solubility in aqueous solutions. To our knowledge, K-bioA-WLA-YRPKam (22), with a $K_d$ value of 27 nM, has by far the highest binding affinity among the EphA2-targeting peptides reported to date. In addition, dimerization and immobilization on the surface of nanoparticles can further increase EphA2 targeting potency through avidity effects, as well as confer or potentiate agonistic properties (5,14,33) (unpublished data). Although YSA derivatives appear to be rather stable in cell culture medium and plasma if they have an unnatural amino acid at their N-terminus (4,11,13), additional modifications will likely be needed to slow down the rapid excretion through the kidneys that is typically observed for small peptides in the blood circulation. Thus, further work will be needed to achieve a long peptide half-life in vivo in the blood circulation. Regardless, the engineering of the new peptides is an important first step on the path to new modulators of EphA2 that could be used in a clinical setting (3,7). The high-resolution structures presented here can guide the future optimization of these modulators.

Experimental procedures

Peptides

All the peptides were purchased from GenScript. Peptide identity and purity were documented by mass spectrometry and high-performance liquid chromatography (HPLC) (Table S3). The peptide solubility values in PBS, H2O or DMSO reported in Table 1 were determined by GenScript. For solubility tests, about 1.0 mg peptide were weighted in a centrifuge tube and 50 µl ultrapure water (or DPBS pH 7.1 ± 0.1 or DMSO) were added to the tube and the tube was shaken to see if the solution was clear and transparent. If the peptide was undissolved, additional solvent was added in 50 µl increments until the peptide was dissolved or the calculated peptide concentration was below 0.1 mg/ml. If the peptide was dissolved, the maximum concentration was calculated. If the peptide was not completely dissolved at a concentration below 0.1 mg/ml, the peptide was considered undissolved. An
ultrasonic instrument was used, if necessary, to help
dissolve the peptides.

All concentrated peptide stocks were prepared
in DMSO, with the exception of the stocks of YSA-
GSGSK-bio (2) used in some experiments, which
were prepared in H₂O. Peptide stocks were stored
frozen in aliquots at -80°C.

EphA2 LBD expression and purification

The DNA sequence coding for the EphA2
LBD (residues 28–200) with an additional C-
terminal Ala-6xHis-tag sequence was cloned into a
modified version of a pETNKI-LIC vector (34) that
encodes a N-terminal MASQGPG sequence in a
pET29 vector backbone. The EphA2 LBD was
expressed in E. coli Origami 2(DE3) (Novagen)
grown in 2xYT medium (BD Difco) at 20°C
overnight and purified using Ni-NTA agarose
(Qiagen) followed by size-exclusion chromatography on a Superdex 75 10/300 GL
column (GE Healthcare) equilibrated in 100 mM
NaCl, 10 mM HEPES pH 7.9. The EphA2 LBD was
concentrated to 5-7 mg/ml, flash frozen in aliquots,
and stored at -80°C.

Crystallization and structure solution

EphA2 LBD (7 mg/ml) was mixed with a 2-
fold molar excess of YSA-GSGSK-bio (2) peptide
dissolved to 2.9 mM in water, and initial crystals
were obtained with the Hampton Index HT screen.
Crystals were optimized with the Hampton
Additive Screen HT, changes in the ratio of protein
to precipitant volume, and two rounds of crush
seeding. Final crystals for structure solution were
obtained by mixing 2.8 µl protein solution with 1 µl
reservoir solution (0.09 M BIS-TRIS pH 5.5, 22.5%
w/v PEG 3,350, 3% w/v 6-aminohexanoic acid) and
equilibration against 50 µl reservoir solution at
20°C in sitting-drop MRC 48-well plates (Molecular Dimensions). Clusters of plate-shaped
crystals appeared overnight. Crystals were
cryoprotected by step-wise transfer to reservoir
solutions with 5-15% glycerol and cryocooled in a
nitrogen stream at 100 K. Diffraction data were
collected on a rotating anode X-ray generator
(Rigaku FR-E superbright) at 100 K and processed
in XDS (35) and with software from the CCP4 suite
(36). Phases were obtained using molecular replacement in Phaser (37) with chain A of PDB ID
3HEI (12) as search model. Model building and refinement were respectively performed in Coot
(38) and Refmac (39) or Phenix (40). The final
model was validated using MolProbity (41). Data
collection and refinement statistics are reported in
Table S1. All structural figures were generated
using PyMOL (Schrodinger, LLC). Peptide polder
OMIT electron density maps in Fig. S1 were
generated according to (42) and the LigPlot+
peptide–EphA2 interaction diagrams in Figs. S1C
and S2 were generated according to (43). Interaction surfaces were calculated using
PDBePISA (44).

For the other structures, peptides were dissolved in DMSO and added to the EphA2 LBD
at a 2-fold molar excess with a final concentration of less than 2% v/v DMSO. Crystals for two of the
other four EphA2–peptide complexes were grown
in the same conditions as described for the YSA-
GSGSK-bio peptide, whereas the two structures of
the EphA2–βA-WLA-YPK-bio (17) complex
formed in a similar condition with 0.09 M sodium-
acetate pH 4.5, instead of Bis-Tris pH 5.5 (Table
S1). The protein-to-precipitant drop ratio was in the
range of 1.8-2.6 µl protein to 1 µl precipitant for these
crystals. Despite these similarities, the
different complexes crystallized in different space
groups (Table S1), each with two EphA2-peptide
complexes in the asymmetric unit.

Isothermal titration calorimetry (ITC)

For ITC, all peptides were dissolved in DMSO
and both the EphA2 LBD and the peptides were
diluted to obtain a final buffer containing 9.5 mM
HEPES, pH 7.9, 95 mM NaCl, and 5% DMSO. The
experiments were carried out at 296 K (23°C) using
an ITC200 calorimeter (Microcal). Two-microliter
aliquots of a peptide solution were injected into the
cell containing 205 µl EphA2 LBD. 200-400 µM
peptide were titrated into 20-40 µM EphA2 LBD.
Experimental data were analyzed using the Origin
software package (Microcal). The integrated values
for the reaction heats were normalized to the
amount of injected peptide after blank subtraction.

ELISAs

To measure inhibition of EphA2-ephrin-A5
binding by the peptides, EphA2 Fc (#639-A2, R&D
Systems) was immobilized at 1 µg/ml on protein A
coated 96-well plates (#15132, Pierce-Thermo
Scientific) for 1 hour at room temperature in TBST
(150 mM NaCl, 50 mM Tris HCl pH 7.5, containing
0.01% Tween-20). The plates were washed 3 times
with TBST and incubated for 1 hour at room
temperature with 0.05 nM ephrin-A5 alkaline
phosphatase (AP) and different peptide concentrations in 40 µl TBST/well. After washing away unbound ephrin-A5 AP, the amount of bound ephrin-A5 AP was quantified by using p-nitrophenyl phosphate substrate (#34045, Pierce-Thermo Scientific) diluted in SEAP buffer (105 mM diethanolamine, 0.5 mM MgCl2, pH 9.8). Approximately 1 hour incubation at 37 °C, optical density at 405 nm (OD405) was measured, and OD405 from wells coated with Fc alone was subtracted as background.

**Immunoblotting**

PC3 prostate cancer cells (ATCC #CRL-1435) were cultured in RPMI 1640 (ThermoFisher Scientific/Gibco 11875-093) containing 10% fetal bovine serum with 1% Antibiotics Antimycotic solution (Corning #30-004-CI). Once they reached 70-80% confluence, the cells were starved overnight in the same medium without serum and then treated for 5 or 15 minutes with peptides. The cells were then rinsed once with cold PBS and collected in SDS-containing sample buffer. Lysates were heated at 95°C for 2 min, briefly sonicated and run on SDS-PAGE gels. After semi-dry transfer, the immobilon membranes were blocked with 5% bovine serum albumin in 0.1% Tween-20 in TBS (150 mM NaCl, 50 mM TrisHCl pH 7.5) for 1 hour and then incubated in the cold overnight with antibodies from Cell Signaling Technology recognizing EphA2 pY588 (#12677, at a 1:2,000 dilution), EphA2 (#6997 at 1:1,000 dilution), and AKT pS473 (#4060, at a 1:2,000 dilution). After washing, the membranes were incubated with an HRP-conjugated anti-rabbit secondary antibody (Invitrogen anti-rabbit A16110, at a 1:3,000 dilution). The chemiluminescence signal was captured using ChemiDoc Touch Imaging System (Bio-Rad), quantified using Image Lab (Bio-Rad) and analyzed using Prism software (GraphPad).

**Förster Resonance Energy Transfer (FRET)**

The human embryonic kidney cells (HEK293T) used in the FRET experiments were from ATCC (#CRL-3216). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, ThermoFisher Scientific, 31600034) with 10% fetal bovine serum (FBS, Hyclone, SH30070.03) at 37°C in the presence of 5% CO2.

For the FRET experiments, the cells were seeded (2.5 x 10^5 cells/dish) on collagen coated, glass bottom petri dishes (MatTek, P35GCOL-1.5-14-C). The cells were transfected at 60% confluency using Fugene HD (Promega, E2311) according to the manufacturer’s recommendations. The cells were co-transfected with different ratios of EphA2-mTURQ DNA and EphA2-EYFP. Co-transfected dishes contained a total of 2 µg DNA, with varying donor to acceptor ratios. Twenty-four hours after transfection, the cells were rinsed twice with phenol red and serum free medium, and starved in the same medium for 12 hours prior to imaging, as described (45).

Imaging was performed in the presence or in the absence of 50 µM YSA-GSG5K-bio (2) peptide using a two-photon laser (Spectra-Physics, Santa Clara) was used to generate femtosecond mode locked pulses, and two images were acquired for each cell: one where the donor is primarily excited (at 800 nm) and a second where the acceptor is primarily excited (at 960 nm) (46,47). The FSI-FRET method was used to measure FRET efficiency, donor (EphA2-mTURQ) concentration, and acceptor (EphA2-EYFP) concentration in small plasma membrane areas of each cell. To convert pixel level intensities of the images to concentrations, calibration solutions of purified fluorescent proteins were used. A detailed description of the FSI-FRET methodology has been published (25). Briefly, unique features of the methodology are: (i) Receptor concentrations in the cells vary over a wide range in the transient transfection experiments, (ii) Donor-labeled and acceptor-labeled receptor concentrations are directly measured in the plasma membranes, along with FRET efficiencies, and (iii) more than 300 cells are analyzed for each condition to construct a binding curve and determine the two-dimensional dissociation constant.

**Acknowledgments:** We thank Dave Boucher for generating the EphA2 LBD construct, Andrey Bobkov for performing the ITC experiments, Malgorzata Dobaczewska for technical assistance, and Denis Wirtz for providing HEK293T cells. This work was supported by NIH grants R01NS087070 (to EBP), R01GM131374 (to EBP and KH), and NCI Cancer Center Support Grant P30CA030199 (supporting SBP.
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Core Facilities), and Institutional funding (to EBP). Crystal structures and data are deposited in the Protein Data Bank (PDB) with accession codes 6NJZ: EphA2 LBD with YSA-GSGSK-bio (2) peptide; 6NK0: EphA2 LBD with βA-WLA-Yam (9) peptide; 6NK: EphA2 LBD with βA-WLA-YSK-bio (11) peptide; 6NK1: EphA2 LBD with βA-WLA-YRPK-bio (17) peptide in SG P1; and 6NK2: EphA2 LBD with βA-WLA-YRPK-bio (17) peptide in SG P6.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

References

1. Miao, H., and Wang, B. (2009) Eph/ephrin signaling in epithelial development and homeostasis. *Int J Biochem Cell Biol* **41**, 762-770
2. Pasquale, E. B. (2010) Eph receptors and ephrins in cancer: bidirectional signalling and beyond. *Nat Rev Cancer* **10**, 165-180
3. Barquilla, A., and Pasquale, E. B. (2015) Eph receptors and ephrins: therapeutic opportunities. *Annu Rev Pharmacol Toxicol* **55**, 465-487
4. Barile, E., Wang, S., Das, S. K., Noberini, R., Dahl, R., Stebbins, J. L., Pasquale, E. B., Fisher, P. B., and Pellecchia, M. (2014) Design, synthesis and bioevaluation of an EphA2 receptor-based targeted delivery system. *ChemMedChem* **9**, 1403-1412
5. Riedl, S. J., and Pasquale, E. B. (2015) Targeting the Eph system with peptides and peptide conjugates. *Current drug targets* **16**, 1031-1047
6. Boyd, A. W., Bartlett, P. F., and Lackmann, M. (2014) Therapeutic targeting of EPH receptors and their ligands. *Nat Rev Drug Discov* **13**, 39-62
7. Zhang, H., Li, Y., Wang, H. B., Zhang, A., Chen, M. L., Fang, Z. X., Dong, X. D., Li, S. B., Du, Y., Xiong, D., He, J. Y., Li, M. Z., Liu, Y. M., Zhou, A. J., Zhong, Q., Zeng, Y. X., Kieff, E., Zhang, Z., Gewurz, B. E., Zhao, B., and Zeng, M. S. (2018) Ephrin receptor A2 is an epithelial cell receptor for Epstein-Barr virus entry. *Nat Microbiol* **3**, 164-171
8. Noberini, R., Lamberto, I., and Pasquale, E. B. (2012) Targeting Eph receptors with peptides and small molecules: progress and challenges. *Semin Cell Dev Biol* **23**, 51-57
9. Koolpe, M., Dail, M., and Pasquale, E. B. (2002) An ephrin mimetic peptide that selectively targets the EphA2 receptor. *J Biol Chem* **277**, 46974-46979
10. Wang, S., Placzek, W. J., Stebbins, J. L., Mitra, S., Noberini, R., Koolpe, M., Zhang, Z., Dahl, R., Pasquale, E. B., and Pellecchia, M. (2012) Novel targeted system to deliver chemotherapeutic drugs to EphA2-expressing cancer cells. *Journal of medicinal chemistry* **55**, 2427-2436
11. Wang, S., Noberini, R., Stebbins, J. L., Das, S., Zhang, Z., Wu, B., Mitra, S., Billot, S., Fernandez, A., Bhowmick, N. A., Kitada, S., Pasquale, E. B., Fisher, P. B., and Pellecchia, M. (2013) Targeted Delivery of Paclitaxel to EphA2-Expressing Cancer Cells. *Clin Cancer Res* **19**, 128-137
12. Himanen, J. P., Goldgur, Y., Miao, H., Myshkin, E., Guo, H., Buck, M., Nguyen, M., Rajashankar, K. R., Wang, B., and Nikolov, D. B. (2009) Ligand recognition by A-class Eph receptors: crystal structures of the EphA2 ligand-binding domain and the EphA2/ephrin-A1 complex. *EMBO reports* **10**, 722-728
13. Mitra, S., Duggineni, S., Koolpe, M., Zhu, X., Huang, Z., and Pasquale, E. B. (2010) Structure-activity relationship analysis of peptides targeting the EphA2 receptor. *Biochemistry* **49**, 6687-6695
14. Duggineni, S., Mitra, S., Lamberto, I., Han, X., Xu, Y., An, J., Pasquale, E. B., and Huang, Z. (2013) Design and synthesis of potent bivalent peptide agonists targeting the EphA2 receptor. *ACS medicinal chemistry letters* **4**, 344-348
Nanomolar peptides that activate or inhibit EphA2 signaling

15. Lamberto, I., Lechtenberg, B. C., Olson, E. J., Mace, P. D., Dawson, P. E., Riedl, S. J., and Pasquale, E. B. (2014) Development and Structural Analysis of a Nanomolar Cyclic Peptide Antagonist for the EphA4 Receptor. ACS Chem Biol 9, 2787-2795

16. Pan, Y., Cheng, K., Mao, J., Liu, F., Liu, J., Ye, M., and Zou, H. (2014) Quantitative proteomics reveals the kinetics of trypsin-catalyzed protein digestion. Analytical and bioanalytical chemistry 406, 6247-6256

17. Gambini, L., Salem, A. F., Udompholkul, P., Tan, X. F., Baggio, C., Shah, N., Aronson, A., Song, J., and Pellecchia, M. (2018) Structure-Based Design of Novel EphA2 Agonistic Agents with Nanomolar Affinity in Vitro and in Cell. ACS Chem Biol 13, 2633-2644

18. Barquilla, A., Lamberto, I., Noberini, R., Heynen-Genel, S., Brill, L. M., and Pasquale, E. B. (2016) Protein kinase A can block EphA2 receptor-mediated cell repulsion by increasing EphA2 S897 phosphorylation. Mol Biol Cell 27, 2757-2770

19. Himanen, J. P., Yermekbayeva, L., Janes, P. W., Walker, J. R., Xu, K., Atapattu, L., Rajashankar, K. R., Mensinga, A., Lackmann, M., Nikolov, D. B., and Dhe-Paganon, S. (2010) Architecture of Eph receptor clusters. Proc Natl Acad Sci U S A 107, 10860-10865

20. Seiradake, E., Harlos, K., Sutton, G., Aricescu, A. R., and Jones, E. Y. (2010) An extracellular steric seeding mechanism for Eph-ephrin signaling platform assembly. Nat Struct Mol Biol 17, 398-402

21. Yang, N. Y., Fernandez, C., Richter, M., Xiao, Z., Valencia, F., Tice, D. A., and Pasquale, E. B. (2011) Crosstalk of the EphA2 receptor with a serine/threonine phosphatase suppresses the Akt-mTORC1 pathway in cancer cells. Cell Signal 23, 201-212

22. Singh, D. R., Cao, Q., King, C., Salotto, M., Ahmed, F., Zhou, X. Y., Pasquale, E. B., and Hristova, K. (2015) Unliganded EphA3 dimerization promoted by the SAM domain. Biochem J 471, 101-109

23. Singh, D. R., Kanvinde, P., King, C., Pasquale, E. B., and Hristova, K. (2018) The EphA2 receptor is activated through induction of distinct, ligand-dependent oligomeric structures. Commun Biol 1, 15

24. Singh, D. R., Pasquale, E. B., and Hristova, K. (2016) A small peptide promotes EphA2 kinase-dependent signaling by stabilizing EphA2 dimers. Biochim Biophys Acta 1860, 1922-1928

25. King, C., Raicu, V., and Hristova, K. (2017) Understanding the FRET signatures of interacting membrane proteins. J Biol Chem 292, 5291-5310

26. Singh, D. R., Ahmed, F., King, C., Gupta, N., Salotto, M., Pasquale, E. B., and Hristova, K. (2015) EphA2 Receptor Unliganded Dimers Suppress EphA2 Pro-tumorigenic Signaling. J Biol Chem 290, 27271-27279

27. Chodera, J. D., and Mobley, D. L. (2013) Entropy-enthalpy compensation: role and ramifications in biomolecular ligand recognition and design. Annu Rev Biophys 42, 121-142

28. Seiradake, E., Schaupp, A., del Toro Ruiz, D., Kaufmann, R., Mitakidis, N., Harlos, K., Aricescu, A. R., Klein, R., and Jones, E. Y. (2013) Structurally encoded intraclass differences in EphA clusters drive distinct cell responses. Nat Struct Mol Biol 20, 958-964

29. Murai, K. K., Nguyen, L. N., Koolpe, M., McLennan, R., Krull, C. E., and Pasquale, E. B. (2003) Targeting the EphA4 receptor in the nervous system with biologically active peptides. Mol Cell Neurosci 24, 1000-1011

30. Koolpe, M., Burgess, R., Dail, M., and Pasquale, E. B. (2005) EphB receptor-binding peptides identified by phage display enable design of an antagonist with ephrin-like affinity. J Biol Chem 280, 17301-17311

31. Wykosky, J., Palma, E., Gibo, D. M., Ringler, S., Turner, C. P., and Debinski, W. (2008) Soluble monomeric EphpinA1 is released from tumor cells and is a functional ligand for the EphA2 receptor. Oncogene 27, 7260-7273

32. Nikolov, D. B., Xu, K., and Himanen, J. P. (2014) Homotypic receptor-receptor interactions regulating Eph signaling. Cell Adh Migr 8, 360-365
Nanomolar peptides that activate or inhibit EphA2 signaling

33. Salem, A. F., Wang, S., Billet, S., Chen, J. F., Udompholkul, P., Gambini, L., Baggio, C., Tseng, H. R., Posadas, E. M., Bhowmick, N. A., and Pellecchia, M. (2018) Reduction of Circulating Cancer Cells and Metastases in Breast-Cancer Models by a Potent EphA2-Agonistic Peptide-Drug Conjugate. *Journal of medicinal chemistry* **61**, 2052-2061

34. Luna-Vargas, M. P., Christodoulou, E., Alfieri, A., van Dijk, W. J., Stadnik, M., Hibbert, R. G., Sahtoe, D. D., Clerici, M., Marco, V. D., Littler, D., Celie, P. H., Sixma, T. K., and Perrakis, A. (2011) Enabling high-throughput ligation-independent cloning and protein expression for the family of ubiquitin specific proteases. *J Struct Biol* **175**, 113-119

35. Kabsch, W. (2010) Xds. *Acta Crystallogr D Biol Crystallogr* **66**, 125-132

36. Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. M., Krissinel, E. B., Leslie, A. G., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterton, E. A., Powell, H. R., Read, R. J., Vagin, A., and Wilson, K. S. (2011) Overview of the CCP4 suite and current developments. *Acta Crystallogr D Biol Crystallogr* **67**, 235-242

37. McCoy, A., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J Appl Crystallogr* **40**, 658-674

38. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **66**, 486-501

39. Murshudov, G. N., Skubak, P., Lebedev, A. A., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F., and Vagin, A. A. (2011) REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr D Biol Crystallogr* **67**, 355-367

40. Adams, P. D., Afonine, P. V., Bunkoczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213-221

41. Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr* **66**, 12-21

42. Liebschner, D., Afonine, P. V., Moriarty, N. W., Poon, B. K., Sobolev, O. V., Terwilliger, T. C., and Adams, P. D. (2017) Polder maps: improving OMIT maps by excluding bulk solvent. *Acta Crystallogr D Struct Biol* **73**, 148-157

43. Laskowski, R. A., and Swindells, M. B. (2011) LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. *J Chem Inf Model* **51**, 2778-2786

44. Krissinel, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline state. *Journal of molecular biology* **372**, 774-797

45. King, C., Stoneman, M., Raicu, V., and Hristova, K. (2016) Fully quantified spectral imaging reveals in vivo membrane protein interactions. *Integr Biol (Camb)* **8**, 216-229

46. Biener, G., Stoneman, M. R., Acbas, G., Holz, J. D., Orlova, M., Komarova, L., Kuchin, S., and Raicu, V. (2013) Development and experimental testing of an optical micro-spectroscopic technique incorporating true line-scan excitation. *Int J Mol Sci* **15**, 261-276

47. Raicu, V., Stoneman, M. R., Fung, R., Melnichuk, M., Jansma, D. B., Pisterzi, L. F., Rath, S., Fox, M., Wells, J. W., and Saldin, D. K. (2009) Determination of supramolecular structure and spatial distribution of protein complexes in living cells. *Nature Photonics* **3**, 107-113
## Table 1. EphA2 Targeting Peptides

| Identifier number | Name$^1$ | Sequence$^2$ | $I_{C50}$ (nM) by ELISA$^3$ | $K_d$ (nM) by ITC$^4$ | $E_{max}$ pY588 | $E_{C50}$ / $I_{C50}$ pY588 (nM)$^4$ |
|------------------|---------|--------------|----------------------------|------------------------|-----------------|----------------------------------|
| 1                | YSA     | YSAYPDSVPMMK | 3,500 ± 300 (3)            | nd                     | +/–              | nd                               |
| 2                | YSA-GSGSK-bio | YSAYPDSVPMMKGGSK-bio | 790 ± 360 (40) | 9,800 ± 0 (2) | +++ | 3,400 [2,700-4,600] (5) |
| 3                | YSA-GSGSK | YSAYPDSVPMMKGGSK | 4,500 ± 1,000 (3)       | 8,000 ± 1,700 (3) | + | nd                           |
| 4                | YSA-K-bio | YSAYPDSVPMMK-bio | 2,500 ± 600 (3)        | nd                     | +++ | 9,000 [6,700-13,300] (3) |
| 5                | YSA1-10am | YSAYPDSVP | 3,400 ± 1,000 (3)       | 8,000 ± 1,700 (3) | + | nd                           |
| 6                | SWL     | SWL | 3,200 ± 500 (3)        | nd                     | +/-              | nd                               |
| 7                | WLAam   | WLAam | 1,500 ± 300 (8)       | nd                     | +/–              | nd                               |
| 8                | βA-WLAam | βA-WLAam | 840 ± 260 (11)        | nd                     | +/-              | nd                               |
| 9                | βA-WLA-Yam | βA-WLA-Yam | 410 ± 150 (9)        | nd                     | +               | nd                               |
| 10               | βA-WLA-YK-bio | βA-WLA-YK-bio | 730 ± 160 (3)        | nd                     | +++ | 1,400 [1,100-1,700] (3) |
| 11               | βA-WLA-YSK-bio | βA-WLA-YSK-bio | 300 ± 110 (6)       | nd                     | +++ | 1,400 [900-2,000] (6) |
| 12               | βA-WLA-YSK | βA-WLA-YSK | 380 ± 250 (3)       | nd                     | –               | nd                               |
| 13               | βA-WLA-YGSK-bio | βA-WLA-YGSK-bio | 400 ± 120 (3)       | nd                     | +++ | 2,200 [1,500-3,600] (2) |
| 14               | βA-WLA-YGSK-bio | βA-WLA-YGSK-bio | 330 ± 60 (3)       | nd                     | +++ | 1,000 [700-1,300] (5) |
| 15               | βA-WLA-YR | βA-WLA-YR | 390 ± 50 (3)        | nd                     | +               | nd                               |
| 16               | βA-WLA-YRPK | βA-WLA-YRPK | 55 ± 13 (6)        | 190 ± 0 (2)            | ++ | 300 [210-430] (5) |
| 17               | βA-WLA-YRPK-bio | βA-WLA-YRPK-bio | 41 ± 13 (10)       | 220 ± 10 (2)           | +++ | 34 [26-48] (7) |
| 18               | βA-WLA-YSPK-bio | βA-WLA-YSPK-bio | 62 ± 5 (3)        | nd                     | +++ | 37 [24-62] (4) |
| 19               | βA-WLA-YRPKam | βA-WLA-YRPKam | 19 ± 5 (6)        | 110 ± 0 (2)            | +/– nd | 3,200 [1,600-7,000] (7) |
| 20               | βA-WLA-YRPKam | βA-WLA-YRPKam | 18 ± 2 (3)        | nd                     | +++ | 100 [82-130] (7) |
| 21               | βA-WLA-YRPacK | βA-WLA-YRPacK | 82 ± 18 (3)       | nd                     | ++ | 190 [150-240] (3) |
| 22               | K-bioA-WLA-YRPKam | K-bioA-WLA-YRPKam | 13 ± 2 (3)       | 27 ± 4                 | +/– nd | 810 [440-1,500] (7) |

$^1$Peptides crystallized in complex with the EphA2 LBD are in bold; "am" indicates an amidated C-terminus.

$^2$YSA residues are in black, SWL unique residues are in orange and other residues are in red.

$^3$Averages ± SD are shown. The number of experiments is indicated in parentheses.

$^4$Averages with the 95% confidence intervals (in square brackets) are shown. The number of experiments is indicated in parentheses. $EC_{50}$ values for Nanomolar peptides that activate or inhibit EphA2 signaling.
Figure 1. The YSA-GSGSK-bio (2) peptide binds to the ephrin-binding pocket of EphA2 and mimics part of the ephrin-A1 G-H loop. (A) YSA-GSGSK-bio (2) peptide in complex with the EphA2 LBD. The LBD is in grey and the portion of the peptide visible in the crystal structure (YSAYPDSVPM, corresponding to residues 1-10) and the biotin are in dark red. The DE, GH and JK loops of EphA2, which line the ephrin-binding pocket, are in dark grey. EphA2 is shown in ribbon representation and the peptide in stick representation (PDBID: 6NJZ). (B) Ephrin-A1 G-H loop in complex with the EphA2 LBD (PDBID: 3HEI). EphA2 is shown as in A and ephrin-A1 is in pink. The GH loop of ephrin-A1, which binds to the ephrin-binding pocket, is indicated. (C) YSA-GSGSK-bio (2) peptide in complex with the EphA2 LBD as in panel A, but shown in surface representation and without the biotin. The peptide-EphA2 interface covers 777 Å². (D) Ephrin-A1 G-H loop in complex with the EphA2 LBD as in panel B, but shown in surface representation. Only ephrin residues of the GH loop interacting with EphA2 (residues 111-119) are shown. Their buried interface covers 603 Å². (E) YSA-GSGSK-bio (2) (the peptide backbone is shown as ribbon and side chains as sticks) in complex with the EphA2 LBD (in surface representation). N-ter indicates the N-terminus of the peptide. (F) Overlay of the structure of the YSA-GSGSK-bio (2) and the ephrin-A1 G-H loop in ribbon representation. The side chains of the Y₁SAY₄ sequence of the peptide and the F₁₁₁TPF₁₁₄ sequence of ephrin-A1 are shown as sticks to highlight the extensive overlap of these regions. (G) Alignment of the YSA (1) and SWL (6) peptides with the G-H loop of ephrin-A ligands. Asterisks mark the aromatic residues of the conserved ΩXXΩ motif. The peptide residues closely interacting with EphA2 in the crystal structure are colored in red. Ephrin-A1 residues shown in panels D and F are colored in pink. (H) Detailed interactions of YSA-GSGSK-bio (2) (sticks) with EphA2 (cartoon and surface with interacting residues shown as grey sticks). Polar interactions are indicated by green dashes. Key interacting residues are labeled. (I) Bivalent interaction of YSA-GSGSK-bio (2) with two EphA2 LBD molecules as observed in the crystal structure. The N-terminus of YSA-GSGSK-bio (2) molecule A interacts with the ephrin-binding pocket of EphA2 LBD molecule A while the biotin moiety interacts with residues in EphA2 LBD molecule B. Only the biotin of peptide molecule A is visible in the crystal structure. (J) Schematic representation of two EphA2 LBDs with two bound YSA-GSGSK-bio (2) peptides.
**Figure 2. Potency and selectivity of EphA2-targeting peptides.** (A) Representative ELISAs comparing the ability of the peptides to inhibit binding of ephrin-A5 fused to alkaline phosphatase (ephrin-A5 AP) to the immobilized EphA2 extracellular domain fused to the Fc portion of an antibody (EphA2 Fc). The graphs show averages ± SD from triplicate measurements from a representative experiment. IC\(_{50}\) values calculated from the fitted curves in each experiment are shown. Average IC\(_{50}\) values from multiple experiments are shown in Table 1. All concentrations are nM and the 100 nM peptide concentration is outlined in red. (B) Ephrin-A5 AP binding to the indicated EphA receptors and ephrin-B2 AP binding to the indicated EphB receptors in the presence of the indicated peptide, normalized to ephrin binding without peptide. The bars show averages ± SD from triplicate measurements (each measurement is shown as a dot).
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Figure 3. Structures of three YSA derivative peptides bound to the EphA2 LBD. (A) Structure of the EphA2 LBD (grey surface with the DE, GH and JK loops that line the ephrin-binding pocket in dark gray) with the βA-WLA-Yam (9) peptide (peptide backbone shown as purple ribbon and side-chains as sticks). The peptide-EphA2 interface covers 800 Å² (PDBID: 6NK0). (B) Detailed interactions of βA-WLA-Yam (9) peptide (magenta sticks) with the EphA2 LBD (interacting residues shown as grey sticks). Polar interactions are indicated by green dashes and key interacting residues are labeled. (C, D) Structure of the EphA2 LBD with the βA-WLA-YSK-bio (11) peptide (mint green) in representations similar to A and B. The peptide-EphA2 interface covers 822 Å² (PDBID: 6NKP). (E, F) Structure of the EphA2 LBD with the βA-WLA-YRPK-bio (17) peptide (orange) in representations similar to A and B. The peptide-EphA2 interface covers 878 Å² (PDBID: 6NK1). The loop containing Tyr48 is observed in two different conformations, suggesting that it is flexible and that Arg12 may induce a conformational change in this loop.
Figure 4. Different YSA derivative peptides vary greatly in their ability to induce EphA2 signaling. PC3 cells were treated for 15 min with different concentrations of the indicated peptides. The immunoblots show EphA2 autophosphorylation on tyrosine 588 (pY588, indicative of receptor activation), total EphA2 levels, and AKT phosphorylation on S473 (pAKT, indicative of AKT activation). Y, lysates from cells treated with 50 µM YSA-GSGSK-bio (2) and run on the same gel for comparison (lane Y is the same in Figs. 4E and F since the lysates shown in the two panels were run on the same gel). The molecular weight standards are labeled in panel A and their positions are indicated by dashes in the other panels. Maximal Y588 phosphorylation was similar for all biotinylated peptides, and thus the values were further normalized to the pY588 value obtained with the highest peptide concentration. pY588 values for the non-biotinylated peptides were normalized to the value obtained with 50 µM YSA-GSGSK-bio (2) in the same blot. pAKT values were further normalized to the highest value observed (without peptide or with low concentration of peptide). Calculated EC_{50} and IC_{50} values and maximal (E_{max}) pY588 values for the non-biotinylated peptides relative to YSA-GSGSK-bio (2) are also shown. The graphs show quantification of pY588 and pAKT from multiple blots (averages ± SE), normalized to total EphA2 levels. All concentrations in the graphs are nM and the 1,000 nM peptide concentration is outlined in red.
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Figure 5. YSA-GSGSK-bio (2) induces EphA2 dimerization through the “dimerization” interface. Dimerization curves obtained by fitting quantitative FRET data to a monomer-dimer model for comparison of EphA2 WT (A), L223R/L254R/V255 (B), and G131Y (C) with and without 50 μM YSA-GSGSK-bio (2) peptide. (D) Comparison of dimerization curves for EphA2 WT, EphA2 L223R/L254R/V255R and G131Y in the absence (D) and in the presence (E) of YSA-GSGSK-bio (2). (F) Two-dimensional dissociation constant values ($K_{\text{diss}}$) and dimerization free energy values ($\Delta G = -RT\ln(10^6/K_{\text{diss}})$) calculated from the curves shown in the other panels. Shown are the best fit values and the 68% confidence intervals. $K_{\text{diss}}$ values for all curves were compared to each other using one-way ANOVA followed by Tukey’s multiple comparison test; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, not significant.
Figure 6. YSA derivative peptides that do not activate EphA2 function as antagonists. PC3 cells were treated for 20 min with different concentrations of the indicated peptides and then activated with 0.1 µg/ml ephrin-A1 Fc for 10 min. The immunoblots show EphA2 auto-phosphorylation on tyrosine 588 (pY588, indicative of receptor activation), total EphA2 levels, and AKT phosphorylation on S473 (indicative of AKT activation). pY588 values were normalized to the value obtained in the same blot with ephrin-A1 Fc treatment in the absence of peptide. pAKT values were normalized to the value obtained in the same blot without any treatment. Calculated EC\textsubscript{50} and IC\textsubscript{50} values are shown. The graphs show quantification of pY588 and pAKT (averages ± SE), normalized to total EphA2 levels.
Engineering Nanomolar Peptide Ligands That Differentially Modulate EphA2 Receptor Signaling
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*J. Biol. Chem.* published online April 23, 2019

Access the most updated version of this article at doi: [10.1074/jbc.RA119.008213](https://doi.org/10.1074/jbc.RA119.008213)

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