Three Distinct Molecular Surfaces in Ephrin-A5 Are Essential for a Functional Interaction with EphA3

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Eph receptor tyrosine kinases (Ephs) function as molecular relays that interact with cell surface-bound ephrin ligands to direct the position of migrating cells. Structural studies revealed that, through two distinct contact surfaces on opposite sites of each protein, Eph and ephrin binding domains assemble into symmetric, circular heterotetramers. However, Eph signal initiation requires the assembly of higher order oligomers, suggesting additional points of contact. By screening a random library of EphA3 binding-compromised ephrin-A5 mutants, we have now determined ephrin-A5 residues that are essential for the assembly of high affinity EphA3 signaling complexes. In addition to the two interfaces predicted from the crystal structure of the homologous EphB2-ephrin-B2 complex, we identified a cluster of 10 residues on the ephrin-A5 E-helix, the E-F loop, the underlying H-beta-strand, as well as the nearby B-C loop, which define a distinct third surface required for oligomerization and activation of EphA3 signaling. Together with a corresponding third surface region identified recently outside of the minimal ephrin binding domain of EphA3, our findings provide experimental evidence for the essential contribution of three distinct protein-interaction interfaces to assemble functional EphA3 signaling complexes.

Signaling by Eph receptors (Ephs) and their cell surface-associated ephrin ligands forms an essential part of a highly conserved molecular mechanism coordinating cell migration and positioning during normal and oncogenic tissue development (1). In general, the path of Eph-expressing cells or axons is directed through contact-dependent cell-cell adhesion or repulsion (2), whereby competing interactions of neighboring Eph-expressing cells for ephrin targets govern the final cell position as the biological outcome (3). Many biological effects attributed to Eph function require concurrent “forward” signaling in Eph-expressing cells and “reverse” signaling in ephrin-expressing cells (4, 5). In contrast to the prototypical activation mechanism of receptor tyrosine kinases, Eph signaling is activated by the assembly of ephrin/Eph oligomers into large clusters. Ephs are composed of conserved structural modules. They include a unique N-terminal ephrin binding domain (6–8) forming a globular beta-barrel (9), a cysteine-rich linker and epidermal growth factor-like region, and two fibronectin type III repeats. The cytoplasmic part contains an uninterrupted, tyrosine kinase domain (10) and several protein-protein interaction modules, including Src homology 2-docking sites, a sterile-alpha-motif, and a C-terminal PDZ binding motif (11). Structural features broadly classify six glycoprophosphatidylinositol membrane-anchored ephrins as A-type, which “promiscuously” can bind and activate nine type-A Ephs, as well as three transmembrane ephrins as B-type, which contain conserved cytoplasmic domains and activate six type-B Ephs (12). It is now clear that this grouping is likely an oversimplification, and in particular, EphA4 and EphB2 bind and become activated by both A- and B-type ephrins (13, 14). This characteristic promiscuity of functionally relevant Eph/ephrin interactions is possibly because of the high structural conservation of Eph and ephrin binding domains. Crystal structures of the interacting domains of EphB2, ephrin-B2, and ephrin-A5 and of their complexes (8, 9, 14, 15) revealed that the initial 1:1 Eph/ephrin contacts (16) are provided by a deep Eph surface channel formed by beta-strands (D, E, G, J, M), which buries the extended, hydrophobic ephrin G–H loop (8). In the crystal structure of the EphB2-ephrin-B2 complex, a second lower affinity heterotetramerization interface facilitates formation of a 2:2 cyclic complex comprising two Eph/ephrin heterodimers (see Fig. 3). Although comparatively small, the tetramerization interface is critical for the assembly of stable, signaling-competent Eph clusters (17), and in agreement with its postulated role of providing subclass binding specificities (8), is not present in the structure of the EphB2-ephrin-A5 complex (14).

Although there is little doubt that the Eph/ephrin heterotetramers are the essential building block of Eph signaling complexes, downstream signaling requires the assembly of higher order oligomers (18). In vitro, this is routinely achieved through Eph activation by preclustered, tetravalent ephrin-Fc fusion proteins (19). The available crystal structures leave unclear how Ephs and ephrins assemble into the oligomeric signaling...
complexes that are required for biological responses (11, 18) and suggest the involvement of Eph/ephrin contact regions outside the crystallized domains. Indeed, earlier studies indicated the presence of ephrin-independent Eph/Eph contacts located C-terminally of the globular domain that are important for EphA3 function (7). Furthermore, a recent analysis of ephrin-A5 binding-compromised EphA3 mutants revealed, in addition to the two structurally defined ephrin binding sites, a third functional binding interface outside the crystallized domain (17). This site, although contributing only modestly to ligand binding, is essential for receptor phosphorylation, recruitment of signaling molecules, and downstream responses, supporting the notion that the tetrameric Eph/ephrin complex observed in the crystal structure is necessary (but not sufficient) for signaling. The position of the newly identified binding site within the cysteine-rich linker that connects the ephrin binding and Eph-Eph dimerization domains suggests that ephrin binding may cause a reorientation of Ephs that facilitates their assembly into oligomeric clusters (17).

We have now applied the same random mutagenesis approach previously used to identify ephrin-A5-interacting residues in EphA3 to assign the molecular determinants of ephrin-A5 that mediate EphA3 binding. In this case, the recently elucidated crystal structure of ephrin-A5 in complex with EphB2 (14) allowed the selection, structural alignment, and functional analysis of EphA3 binding-compromised ephrin-A5 mutants from a library of random point mutants spanning the whole N-terminal receptor binding domain of ephrins. Our analysis revealed a number of critical residues that confirm the two Eph binding sites shown in the crystal structure of the EphB2/ephrin-B2 complex (8). We also uncover a potential third Eph-interacting surface, the existence of which had been implied by the EphA3 mutagenesis study (17). This new site, which likely mediates Eph/ephrin clustering interactions, includes the protruding ephrin-A5 E α-helix and E–F loop, the underlying H β-strand, as well as the nearby B–C loop. Its location, between the dimerization and tetramerization sites, is consistent with a corresponding interaction surface on ephrin-A5 that is closely adjacent to the Eph globular domain. Kinetic and functional analysis of representative mutants confirms the notion that engagement of each of the three identified Eph binding sites of ephrin-A5 is required to elicit cell-morphological responses in EphA3-expressing cells.

**MATERIALS AND METHODS**

**Reagents**

The anti-EphA3 monoclonal antibody, IIIA4, and the affinity-purified rabbit polyclonal antibodies were previously described (16, 20). Other antibodies and reagents were from Transduction Laboratories (anti-CrkII), Upstate Biotechnology (anti-CrkII), Upstate Biotechnology (4G10), New England Biolabs (anti-human IgG horseradish peroxidase) and by BIAcore analysis using primers corresponding to nucleotides 344–362 and 862–879 and cloned into a modified (17) YEpFLAG-1 (Sigma) yeast expression vector. This expression vector positions the ephrin coding sequence between N- and C-terminal FLAG and Myc epitopes, respectively, allowing detection of the full-length protein with anti-FLAG antibody (M2, Sigma) and/or the anti-Myc antibody (9E10, a generous gift from Dr. D. Huang, Walter and Eliza Hall Institute, Melbourne, Australia).

**Random Mutagenesis**

Random point mutants of the ephrin-A5 extracellular domain were created using non-stringent PCR (17, 23). Conditions were optimized to yield 3–4 mutations or 1–3 amino acid changes/clone, so that 500 independent clones with some 1000 mutated amino acid residues provided a four-fold mutational coverage of the target sequence. A library of random point mutants was initially prepared in Escherichia coli. The plasmid cDNAs from pooled colonies (~17,000) were then transfected into the Saccharomyces cerevisiae strain BJ3505, and yeast colonies expressing mutant ephrin-A5 proteins were screened with monoclonal antibodies against the Myc (9E10) and FLAG (M2) epitopes and with EphA3-Fc as described previously (17). Individual filters from each set of replicates were probed with horseradish peroxidase-conjugated antimouse and anti-human IgG antibodies (Dako). Colonies showing reduced EphA3-Fc binding were recovered and DNA extracted for sequence analysis of the ephrin-A5 XhoI-BglII inserts.

**Site-directed Mutagenesis and Transient Protein Expression**

To determine relevant mutations in critical ephrin-A5 clones containing >1 amino acid change, individual mutations were introduced into the ephrin-A5/TEV-Fc mammalian expression vector by site-directed mutagenesis (QuickChange mutagenesis kit, Stratagene). Following transient transfection into HEK293T cells (FuGENE 6, Roche Applied Sciences), mutant and wild-type (w/t) ephrin-A5/TEV-Fc proteins were purified from culture supernatants using protein-A and ion exchange chromatography as described under “EphA3 and Ephrin-A5-Fc Fusion Proteins.” Protein expression was assessed by Western blot (anti-human IgG horseradish peroxidase) and by BIAcore analysis using sensor chips with parallel surfaces containing immobilized EphA3, anti-human IgG antibody, and single chain ephrin-A5.

**Surface Plasmon Resonance Analysis**

Analysis of protein interactions by surface plasmon resonance was carried out on a BIAcore 2000 biosensor (BIAcore) as described previously (17). Purified wild-type and mutant ephrin-A5 extracellular domain proteins were analyzed on parallel EphA3-Fc- and ephrin-A5-derivatized CM5 sensor chips (BIAcore 2000 optical biosensor, BIAcore AB, Sweden). The concentrations of high pressure liquid chromatography-purified proteins were determined from absorbance measurements at 215 nm. Samples of purified ephrin-A5 extracellular domain (62.5–1000 ng/ml) were analyzed in each assay. Interaction kinetics were evaluated from seven serial dilutions of each sample by Global Analysis using the BIAevaluation software (version 3.1).

### Table I

| FLAG/Myc epitope present | EphA3 binding | No. of mutations analyzed | % of total mutations | No. of colonies | % of total colonies analyzed |
|--------------------------|---------------|--------------------------|----------------------|----------------|----------------------------|
| +                        | +             | 77                       | 48.5                 | 40             | 48                         |
| +                        | +             | 52                       | 51.5                 | 44             | 52                         |

Yeast colonies transfected with YEpFLAG-1 encoding mutant, soluble ephrin-A5 proteins were selected by probing three consecutive nitrocellulose filters containing absorbed proteins from individual yeast colonies with anti-FLAG and anti-Myc antibodies, EphA3-Fc, and with appropriate secondary antibodies. Only clones secreting ephrins with N- and C-terminal FLAG and Myc epitopes, respectively, were further considered. Approximately similar numbers of colonies secreting EphA3-binding (+) or binding-compromised (−) mutant ephrins were analyzed. As some clones express ephrin-A5 proteins with more than one mutation (see “Materials and Methods”), the number of mutations is > the number of colonies.

acectate hollow fiber bioreactors (Cellex Biosciences, Minneapolis, MN) as described previously (14).
FIG. 1. Location of ephrin-A5 point mutants within the amino acid sequences of human A-type ephrins and ephrin-B2. a, the alignment gaps were adopted from a ClustalX (1.81) multiple sequence alignment of all known ephrin sequences, excluding *Drosophila* ephrin. Residues that are conserved in four or more ephrins are shaded gray. Ephrin-A5 point mutants not affecting binding and mutants that compromise

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The HEK293 cells that were stably transfected with full-length EphA3 have been described previously (24, 25). Following stimulation of these cells for 10 min with 1.5 µg/ml preclustered, w/t, or mutant ephrin-A5-Fc, Triton X-100 cell lysates were prepared, and even portions (50% v/v) were subjected to immunoprecipitation (IP) with anti-EphA3 monoclonal antibody IIIA4 affinity beads or anti-CrkII monoclonal antibody/protein-A-Sepharose as described previously (24). The anti-CrkII IPs and ½ of the anti-EphA3 IPs were probed with anti-EphA3 and anti-phosphotyrosine antibodies, respectively. To assess even gel loading, 8% (v/v) of each anti-EphA3 IP was probed with anti-EphA3 antibodies.

### RESULTS

#### Expression Library Screen for EphA3 Binding-defective Ephrin-A5 Mutants

To define critical Eph binding and signal initiation residues in ephrin-A5, we subjected most of the extracellular domain (residues Gln21–Ser199) to random mutagenesis, excluding only the N-terminal signal sequence and C-terminal unstructured residues. The experimental conditions, previously optimized to assign critical EphA3 surfaces (17), resulted in a four-fold mutational coverage of the ephrin-A5 sequence. This corresponds to a library of some 1000 mutants, which are randomly distributed across the entire target sequence. Only mutations that had generated full-length secreted proteins containing both the N-terminal FLAG and the C-terminal Myc tags (assyayed with anti-FLAG and anti-antibodies) were selected for further analysis (Table I). A total of 84 colonies with an average of two amino acid substitutions were sequenced. The EphA3 proteins secreted from these colonies were further assayed for binding of EphA3-Fc, revealing that 44 clones produced ephrins that retained EphA3 binding capacity, whereas 40 mutants had lost this capacity.

The EphA3 Binding Mutants Define Three Distinct Contact Surfaces

For an initial assessment of their functional relevance, we mapped these mutations onto the wild-type sequences of human A-type ephrins, as well as ephrin-B2 (Fig. 1a), all of which bind with varying affinities to EphA3 (16, 26). A total of 33 amino acid substitutions that affect the EphA3 binding map to the crystallized portion of ephrin-A5, whereby most (21/33) of the corresponding residues are conserved in five or all of the six aligned ephrins. Some 19 of these 21 mutations are located within (or immediately adjacent to) the proposed heterodimerization (12/33) or heterotetramerization (7/33) molecular surfaces. Importantly, a cluster of 5 mutations that compromised EphA3 binding was found within the highly conserved sequence motif YY(F/Y)I(I/S) in the “H” α-strand. This strand packs against the protruding E-helix and the long E–F loop that harbor 4 additional mutations. Together with the nearby B–C loop, containing one mutation, these secondary structure elements generate an ephrin surface positioned between the dimerization and tetramerization interfaces (Fig. 1b; see also Fig. 3), which is not in direct Eph contact in the Eph-B2/ephrin-B2 structure, (14). The clustered localization of these residues suggests that they define a distinct third interaction site of the EphA3-ephrin-A5 complex.

For comparison, we also mapped the 44 amino acid substitutions that did not alter the binding to EphA3. Only 9 of the 44 affected conserved residues (in at least four of the aligned ephrins), and 7 of these were conservative substitutions (Asp → Asn, Ser → Thr, Leu → Ile, or Phe → Tyr). Notably, mutations in 16 (of the 24) juxtamembrane ephrin-A5 residues did not affect EphA3 binding, confirming that this unstructured region is not involved in Eph/ephrin interactions.

#### Kinetic Analysis of Selected Ephrin-A5 Mutants

The mapping of the amino acid substitutions onto the ephrin-A5 structure in the context of known Eph/ephrin contacts allowed us to select six mutants for functional analysis, including representatives for each of the previously defined interfaces. We introduced these mutations into an expression vector encoding ephrin-A5 fused to the Fc part of human IgG and separated by an engineered TEV protease cleavage site, allowing production of monomeric ephrin-A5 as well as dimeric Fc-fusion derivatives from the same expression construct. For surface plasmon resonance analysis, monomeric ephrins were released by TEV cleavage (Fig. 2a, inset), and their binding to the EphA3 ectodomain or bovine serum albumin as non-relevant control proteins was tested on parallel BIACore sensor surfaces (Fig. 2a).

The kinetic analysis of the ephrin-A5 mutants revealed that a substitution of the G–H loop residue Thr125 with an alanine profoundly reduces the association rate and increases the dissociation rate of the EphA3/ephrin-A5 interaction (Table II), resulting in marginal micromolar affinity binding (Fig. 2a). This observation confirms previous findings, indicating that the major contribution to the binding affinity is provided by docking of the ephrin G–H loop into the ligand binding Eph channel at the heterodimerization interface (8, 17). By comparison, amino acid substitutions on the ephrin “D” β-strand (Gly366 → Arg and Lys368 → Asn), which binds along the upper convex surface of the receptor at the Eph/ephrin dimerization interface, affect EphA3/ephrin-A5 interaction less dramatically, yielding only a 3–4-fold decrease in the binding affinity (41 and 60 nM, respectively).

Not surprisingly, a similarly pronounced effect upon EphA3 recognition and binding was observed for the mutation Asn37 → Ile, located at the base of the ephrin A–B loop at the tetramerization interface. Its almost 10-fold affinity drop from 13 to 122 nM was primarily the result of a substantially reduced association rate of the mutant protein (2.8 × 10^4 M s^-1) compared with w/t ephrin-A5 (1.1 × 10^5 M s^-1, Table II).

Importantly, two of the point mutations, Phe137 → Ser and Ile139 → Thr, located between the previously characterized dimerization and tetramerization surfaces (see Fig. 1b), affect EphA3 binding to an extent comparable with that of the dimerization interface mutations Gly366 → Arg and Lys368 → Asn. Specifically, the mutated residues map to the conserved H-strand, where they mediate its packing against the “E” helix at the ephrin-A5 molecular surface. Phe137 and Ile139 are far from the dimerization (~20 Å of distance to the tip of the G–H loop) and tetramerization interfaces, and it is doubtful their mutation would affect the structure of these ephrin binding sites. More likely, they would only affect the local molecular surface and, in particular, the positioning and/or conformation of the protruding E–F loop and E helix relative to the underlying β-barrel scaffold. We postulate that this surface region, to...
together with the nearby B–C loop constitutes an additional (third) Eph/ephrin interface (Fig. 1b, green) that was not apparent in the crystal structures of the complex of their minimal interaction domains.

**Reduced EphA3 Binding Affinities Affect Ephrin-A5-induced Biological Responses**—We evaluated whether the reduced binding affinities of the ephrin-A5 mutants cause corresponding effects on EphA3 signaling and downstream cell-morphological responses by assaying typical ephrin-A5-triggered responses, including cell rounding and detachment (Fig. 2b, c), as well as EphA3 phosphorylation and recruitment of CrkII (Fig. 2d). Purified w/t or mutant ephrin-A5-Fc fusion proteins were used for these experiments, and their binding avidities to EphA3 were confirmed by plasmon resonance analysis (Table II).

As described previously (24), exposure of stably EphA3-transfected HEK293 (EphA3/HEK293) cells to preclustered ephrin-A5-Fc results in dose-dependent cell rounding (Fig. 2b) and detachment (Fig. 2c). By contrast, treatment of parallel cultures of EphA3/HEK293 cells with the most severely affected mutant, Thr122→Ala, changed neither the cell morphology nor cell adhesion, as compared with untreated control cells. In agreement, CrkII recruitment was not noticeable in these cells. Interestingly, EphA3 phosphorylation was only reduced but not ablated, in agreement with previous findings that EphA3 phosphorylation and Crk recruitment are not necessarily linked (24). Similarly, exposure of cells to ephrin-A5-Fc mutated in the tetramerization interface (Asn187→Ile) or the H-β-strand (Phe187→Ser) only weakly modulated cell morphology and cell adhesion. CrkII recruitment and EphA3 phosphorylation were affected for both mutants to similar degrees, suggesting a possible role also of the third EphA3 interface for the overall stability of the Eph/ephrin signaling complex. Together, these findings confirm the functional importance of the heterodimerization and the heterotetramerization interfaces for EphA3 binding and activation by ephrin-A5 and, in addition, suggest the essential involvement of a third previously unidentified Eph/ephrin contact site.

**DISCUSSION**

A unique feature of Eph signaling is the assembly of oligomeric signaling complexes (18, 21), which are required to translate cell surface densities of cognate ephrins into graded cell-morphological responses of Eph-expressing cells (2, 3). Crystallographic analysis of the Eph and ephrin interaction domains unraveled a heterotetrameric complex as an essential building block (8) but also suggested that additional molecular contacts, not apparent in the crystal structures, must be formed to assemble functional signaling clusters.

Here we have identified a cluster of 10 ephrin-A5 residues that are part of the E α-helix and E–F loop, the underlying H-β-strand, as well as the nearby B–C loop. Together they form a rugged, convex surface between the heterodimerization and the heterotetramerization domains, providing a third EphA3 interaction site that seems essential for EphA3 signal initiation. In our study, we exploited a library screening approach, designed previously to assign the molecular determinants of EphA3 required for high affinity ephrin binding and formation of functional signaling complexes (17). The strategy is based on the design of a library of mutations randomly distributed across the target sequence, providing an unbiased mutational coverage of the target protein. We argue that, in combination with antibody and protein binding assays as functional read-outs for secreted and (by inference) correctly folded mutant proteins with binding defects for the interaction partner, this approach ensures identification of all relevant interaction sites. Indeed, repeated amino acid substitutions of the same residue through-

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**Fig. 2. Functional analysis of selected EphA3 binding-compromised ephrin-A5 mutants.** Ephrin-A5-Fc fusion proteins (w/t or containing indicated amino acid substitutions) were purified on protein-A-Sepharose from supernatants of transiently expressed HEK293 cells and subjected to kinetic analysis by surface plasmon resonance (a), or to cell-based functional analysis (b–d). a, for BIAcore assays, TEV-cleaved proteins were purified by ion exchange high pressure liquid chromatography and analyzed by SDS-PAGE/silver staining (inset) prior to analysis on a sensor chip containing the EphA3 exodomain. BIAcore sensograms illustrate binding of ephrin-A5 (w/t or mutant as indicated) at the maximal tested concentration (50 nM, in color) and the calculated theoretical fit (stipped line) to a Langmuir interaction. b, EphA3-HEK293 cells cultured on fibronectin-coated glass slides were exposed to preclustered, w/t, and mutant ephrin-A5-Fc for 10 min. Cell-morphological and actin-cytoskeletal responses of fixed rhodamine/phalloidin-stained cells were monitored by confocal microscopy. Scale bar = 20 μm. c, adhesion of cells exposed to preclustered w/t ephrin-A5-Fc or mutants, as indicated, was determined from the area covered by cells in a minimum of three microscopic fields (selected by a blinded observer). Mean and standard deviation from a minimum of three independent experiments is shown. d, in parallel experiments, cells were exposed to w/t or mutant ephrin-Fc, equal portions of cell lysates subjected to IPs with anti-EphA3 or anti-CrkII antibodies and probed with anti-EphA3 and anti-phosphotyrosine antibodies as indicated under “Materials and Methods.” The immunoblots (W/B) from three independent experiments were scanned, and relative intensities (mean and S.D.) of individual bands are illustrated together with representative examples of the immunoblots. To assess even gel loading, a sample of the anti-EphA3 IPs was probed with anti-EphA3 antibodies as indicated in the bottom panel.
The assignment of seven function-affecting ephrin-A5 mutations to positions corresponding to the heterotetramerization interface of ephrin-B2 (Fig. 3) verifies that the high affinity ephrin-A5-EphA3 complex has the same heterotetrameric architecture as the structurally elucidated EphB2-ephrin-B2 complex. In agreement with this conclusion, the reduction in EphA3 binding affinity and biological activity observed for the heterotetramerization surface substitution, Asn\textsuperscript{87} \rightarrow Ile (\sim 9-fold reduced affinity, reduced EphA3 activation), and the dimerization surface substitution, Thr\textsuperscript{122} \rightarrow Ala (\sim 50-fold reduced affinity, loss of EphA3 activation), match the effects that would be expected for mutations of these low and high affinity binding sites, respectively. In this context, it is interesting to note that, despite only moderate effects on binding affinity, mutations in the newly identified (third) interaction site substantially reduce the capacity of ephrin-A5 to trigger EphA3 phosphorylation, Crk recruitment, and cell rounding.

We propose that the structural role of the newly identified interaction surface in ephrins is to bind the cysteine-rich domain of an Eph receptor from an adjacent Eph/ephrin tetramer (Fig. 3), thus assembling higher order signaling clusters. The fairly mild effect of the analyzed mutations on EphA3 binding suggests that engagement of this interface, similar to the tetramerization interface, relies on pre-existing, high affinity Eph/ephrin contacts. We speculate that the additional contact through the third interface triggers an EphA3 conformation that favors Eph/Eph oligomerization. It should be noted that it is also possible to model an Ephrephin tetrameric complex where all three contact sites are contained within the four interacting Eph and ephrin molecules. In this case, formation of higher order clusters would rely solely on Eph-Eph interactions, a notion supported by the finding that Eph receptors lacking the whole ephrin binding domain are effectively recruited into Eph signaling clusters (25). Thus, a detailed understanding of the precise molecular architecture of the functional Eph/ephrin signaling clusters requires crystallographic analysis of a complex between their complete extracellular domains.

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