Distinct Subdomains of the EphA3 Receptor Mediate Ligand Binding and Receptor Dimerization

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Eph receptor tyrosine kinases and their ligands (ephrins) are highly conserved protein families implicated in patterning events during development, particularly in the nervous system. In a number of functional studies, strict conservation of structure and function across distantly related vertebrate species has been confirmed. In this study we make use of the observation that soluble human EphA3 (HEK) exerts a dominant negative effect on somite formation and axial organization during zebrafish embryogenesis to probe receptor function. Based on exon structure we have dissected the extracellular region of EphA3 receptor into evolutionarily conserved subdomains and used kinetic BIACore analysis, mRNA injection into zebrafish embryos, and receptor transphosphorylation analysis to study their function. We show that ligand binding is restricted to the N-terminal region encoded by exon III, and we identify an independent, C-terminal receptor-dimerization domain. Recombinant proteins encoding either region in isolation can function as receptor antagonists in zebrafish. We propose a two-step mechanism of Eph receptor activation with distinct ligand binding and ligand-independent receptor-receptor oligomerization events.

The Eph family of receptors signal by binding cell-surface proteins known as ephrins. Cell contact is thought essential for this process, as only membrane-associated or artificially clustered forms of the ephrins, which mimic cell-cell apposition, can cause receptor transphosphorylation and activation (1–3). Inferred from sequence homologies, the structure of the Eph family is typified by an extracellular domain (ECD)1 comprising an N-terminal, cysteine-rich region, an EGF-like motif, and two fibronectin III repeats (4–6); however, the structural requirements and mechanism of receptor activation remain to be elucidated.

Studies measuring Eph/ephrin binding affinities using artificially clustered receptor ECDs suggest that Eph receptors and ephrins fall into the following two groups: EphA receptors interact preferentially with glycosylphosphatidylinositol-linked ephrins (ephrin-A), whereas those interacting preferentially with transmembrane ligands (ephrin-B) are called EphB receptors (7). Within each group, Eph receptors display cross-reactivity with multiple ephrins (1, 8–12). However, receptors and ligands within a class do not show equivalent affinities, but rather display a distinct ordering (3, 12–14). These findings are in keeping with the specialized roles in the development of the visual system, observed for EphA3 receptors (MEK4/CEK4) and ephrin-A2 (ELF1) and -A5 ligands (AL1/RAGS) (14–18). Very similar functional and structural characteristics have been described for the zebrafish ephrin zEphL4, suggesting it as the orthologue of ephrin-A5 (19).

The activation mechanisms for a number of other RTK subfamilies have been elucidated. These include dimerization/activation of individual class I receptor chains through conformational changes upon binding of soluble ligands, ligand-induced activation of preassociated, disulfide-stabilized heterotetrameric type II receptors, and ligand dimer-induced activation of type III receptors (reviewed in Ref. 20). Both monomeric and dimeric ligands are known to induce rapid receptor dimerization, and for some monovalent RTK ligands, the critical role of an intrinsic receptor dimerization interface for receptor activation and biological function has been demonstrated (21). Little is known about the composition of Eph-ephrin signaling complexes. Importantly, the demonstration of stable human EphA3-ephrin-A5 complexes in solution, revealing a strict 1:1 stoichiometry (3), the ability of soluble forms of ephrin-A5 to act as a signaling antagonist (2), and the notion of distinct signaling pathways for dimeric and higher oligomeric receptor complexes (22) suggest that models of dimerization/activation for other RTK may not adequately describe the formation of active signaling complexes for Eph receptors.

To understand further Eph/ephrin interactions and the mechanisms of Eph receptor activation, we dissected the human EphA3 (h-EphA3) ECD into structural subdomains, and through BIACore analysis we identified a unique N-terminal domain that is sufficient for h-ephrin-A5 (LERK7) binding. By adopting a dominant negative approach to disrupt zebrafish embryogenesis (23, 24), we confirmed the function of the ligand-binding domain in vivo. The same approach allowed characterization of a distinct C-terminal domain which mediates ligand-independent dimerization of the EphA3 ECD. The role...
of these two domains in receptor activation was confirmed in transphosphorylation assays. By taking into account the 1:1 stoichiometry of the h-EphA3/h-ephrin-A5 interaction (3), the “mass action” model suggested by Nakamoto et al. (17) from functional studies, the receptor dimerization mechanism of the PDGF receptors (25), and the data presented here, we propose a stepwise receptor activation mechanism. In our model, high affinity interactions between ephrin-A on the leading edge of migrating cells and the N-terminal ligand binding domain of EphA receptors on opposing cells leads to clustering of EphA receptors. A sufficiently high local concentration of ephrin-A will facilitate accumulation of receptors to a critical concentration that triggers oligomerization through their C-terminal receptor/receptor interaction domain and leads to receptor transphosphorylation and signal transduction.

MATERIALS AND METHODS

Isolation and Mapping of hEphA3 Genomic Clones—The h-EphA3 cDNA probes used to screen the human genomic library were PCR fragments amplified from plasmids containing full-length h-EphA3 cDNA. The primers used were probe A (spans 74–1161 bp as described by Wickes et al. (58) GTAGGATCCCTCTTCTAGGTCGACC and GTAGGGATCCGGCCTCCTGTTCCAA) and probe B (1052–1124 bp) GTAGGATCCGGCCTCCTGTTCCAA, and probe D (spans 909–1404 bp) GTAGGATCCCTCTTCTAGGTCGACC and GTAGGGATCCGGCCTCCTGTTCCAA, and probe D (spans 909–1404 bp) GTAGGATCCCTCTTCTAGGTCGACC and GTAGGGATCCGGCCTCCTGTTCCAA. PROEXCEL (ECL, Amersham).

PAGE and Western blot analysis using M2 anti-FLAG mAb and rabbit anti-mouse AP-tagged mAb for detection by enhanced chemiluminescence (ECL, Amersham).

Deletion mutants were purified on M2 anti-FLAG affinity columns and eluted with FLAG peptide according to the manufacturer’s instructions. Homogenous preparations (> 95% by SDS-PAGE and silver staining) were obtained by anion exchange (Mono Q, 5 × 50 mm, Pharmacia, Uppsala, Sweden) and size exclusion chromatography (Superose 12, 10 × 300 mm, Pharmacia, Uppsala, Sweden). The identity and concentrations of the purified h-EphA3 proteins in the fractions were confirmed by N-terminal amino acid sequence analysis and amino acid analysis, and where applicable, their native conformation was confirmed on the BIAcore as described (28).

Production of FLAG-tagged Ephrin-A5—h-Ephrin-A5 containing a N-terminal FLAG peptide was purified from transfected CHO cells and tested for its specific binding to sh-EphA3 as described (3).

Synthesis of sh-EphA3-derived Peptides—The peptides according to the amino acid sequence encompassing residues Glu1 to Glu13 (h-EphA3 1–31) of sh-EphA3 was assembled by solid-phase peptide synthesis according to standard protocols, purified by reverse phase-high pressure liquid chromatography, and their masses confirmed by mass spectrometry.

Analysis of the Interaction between sh-EphA3 Constructs and Ephrin-A5—The binding of various h-EphA3 constructs and derived peptides was analyzed on the BIAcore optical biosensor (Pharmacia Biosensor, Sweden) using purified h-EphA3 ECD or ephrin-A5–FLAG derivatized CM 5 sensor chips, and the interaction kinetics were determined as described (3). For the analysis of the binding of the h-EphA3 constructs and derived peptides to ephrin-A5, the CM 5 sensor chips were derivated by subtraction of the response on a parallel channel containing a non-relevant protein as described (28). The effect of h-EphA3-derived peptides on the interaction of h-EphA3 with ephrin-A5 was tested by incubating a constant concentration of the ligand with increasing amounts of peptide prior to analysis on a h-EphA3 ECD-derivatized sensorchip. The affinity surface was regenerated between subsequent injections of samples with a 35-μl injection of 50 mM 1,2-diethylamylene, 0.1% Triton X-100, followed by two washes with BIAcore running buffer (Hepes buffered saline, 0.005% Tween 20).

Fish Care and Embryo Collection—Wild type zebrafish were obtained from St. Kilda Aquarium (Melbourne, Australia) and were kept essentially as described (30). Embryos were obtained by natural spawning between a small number (4–10) of male and female fish. Embryos were removed from the spawning tanks within 20 min of fertilization, cleaned in system water, and transferred to the injection apparatus.

RNA Synthesis—Constructs equivalent to full-length h-EphA3 ECD (h-EphA3 I–VII) and h-EphA3 IV–VII were generated by PCR from the constructs described above. In each case the 5′ oligo was based on the interleukin-3 signal sequence and the 3′ oligos were as above except that the reverse primers were used to amplify and PCR products into the pSP64KT vector. mRNA from the h-EphA3 constructs and a control E-GFP cDNA construct were transcribed in vitro using the mMessage mMaker kit (Ambion, Texas) and resuspended in water at a concentration of 0.1 mg/ml in small aliquots. Integrity of the RNA was checked by denaturing gel electrophoresis of the resulting products. Immediately prior to injection, aliquots of h-EphA3 I–III, h-EphA3 I–IV, or h-EphA3 IV–VII cDNAs and E-GFP cDNA were mixed to a final concentration such that either 100, 10, or 1 pg of the receptor mRNA and 5 pg of the E-GFP mRNA were delivered to each embryo.

RNA Microinjection—Approximately 60 pl of RNA dissolved in water at various concentrations was injected into one, two, or four cell embryos under a Wild stereo microscope using Leitz micromanipulators (Leitz, Wetzlar, Germany) and compressed nitrogen. The needle was positioned under the blastoderm in the region of cytoplasmic streaming. Successful injection was judged in the first instance by a visible bolus of fluid in the embryo. Uptake and translation of mRNA by the embryo was measured by including 5 pg of mRNA encoding E-GFP as a marker in each injection. Injection of over 100 pg of E-GFP mRNA per embryo does not cause developmental defects. The translation of the injected h-EphA3 mRNA was measured at intervals during embryogenesis by Western blotting and BIAcore analysis.

Western Blot and BIAcore Analysis of Zebrafish Lysates—Ten embryos per sample were lysed in embryo lysis buffer (0.1 ml, 25 mM Tris-HCl, pH 7.4, 0.5 mM NaCl, 1% Triton X-100), and the lysate was cleared by centrifugation (10 min, 1 × 10^6 g) and stored at −80 °C until use. For comparison, CHO cell conditioned h-EphA3 I–VII and sh-EphA3 I–VII constructs were cloned into pSG5 (Stratagene Cloning Systems, La Jolla), mouse in FIX II vector (Stratagene Cloning Systems, La Jolla), and h-Ephrin-A5 containing an N-terminal FLAG peptide was purified from transfected CHO cells and tested for its specific binding to sh-EphA3 as described (3).

Production of FLAG-tagged Ephrin-A5—h-Ephrin-A5 containing a N-terminal FLAG peptide was purified from transfected CHO cells and tested for its specific binding to sh-EphA3 as described (3).
Distinct Ligand Binding and Dimerization Domains in Eph RTK

We describe the isolation of a novel Eph family member, EphA3, and the characterization of its extracellular domain (ECD). The EphA3 ECD consists of seven exons (I–VII), each containing the EGF-like domain (EGFL) motif (CAVXYXYC) characteristic of the EGFL family of proteins. Sequence alignment suggested no significant homology to other proteins, and a structural basis of the EphA3 receptor/ephrin-A5 interaction and receptor activation was analyzed using the function of isolated subdomains derived from the complete EphA3 ECD.

Expression of Receptor ECD Subdomains—To analyze the structural basis of the EphA3 receptor/ephrin-A5 ligand interaction and receptor activation, we analyzed the function of isolated subdomains derived from the complete EphA3 ECD.

RESULTS

Expression of Receptor ECD Subdomains—To analyze the structural basis of the EphA3 receptor/ephrin-A5 ligand interaction and receptor activation, we analyzed the function of isolated subdomains derived from the complete EphA3 ECD.
For the design of stable subdomains, we analyzed the h-EphA3 gene structure and found that exon-intron boundaries of h-EphA3 ECD genomic clones aligned with clones of the mouse EphA4 (SEK1), EphA5 (BSK), and EphA1 (ESK) genes. Together with data on the chicken EphB2 (CEK5) gene (33) and splice variants of other Eph-like RTK (26, 34), this suggests a highly conserved exon structure within the Eph subfamily (Fig. 1a). Comparison of ECD sequences of h-EphA3 and its mouse (MEK4) and chicken (CEK4) homologues (26) demonstrates the highest amino acid sequence identity (99.5 and 98.3%, respectively) is found in the exon III-encoded domain (Fig. 1b). The structure of the domain encoded by exons II and III was analyzed in detail, addressing previous reports that this region consisted of an N-terminal Ig-like domain and a C-terminal cysteine-rich region. Sequence data base comparisons and alignment of the h-EphA3 exon II and III sequences with known C1, C2, and V set Ig domains and a number of EGF domains using the ALIGN program (35) showed features of the C-terminal half of an EGF domain in the most C-terminal exon III-encoded region, but no homology more N-terminally, whereas homology to an Ig-like motif was not found within this region.

The defined exon boundaries were used to demarcate cDNA domain deletion mutants of the h-EphA3 ECD for expression as FLAG epitope-tagged proteins in CHO cells (Fig. 1c) and, following in vitro transcription into mRNA, for expression in zebrafish embryos (Fig. 2). The proteins are identified throughout by roman numbers according to their corresponding exons. The exon III- and exon IV-encoded portions of the h-EphA3 receptor correspond to domains described as globular and cysteine-rich domains in a recent report on the functional dissection of the EphB2 receptor (36). Thus, the FLAG fusion proteins h-EphA3 I–III, h-EphA3 I–IV, and h-EphA3 VI–VII directly relate to the EphB2-alkaline phosphatase (AP) fusion proteins “280-AP,” “331-AP,” and “CEK5ΔGlob-AP” (which includes an exon III-encoded part), respectively (36).

We obtained homogenous proteins from CHO cell supernatants by anti-FLAG affinity and size exclusion chromatography; SDS-PAGE and silver staining of the purified proteins revealed apparent molecular sizes as predicted from the amino acid sequence and putative glycosylation sites (Fig. 1c). Doublet protein bands for h-EphA3, h-EphA3 I–VII, and h-EphA3 IV–VII are likely due to the glycosylation heterogeneity of these proteins; h-EphA3 derived from transfected glycosylated band for h-EphA3, h-EphA3 I–VII, and h-EphA3 I–III, and h-EphA3 IV–VII were immunoprecipitated with anti-FLAG mAb (M2) agarose and analyzed by Western blots with anti-FLAG mAb (M2) agarose and analyzed by Western blots with anti-FLAG mAb, visualized by enhanced chemiluminescence (lanes 1–3). Zebrafish embryos injected with 10 pg of either h-EphA3 I–VII, h-EphA3 I–III, or h-EphA3 IV–VII mRNA were lysed after 5 or 10 h (10 embryos/0.1 ml) and analyzed in parallel lanes of the gel (lanes 4–9). Lanes 1 and 2, CHO cell h-EphA3 I–VII and h-EphA3 IV–VII, 15 and 10 ng, respectively; lane 3, 10 ng of CHO cell h-EphA3 I–III; lanes 4 and 5, h-EphA3 I–IV; lanes 6 and 7, h-EphA3 I–VII RNA injections, 5 and 10 hpf; lanes 8 and 9, h-EphA3 I–III RNA injections, 5 and 10 hpf, parallel samples of zebrafish lysates from h-EphA3 I–III RNA-injected embryos (100 pg/embryo), collected 5, 10, 15, and 24 h post-fertilization were extracted on M2 agarose, and the FLAG peptide eluate was analyzed on a BIAcore sensorchip which had been derivatized with the conformation-specific anti h-EphA3 mAb IIIA4. The BIAcore response was used to estimate the h-EphA3 I–III abundance by comparison with identically treated samples of CHO cell-derived h-EphA3 I–III at a known concentration.

**Identification of the EphA3 Ligand-binding Domain**—In order to characterize the ligand-binding region of the h-EphA3 receptor, the individual binding affinity of each of the ECD subdomains for the h-EphA3 ligand was assayed using an h-ephrin-A5-derived BIAcore sensorchip. A kinetic analysis demonstrates high affinity interactions for the binding of h-EphA3 I–VII (the FLAG-tagged version of the h-EphA3 ECD encoded by exons I–VII), h-EphA3 I–IV, and h-EphA3 I–III, with affinities in the same range (K<sub>d</sub> 18–72 nM, Fig. 1, d and e) reported previously for the binding of h-ephrin-A5 to sensor chip-immobilized h-EphA3 (3). No binding of h-EphA3 IV–VII was observed at any of the tested concentrations (16–500 nM), localizing the ligand binding site to the exon I–III-encoded N terminus of h-EphA3. Very similar apparent dissociation constants for the h-EphA3 ECD and h-EphA3 I–VII (72 ± 15 and 62 ± 12 nM, Fig. 1e) suggest that an N-terminal addition of the FLAG peptide has no effect on the interaction between h-EphA3 and its ligand. Substantially lower dissociation constants (i.e., higher affinities) of 18–29 nM due to increased association rate constants (Fig. 1d) were observed for the h-EphA3 subdomain constructs h-EphA3 I–IV and h-EphA3 I–III. In solution competition with an exons I- and II-encoded synthetic h-EphA3<sub>1–31</sub> peptide, and with the FLAG-tagged h-EphA3 IV–VII construct, did not inhibit the receptor/ligand interaction at concentrations up to 10 μM, whereas addition of h-EphA3 I–VII or h-EphA3 I–III resulted in a dose-dependent reduction of the BIAcore response (Fig. 1f). Taken together, these results imply that the cysteine-rich domain encoded by exon III of h-EphA3 is necessary and sufficient for ligand binding.

**Soluble EphA3 Ligand Binding Subdomain Induces a Dominant Negative Phenotype in Zebrafish**—To confirm the binding...
studies we analyzed the effect of ectopic expression of receptor constructs h-EphA3 I–III (ligand binding domain) or h-EphA3 IV–VII, which shows no ephrin-A5 binding, on zebrafish development. We have recently characterized developmental defects in the formation of somite boundaries in zebrafish embryos induced by injection of either soluble h-EphA3 ECD or h-ephrin-A5 as signaling antagonists. A similar dominant negative approach has been used earlier to evaluate the role of EphA4 signaling in forebrain and hindbrain formation (23). These authors expressed kinase domain deletion constructs of m-EphA4 in zebrafish embryos to disrupt the function of the endogenous orthologue by forcing heterodimerization with the exogenous mutant receptor (23, 24). In a modification of this approach, we anticipated that expression of the h-EphA3 ligand binding domain during zebrafish development should be sufficient to block endogenous receptor/ligand interactions by competing for binding to endogenous ligand. On the other hand, expression of the regions of the ECD that cannot bind ligand should not mediate these antagonistic effects.

Thus, the effects of constructs h-EphA3 I–VII, containing the entire ECD, h-EphA3 I–III, encompassing the ligand binding domain, and h-EphA3 IV–VII, encompassing the remainder of the ECD (and incapable of h-ephrin-A5 binding) on zebrafish development were analyzed. The corresponding mRNAs, denoted h-EphA3 I–VII RNA, h-EphA3 I–III RNA, and h-EphA3 IV–VII RNA, respectively, were introduced into zebrafish embryos by microinjection. A widespread distribution of the exogenous proteins throughout the embryo was observed as indicated by the uniform expression pattern of the GFP protein (Fig. 3f). FLAG epitope-containing proteins with the same molecular weights as the corresponding proteins expressed in CHO cells were detected by Western blot (Fig. 2a) at similar abundance of all three constructs in the embryos at 5 and 10 hours post-fertilization. This suggests equivalent expression of all mRNAs throughout the time frame of the experiment. By using the native conformation-specific, anti-h-EphA3 mAb, IIIA4 (28, 29), we quantitated the expression level of exogenous, biologically active h-EphA3 I–III by BiAcore analysis of lysates from zebrafish embryos, which had been injected with h-EphA3 I–III RNA (Fig. 2b). As shown previously for h-EphA3 I–VII and h-ephrin-A5, there was an initial steep rise in expression h-EphA3 I–III leading to a plateau after 15 h (Fig. 2b).

Embryos injected with 1 pg or 10 pg of sh-EphA3 I–III RNA per embryo developed a syndrome indistinguishable from that described for the full-length h-EphA3 I–VII RNA (Fig. 3c and d). The defects noticed in embryos between 11 and 15 h post-fertilization (hpf), by comparison with non-injected control embryos (Fig. 3a and b), included most prominently a disruption of somite boundaries (Fig. 3a and c and d) coincident with a reduced height of the dorsal axis from the surface of the yolk cell (Fig. 3c). Furthermore, a disorganized anterior neuraxis and retarded tailbud development were observed (Fig. 3c). To monitor nonspecific effects on embryogenesis, which may have been caused by injection of mRNA and expression of foreign protein, embryos were injected with E-GFP or the soluble, FLAG-tagged ECD of deleted in colon cancer (DCC) (38), a major guidance receptor known to be involved in embryogenesis. Although expression of the soluble FLAG-tagged DCC construct induces a defined nerve guidance defect in Xenopus embryos, and despite high levels of exogenous protein expression, we could not detect any developmental defects in E-GFP or DCC

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5 J. M. Gad and H. M. Cooper, personal communication.
brain (form tight bands marking forebrain (mid- and hindbrain displaced from the midline. An intact 12 hpf showing pax-b and reveal a distinctly twisted midline. gest that the somite boundaries are out of register across the midline present anteriorly. The myoD expressing cells are also symmetrically distributed lateral to the pax-b pressing

injected with h-EphA3 IV–VII RNA, demonstrating normal expression of myoD repeated pattern of myoD in the paraxial mesoderm. A regular, serially repeated pattern of myoD staining illustrates the somites. Cells expressing pax-b, hlx-1, and krox20 have migrated toward the midline and form tight bands marking forebrain (hlx-1, midbrain (pax-b), and hindbrain (krox20). c and d, h-EphA3 I–III RNA (10 pg)-injected embryo at 12 hpf showing normal expression of hlx-1 in the ventral forebrain, pax-b in the midbrain, krox20 in rhombomeres 3 and 5 of the hindbrain and myoD in the paraxial mesoderm. A regular, serially repeated pattern of myoD staining illustrates the somites. Cells expressing pax-b, hlx-1, and krox20 have migrated toward the midline and form tight bands marking forebrain (hlx-1, midbrain (pax-b), and hindbrain (krox20). c and d, h-EphA3 I–III RNA (10 pg)-injected embryo at 12 hpf showing normal expression of hlx-1, pax-b, krox20 (c), and myoD (d). The distinctive twist in the neuraxis of affected embryos is missing and the pax-b and krox20 expressing cells are also symmetrically distributed lateral to the midline.

mRNA-injected embryos as judged by the criteria of our experiments (Fig. 3f and data not shown).

The similarity of the phenotype due to h-EphA3 I–VII RNA and h-EphA3 I–III RNA injection was also evident by analysis of marker gene expression; in situ hybridization with probes to hlx-1 (39), paxb (40), krox20 (41), and myoD (42) revealed abnormal patterns consistent with the morphological defects observed in the live embryos (Fig. 4, c and d). In particular, myoD-expressing cells were disarrayed along the paraxial mesoderm indicating a disruption of somite formation, giving the track formed by myoD-expressing, adaxial mesoderm cells adjacent to the midline a distinctive twist (Fig. 4c). A single axially located stripe of hlx-1 expression suggested an intact ventral forebrain region. Non-injected control embryos (Fig. 4, a and b) or embryos injected with E-GFP alone (not shown) did not show these defects in the expression of myoD. In contrast, no apparent developmental defects were detected in embryos injected with either 1 or 10 pg of h-EphA3 IV–VII RNA per embryo, either by gross morphological criteria (Fig. 3e) or by analysis of marker gene expression (Fig. 4, e and f). Ubiquitous expression of the co-injected E-GFP mRNA (Fig. 3f) and Western blot analysis (Fig. 2a) during the period of development under analysis indicated that the protein was both widely and highly expressed.

The ability of h-EphA3 I–III but not of h-EphA3 IV–VII to mimic the developmental disruption caused by h-EphA3 I–VII implies that the subdomain responsible for mediating the specific, dominant negative effect of the EphA3 ECD on zebrafish development is encoded in the first three exons. This finding is consistent with assignment of the ligand-binding domain to exon III by BIACore analysis of ligand binding affinities.

High Concentration of C-terminal Domain Protein Induces Disruption of Somite Formation—We observed a linear, dose-dependent increase in the number of affected embryos when the amount of injected h-EphA3 I–III RNA was increased from 1 to 100 ng per embryo, whereby the increase in defective embryos paralleled the increase in concentration of the expressed protein (Fig. 5a). As before, only a small number of defective embryos were observed at low and moderate concentrations of h-EphA3 IV–VII RNA (1 and 10 pg/embryo, Fig. 4, e and f and Fig. 5a), whereas at high concentrations, the proportion of affected embryos injected with sh-EphA3 IV–VII RNA was similar to the proportion of defective sh-EphA3 I–III RNA-injected embryos (Fig. 5a, 100 pg/embryo). Importantly, the phenotype of animals injected with 100 pg of sh-EphA3 IV–VII RNA was indistinguishable from the defects resulting from injection of either the entire h-EphA3 ECD or the ligand binding domain alone, as judged by morphology and the expression of marker genes (Fig. 5b). Thus the C-terminal portion of the receptor ECD encoded by exons IV–VII mediates a ligand-independent dominant negative effect on zebrafish somite formation and axial organization.

Transphosphorylation Assays Suggest Ligand-independent h-EphA3 Dimerization—This dominant negative effect at high h-EphA3 IV–VII concentration can be explained by the occurrence of heterodimers between intact endogenous receptors and the h-EphA3 IV–VII protein which are acting to block receptor function. This notion, implying the existence of a dimerization interface outside the ligand-binding region was supported by BIACore analysis of the binding of h-EphA3 I–VII, h-EphA3 I–III, and h-EphA3 IV–VII to a sensorchip derivatized with h-EphA3. We demonstrated binding of h-EphA3 IV–VII at micromolar concentrations that was characterized by a slow off rate, yielding an apparent dissociation constant of 3 μM. Marginally weaker binding was observed for the h-EphA3 I–III construct, whereas the interaction of h-EphA3 I–III had a substantially lower affinity (Table I).

To test further the hypothesis of a dimerization interface, we performed in vitro transphosphorylation assays of h-EphA3, constitutively expressed in LK 63 cells (29). Transphosphorylation of the receptor by cross-linking with anti-h-EphA3 mAb IIIA4 during immunoprecipitation or by incubation with mAb cross-linked ephrin-A5-FLAG complexes has been demonstrated previously (3, 29). In the latter experiments 20–30 nM ephrin-A5-FLAG complex had been used to induce receptor transphosphorylation. The approximately 100-fold lower affinity of the receptor/receptor interaction (Table I) compared with
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Fig. 5. Dose-response of embryonic disruption to injected h-EphA3 I–III or h-EphA3 IV–VII RNA. a, batches of embryos (n = 29–107) were injected with decreasing concentrations (from 100 to 1 pg) of h-EphA3 I–III (●) or h-EphA3 IV–VII RNA (■), and a constant amount of E-GFP mRNA (5 pg). The embryos were allowed to grow for 12 to 13 hpf before fixation. They were then hybridized with pax-b, hlx-1, krox20, and myoD DIG-labeled riboprobes. Embryos were observed under a dissecting microscope and scored for disrupted patterns of gene expression. To control for potential defects due to the genetic background of particular parents in our strain, a number of embryos that were transferred to the injection stage were not injected but were handled identically to the injected embryos and were scored as described for defects. Parallel samples of h-EphA3 I–III RNA-injected embryos were lysed at 10 hpf (10 embryos/100 μl), the exogenous FLAG-tagged protein extracted on M2-agarose, and its concentration in the lysate determined on an anti-h-EphA3 mAb-derivatized BIAcore sensorchip. The concentration of h-EphA3 I–VII per embryo is shown (□). b, in situ hybridization analysis of 100 pg of h-EphA3 IV–VII RNA-injected embryo. An injected embryo at 12–13 h of development was fixed for in situ hybridization with pax-b, hlx-1, krox20, and myoD DIG-labeled riboprobes. The photograph represents an antero-dorsal view with anterior to the top and posterior to the bottom. Disorganization of pax-b and krox20 expressing cells similar to the pattern observed in h-EphA3 I–III RNA-injected embryos (Fig. 4d) is illustrated. Staining with myoD reveals defects in somite organization, whereby open arrowheads indicate missing somites in the right-hand part of the embryo.

As we were unable to provide these high protein concentrations for this competition assay, in an alternative approach we analyzed ligand-independent receptor dimerization by assessing receptor binding of h-EphA5 constructs. Thus, in the following experiments we prepared immune complexes of FLAG peptide-tagged receptor ECD or the derived subdomain deletion constructs with M2 antibody. These preformed, divalent receptor ECD-M2 complexes were used at a low micromolar concentration to induce receptor dimerization and transphosphorylation by the endogenous receptor kinase. Incubation of LK63 cells with h-EphA3 I–VII-M2 mAb complex resulted in a substantial phosphorylation of the endogenous h-EphA3. This confirms that the soluble, exogenous mAb cross-linked receptor dimer binds and tethers endogenous h-EphA3 receptors in the absence of ligand and facilitates their transphosphorylation. The interaction between the endogenous receptors and the exogenous receptor ECD-M2 complex was sufficiently stable to withstand cell lysis and immunoprecipitation with protein A-Sepharose (Fig. 6b, lane C). Importantly, incubation of the cells with the h-EphA3 IV–VII subdomain gave a virtually identical result (lane A), confirming that this domain harbors the suggested receptor dimerization interface. Notably, in both cases no additional phosphorylated h-EphA3 was recovered on immunoprecipitation of the protein A-depleted lysates with anti-h-EphA3 mAb IIIA4 and confirms that only endogenous receptors, which had been dimerized by the h-EphA3 IV–VII-M2 mAb complex, underwent transphosphorylation. Western blot analysis with an anti-h-EphA3 polyclonal antiserum indicated that only a small (virtually undetectable) proportion of the total endogenous receptor population had been captured into the M2-protein A complex. In contrast to these results, analysis of a parallel sample of the cells treated with an identical concentration of h-EphA3 I–III-M2 complex (lane B) revealed phosphorylated h-EphA3 in the anti-h-EphA3 mAb precipitate but not in the protein A precipitate. This indicates that h-EphA3 I–III can induce dimerization at high concentrations, but in keeping with the BIAcore results, this interaction is notably weaker, leading to dissociation of endogenous phosphorylated h-EphA3 from the h-EphA3-M2 complex during cell lysis. In a control experiment, LK63 cells were treated with M2 mAb on its own, but yielded no phosphorylated endogenous receptor in either the protein A or IIIA4 precipitates (lane D). Taken together, our experiments suggest that the exon IV–VII-encoded subdomain with some contribution of the exon III-encoded region provide the dimerization interface of the h-EphA3 receptor, which is functional in the absence of ligand.
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**DISCUSSION**

While Eph receptors have been shown to mediate key functions in embryogenesis, the mechanisms of ligand binding and receptor activation underlying these functions remain to be defined in detail. It is clear that only membrane-bound or artificially dimerized or clustered soluble ligands will mediate activation of Eph receptors (43) including h-EphA3 (3), and soluble monomeric h-ephrin-A5 has been shown to act as an antagonist of receptor activation (2). Recently the role of higher order receptor oligomers has been studied, demonstrating that full biological activation is only achieved once a tetrameric receptor aggregate is generated (22). Our analysis of the stoichiometry of soluble h-ephrin A5 binding to the EphA3 ECD unambiguously confirmed a one-to-one interaction (3). The event(s) that follow ligand binding, leading to receptor oligomerization and cell signaling, remain to be defined. This study sought to further our understanding of this first step of receptor activation through the analysis of functional domains of the h-EphA3 ECD both in vitro and in vivo.

The highly conserved subdomain architecture of the ECD of Eph receptors coincides with a conserved exon structure (this paper and Ref. 33). A study of interspecies sequence homologies between human, murine, and chicken EphA3 revealed high homology throughout but with a ranking of exon sequence identities, the strongest evolutionary constraint (≥99% identity between mouse and human) being on exon III (Fig. 1, a and b). Regions of the ECD demarcated by exon boundaries were expressed in CHO cells, and the proteins were purified to homogeneity. A kinetic analysis, using plasmon resonance detection of binding of the various subdomains to BIACore sensorchip-immobilized h-ephrin-A5 clearly localized the ligand binding sequences to those encoded by exons I–III. The affinity for the interaction of this smaller ligand binding domain of $K_D = 18$ nM was well within the range reported previously for the binding of ephrin-A5 to sensorchip-immobilized h-EphA3 $K_D = 12$ nM (33) but was somewhat higher than the affinity estimated for the binding of the h-EphA3 ECD to sensorchip-immobilized ephrin-A5 ($K_D = 62–72$ nM, Fig. 1c). Correspondingly increased association rates of the smaller subdomain constructs (Fig. 1d) and of the ligand (3) suggest that higher diffusion rates and improved access to the binding interface of the smaller proteins may explain this apparent affinity increase.

In seeking to further narrow the binding region, we note that exon I contributes only seven non-conserved residues, but both exons II and III show very high sequence identity across species (Fig. 1). Attempts to express exon III without exon II sequences were unsuccessful, and expression of protein was not detected. However, in-solution competition experiments with a peptide corresponding to the exon I- and II-encoded residues of the receptor ECD showed no effect on binding. Taken together with the homology data, these results suggest that exon III-encoded sequences are directly involved in ligand binding, whereas exon II is not involved but is required for a stable protein domain structure. Furthermore, attempts to express a C-terminal truncated form of exon III, terminating after cysteine 186 (corresponding to cysteine 191 of EphB2), resulted in low yields of high molecular weight protein aggregates, suggesting the importance of this C-terminal sequence for the structural integrity of this domain. These findings are in general agreement with a recent study of the chicken EphB2 and EphA3 receptors (36). By using a different approach the authors also identified the critical role of the N-terminal region in ligand binding. Although they did not analyze the contribution to protein secretion, stability, or ligand binding of the N-terminal, exon I- and II-encoded subdomain denoted in their report as “signal peptide,” successful expression of N-terminally FLAG-tagged proteins in our study suggests that this signal peptide is not cleaved from the receptor ECD during secretion.

Furthermore, restricted C-terminal truncations of exon III sequences in their study resulted in reduced ligand binding affinity (36), suggesting that most or all of exon III is required for high affinity binding. Together, these findings, emphasizing the structural importance of the N- and C-terminal sequences of the N-terminal half of the EphA3 ECD, support our conclusion that exons II and III encode an integral structure rather than, as previously assumed, discrete globular and cysteine-rich subdomains.

Organization from defined structural building blocks with distinct regions of sequence conservation is a common feature of RTKs (44). As our data imply for EphA receptors, the region of highest sequence conservation within several subfamilies including the fibroblast growth factor receptors delineates the ligand-binding interface. In the case of the fibroblast growth factor receptors, ligand binding is encoded by Ig domains II and III, both of which independently bind fibroblast growth factors. Similarly, the PDGFR, c-Kit, TrkR, and Flt1 RTKs use multiple Ig repeats to bind ligand (21, 45–47), whereas the insulin R ligand-binding site spans two unique N-terminal α-subunit domains (48) and ErbB binds EGF through the region between two Cys-rich domains, with some contribution from the N terminus (49–51). In the Eph family the ligand-binding site is characterized as a single structural domain which, despite reported weak similarities to Ig-like (4, 52) or laminin VI (36) domains, appears to be unique to this family. The notion of a single exon II- and III-encoded protein domain is also supported by an evolutionary argument based on intron phase analysis (53). The 5’ end of exon II and the 3’ end of exon III are phase 1 (i.e., interrupting the coding triplet after one base), whereas the intron between exons II and III is phase 0, implying that it arose through insertion into an ancestral coding sequence.

To analyze further the role of isolated receptor ECD subdomains in vitro, we modified a dominant negative strategy in zebrafish, previously used to study EphA4 signaling in fore- and hindbrain formation during zebrafish embryogenesis (24). Inhibition of RTK signaling by expression of kinase-deleted or truncated forms of the receptor, either in a ligand-dependent (23, 24, 54) or independent manner (55, 56), is well established (reviewed in Ref. 44). We used our observation that expression of exogenous soluble EphA3 or ephrin-A5 resulted in a characteristic developmental defect in somite development and axial organization4 to probe the function of ECD subdomains. As
anticipated from the BIAcore analysis, embryos injected with h-EphA3 I–III RNA show the same phenotype as h-EphA3 I–VII or soluble ephrin-A5 RNA-injected embryos, consistent with a dominant negative effect by the soluble EphA3 ligand binding domain. By contrast, injection of the h-EphA3 I–VII RNA, encoding all regions except the ligand-binding domain, was not expected to alter the normal phenotype. At low and medium concentrations no abnormality in embryo development was observed. Extended dose-response studies revealed that both h-EphA3 I–VII or h-EphA3 I–III exhibited the expected dose-dependent increase in the number of developmentally defective embryos (Fig. 5). Although the severity of the effects also increased (an increasing number of somites were disrupted at higher concentrations of RNA; data not shown), the defects were confined exclusively to those tissues that had been perturbed also at low concentrations of injected RNA. Importantly, at high concentrations of h-EphA3 I–VII RNA and h-EphA3 I–III RNA, injections resulted in a similar proportion of identically defective embryos. The complete overlap of phenotypes resulting from these injections of either h-EphA3 I–VII RNA, h-EphA3 I–III RNA, or high concentrations of h-EphA3 I–VII RNA implied that the same signaling processes had been disrupted by all receptor constructs.

Since the h-EphA3 I–VII protein cannot bind ephrin-A5 (Fig. 1, d and e), this finding suggested that h-EphA3 can bind to endogenous receptor to produce functionless heterodimers, thus disrupting Eph signaling in a ligand-independent manner. An approximation of the abundance of the exogenous receptor proteins on the basis of Western blot and BIAcore data (Figs. 2, a and b, 5a), and by assuming the extracellular space of a 10 hpf embryo as 5 pl (1/100th of the total volume of a 1-mm diameter embryo), suggests a concentration of 10–20 μM h-EphA3 I–VII in 100 pg of mRNA-injected embryos. This high concentration of expressed protein required to achieve the dominant negative effect (Fig. 5, a and b) implies a significantly lower affinity of the receptor/receptor interaction than for the receptor-ligand binding. This notion was confirmed by BIAcore studies of h-EphA3 I–VII or h-EphA3 I–VII binding to h-EphA3 I–VII derivatized sensor surfaces, indicating that ligand-independent receptor dimerization occurred at micromolar concentrations (Table I). Although we were not able to achieve high enough concentrations of monomeric h-EphA3 I–VII to block ligand-induced h-EphA3 transphosphorylation in LK63 cells, we confirmed the ligand-independent receptor dimerization through a direct in vitro binding experiment. Anti-FLAG mAb cross-linked forms of either soluble h-EphA3 I–VII or the ligand binding domain-deficient h-EphA3 I–VII construct (Fig. 6) induced transphosphorylation of endogenous receptors, demonstrating their competence for a ligand-independent interaction. A slow dissociation rate from the h-EphA3 I–VII sensor surface during BIAcore experiments and immunoprecipitation of phosphorylated endogenous h-EphA3 with mAb-dimerized h-EphA3 ECD constructs from a detergent lysate of LK63 cells suggest that the interaction is stable, once the critical receptor concentration is reached. On the other hand, a weak interaction of h-EphA3 I–III with h-EphA3 I–VII inferred from BIAcore and transphosphorylation experiments (Table I, Fig. 6) did not withstand the immunoprecipitation and indicates a minor contribution of this domain to the dimerization. Thus, our experiments provide several lines of evidence suggesting the presence of a low affinity dimerization domain which is encoded by exons IV–VII and functions independently of ligand binding. Exons IV–VII encode an EGF domain and two Fibronectin type III domains, the latter having been implicated in receptor dimerization in the cytokine receptor family (57). Although the data presented in this report do not precisely define the region mediating dimerization, preliminary zebrafish studies with h-EphA3 IV–V and h-EphA3 V–VII suggest that dimerization is mediated by exon IV sequences.

The presence of a ligand-independent dimerization domain in EphA3 invites comparison with activation of the c-Kit and PDGFR. Experiments with c-Kit-specific mAbs which block dimerization, and analysis of c-Kit ECD deletion mutants, define an Ig domain C-terminal to the ligand binding interface which is required for receptor dimerization (21). This dimerization domain is essential for biological effects of the Kit ligand. Of most relevance to the results presented, ligand-independent activation at high receptor density has been demonstrated using high level expression of PDGFR in SF9 cells (25).

The identification of distinct receptor subdomains that mediate ligand-binding and receptor dimerization at different concentrations suggests a stepwise mechanism of receptor activation for EphA receptors and ephrin-A ligands. In our model, an EphA-expressing cell or axon moving into a gradient generated by differentially expressing ephrin-A-positive cells encounters progressively higher Eph-A concentrations. As EphA receptors engage ephrin through high affinity interaction of the N-terminal ligand-binding domain at the cell-cell interface (step 1), the reduced mobility of the receptor-ligand complexes versus free receptor or ligand results in their accumulation at the interface. At some position in the ephrin-A gradient, a critical receptor concentration (dependent on receptor-ligand affinity) obtains at the interface between the cells such that receptor-receptor interaction through the C-terminal dimerization domain (step 2) allows the generation of multimeric complexes. EphA signaling is activated by transphosphorylation of the oligomerized receptors (step 3), and the resulting signal halts migration of the EphA-expressing cell. Recent observations indicating that multimeric complexes (tetramers) of EphB1 and EphB2 receptors are required for full biological function (22) is of interest in regard to the experiments presented here, in which transphosphorylation is induced by heterotetramer formation and might involve even higher order aggregates. It seems reasonable to speculate that the low affinity dimerization domain is involved in mediating the formation of these higher order structures and thus provides a critical component of the Eph-receptor signaling system.

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