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The N-terminal globular domain of Eph receptors is sufficient for ligand binding and receptor signaling

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The Eph family of receptor protein-tyrosine kinases (RTKs) have recently been implicated in patterning and wiring events in the developing nervous system. Eph receptors are unique among other RTKs in that they fall into two large subclasses that show distinct ligand specificities and for the fact that they themselves might function as 'ligands', thereby activating bidirectional signaling. To gain insight into the mechanisms of ligand–receptor interaction, we have mapped the ligand binding domain in Eph receptors. By using a series of deletion and domain substitution mutants, we now report that an N-terminal globular domain of the Nuk/Cek5 receptor is the ligand binding domain of the transmembrane ligand Lerk2. Using focus formation assays, we show that the Cek5 globular domain is sufficient to confer Lerk2-dependent transforming activity on the Cek9 orphan receptor. Extending our binding studies to other members of both subclasses of receptors, it became apparent that the same domain is used for binding of both transmembrane and glycosylphosphatidyl-anchored ligands. Our studies have determined the first structural elements involved in ligand–receptor interaction and will allow more fine-tuned genetic experiments to elucidate the mechanism of action of these important guidance molecules.
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Introduction

Axonal guidance during the development of the nervous system is controlled by both soluble (long-range) and surface-bound (short-range) cues located in the trajectories of navigating axons (Tessier-Lavigne and Goodman, 1996). The Eph family of receptor tyrosine kinases and their cell surface-bound ligands have recently been implicated in short-range control of axon guidance during retinotectal map formation and in guidance of commissural axon projections across the midline (Cheng *et al.*, 1995; Drescher *et al.*, 1995; Henkemeyer *et al.*, 1996; Nakamoto *et al.*, 1996; Orioli *et al.*, 1996). In addition, they have roles in axon fasciculation (Winslow *et al.*, 1995; Orioli *et al.*, 1996) and in patterning of forebrain and hindbrain structures (Xu *et al.*, 1995, 1996).

The Eph receptor family falls into two subclasses based on their interactions with ligands that are tethered to the cell surface either by a single transmembrane domain (TM) or by a glycosylphosphatidyl (GPI) anchor (Brambilla and Klein, 1995; Brambilla *et al.*, 1995; Gale *et al.*, 1996). Whereas cross-reactive binding between the two subclasses is rare and of low affinity, ligand–receptor interactions within a subclass are rather promiscuous (Brambilla *et al.*, 1996; Gale *et al.*, 1996). However, small differences in binding affinities observed *in vitro* may result in different biological responses *in vivo*. Genetic evidence suggests that this is indeed the case. The two Eph receptors Nuk (Henkemeyer *et al.*, 1994) and Sek4 (Becker *et al.*, 1994; Ciossek *et al.*, 1995) (in this report referred to as Cek5 and Cek10, respectively) both bind the transmembrane ligands Lerk2 (Beckmann *et al.*, 1994; Shao *et al.*, 1994; Brambilla *et al.*, 1995) and Elf2 (also referred to as Lerk5 or Htk-L) (Bennett *et al.*, 1995; Bergemann *et al.*, 1995; Kozlosky *et al.*, 1995). However, the analysis of Nuk and Sek4-deficient mice has revealed that, despite being co-expressed, each receptor has unique roles in the guidance of commissural axons and that both receptors cooperate in axon guidance and fasciculation, as well as in the development of midline structures outside the nervous system (Henkemeyer *et al.*, 1996; Orioli *et al.*, 1996). These results are consistent with the idea that small differences in ligand interaction may influence in subtle ways the guidance of navigating growth cones *in vivo*.

Despite the rapidly accumulating knowledge of the biological functions of Eph receptors and their ligands, the precise mechanism of guidance is poorly understood. Two GPI-anchored ligands, Rags and Elf1, have been shown to be contact repellents for retinal ganglion cell axons (Drescher *et al.*, 1995; Nakamoto *et al.*, 1996); other ligands may behave in similar ways or be contact attractants for certain cells. Little information is available on the signaling events triggered by activated Eph receptors after ligand-induced receptor autophosphorylation (Brambilla and Klein, 1995; Ellis *et al.*, 1996).

Both genetic and biochemical evidence suggests that TM ligands are also actively involved in signaling during axonal pathfinding. Mice expressing a kinase-defective version of Nuk have a normal anterior commissure, at least in certain genetic backgrounds, suggesting that reverse signaling through TM ligands on the surface of the navigating axon may help to guide it properly across the midline (Henkemeyer *et al.*, 1996). Consistent with the idea of ligand signaling, TM ligands carry within their cytoplasmic domains a set of conserved tyrosine residues, which become phosphorylated after receptor contact (Holland *et al.*, 1996; Brückner *et al.*, 1997). This suggests that receptor contact causes ligand clustering and subsequent phosphorylation by an as yet unknown cytoplasmic tyrosine kinase endogenous to the ligand-expressing cells (Orioli and Klein, 1997).

Elucidating the structural elements involved in ligand–

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receptor interaction is essential for our understanding of the sequence of events which result in bidirectional signaling by Eph receptors and their ligands. In this report, we have determined the domain of Eph receptors responsible for ligand binding by constructing a series of Eph receptor deletion and domain swapping mutants, which were then analyzed for ligand binding and subsequent receptor signaling. We conclude that the same domain is used by all Eph receptors to interact with their respective ligand subclass.

Results

An N-terminal globular domain allows Lerk2 to bind to the Cek5 receptor

Our mapping studies of the ligand interaction domain in Eph receptors were guided by the recently published genomic organization of the chicken Cek5 gene (Connor and Pasquale, 1995) and by information on sequence homologies and structural domains. The C-terminal half of the Cek5 ectodomain encompassing amino acid (aa) residues 332–549 contains two fibronectin type III (FN III) domains whose boundaries can be clearly defined based on high sequence conservation with other FN III domains (O'Bryan *et al.*, 1991). The N-terminal half of the Cek5 ectodomain is encoded by two exons: a large exon 3 (aa 42–279) and exon 4 (aa 280–331), which can be alternatively spliced in other Eph receptors (Valenzuela *et al.*, 1995). The C-terminal portion of exon 3 and exon 4 contain two stretches of cysteine-rich sequences with characteristically spaced cysteine residues bearing significant homology to epidermal growth factor (EGF)-like modules from tenascin and thrombospondin (Connor and Pasquale, 1995) (J.P.Labrador and R.Klein, unpublished observations). The N-terminal portion of exon 3 has previously been proposed to have weak homology to immunoglobulin (Ig)-like domains (O'Bryan *et al.*, 1991; see also Tessier-Lavigne and Goodman, 1996), although this similarity is controversial (Connor and Pasquale, 1995). Secondary structure predictions for this region suggest that it is exclusively composed of β-sheet segments separated by loops (Rost, 1996). This is characteristic not only of Ig-like, but also of several other extracellular globular domains. In the absence of any structural data, we will refer to this domain as the N-terminal globular domain.

We constructed a series of soluble deletion mutants of the Cek5 ectodomain fused to heat-stable alkaline phosphatase (AP) (Flanagan and Leder, 1990). Such Cek5–AP fusion proteins, when expressed and secreted by COS cells, bind to membrane-bound Lerk2 ligand with nanomolar affinity (Brambilla *et al.*, 1995). As indicated in Figure 1, the N-terminal half of the Cek5 ectodomain including the globular domain and cysteine-rich regions (331–AP) specifically bound to NIH 3T3 cells expressing Lerk2, while showing no specific binding to wild-type NIH 3T3 cells (data not shown). The reciprocal deletion mutant containing both FN III domains fused to the Cek5 signal peptide (2FN–AP), despite being efficiently secreted by COS cells (data not shown), did not bind to Lerk2. The amount of binding of 2FN–AP was comparable to that of unfused AP protein (Figure 1). Further removal of the cysteine-rich sequences encoded by the alternatively spliced exon (280–AP) did not affect Lerk2 binding, nor

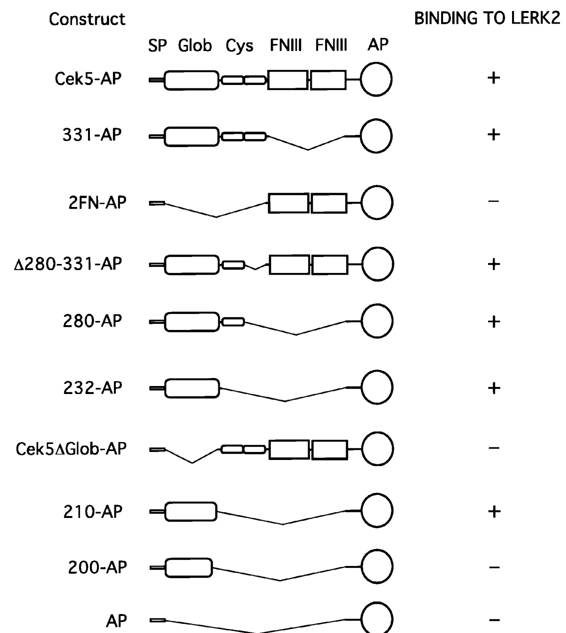

Fig. 1. An N-terminal globular domain is the primary ligand binding determinant in Cek5 receptors. Schematic representation of Cek5 deletion mutants fused to AP. Deleted regions are indicated by thin bent lines. The individual domains are drawn to scale. Mutant names correspond to the most C-terminal aa residue fused to AP, e.g. 200–AP: the most N-terminal 200 aa residues of Cek5 fused to AP. Internal or N-terminal deletions are expressed as Δ followed by the name of the domain or aa residues deleted. The indicated fusion proteins were assayed for binding to wild-type NIH 3T3 cells or NIH 3T3 cells stably expressing Lerk2. Binding activity is expressed qualitatively as + when the binding affinity of the mutant was in the nanomolar range and was scored as – when no binding was detected above background ($K_D > 100$ nM). None of the mutants bound to wild-type NIH 3T3. cys, cysteine-rich region; FN III, fibronectin type III domain; glob, globular domain; SP, signal peptide.

did the specific deletion of this exon in the context of the entire Cek5 ectodomain (Δ280–331–AP). Specific binding to Lerk2-expressing cells was still observed after removal of the entire cysteine-rich region (232–AP) up to residue 210. A reciprocal deletion mutant to 232–AP containing the cysteine-rich regions and both FN III domains fused to the Cek5 signal peptide (Cek5ΔGlob–AP) did not bind to Lerk2. Further C-terminal deletion (200–AP) including the conserved cysteine at position aa 205 abolished Lerk2 binding without affecting secretion of the fusion protein. 200–AP was the smallest peptide that could be expressed as an AP fusion protein. Further C- and N-terminal deletions did not produce active AP fusion proteins, suggesting that these peptides were not properly folded (data not shown).

For those AP fusion proteins that showed specific binding, Scatchard analyses were performed to determine binding affinities. As shown in Figure 2, the entire Cek5–AP fusion protein bound with subnanomolar affinity to Lerk2-expressing NIH 3T3 cells. Cek5 mutants lacking both FN III domains and carrying partial or complete deletions of the cysteine-rich domain showed comparable affinities, with K_D between 2 and 3 nM. Similar values were also observed with the globular domain alone generated as Cek5–TrkB chimeric receptor, when expressed in NIH 3T3 cells and tested with Lerk2–AP fusion proteins (data not shown).

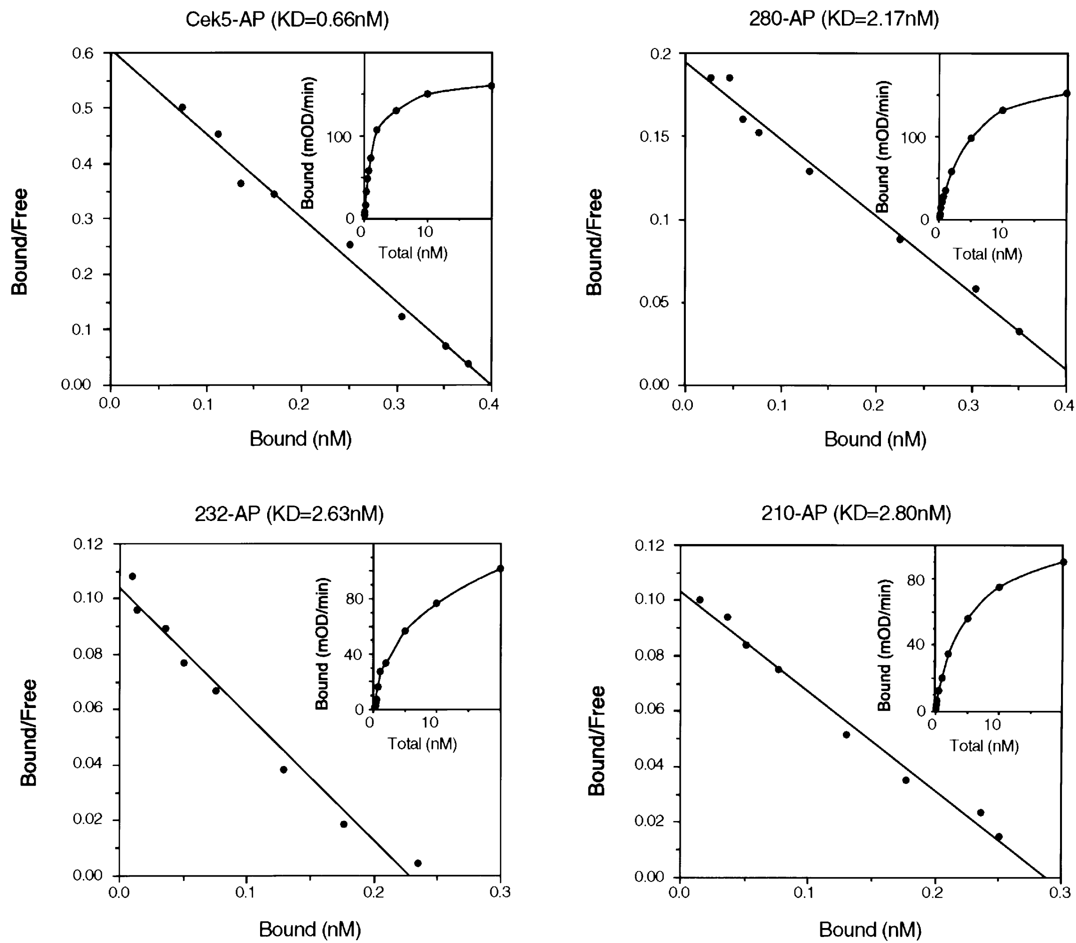

Fig. 2. The globular domain of Cek5 retains full binding activity to Lerk2. Scatchard analyses of the binding of full-length Cek5–AP, and the Cek5 deletion mutants 280–AP, 232–AP and 210–AP to membrane-bound Lerk2 expressed in NIH 3T3 cells. Dissociation constants are indicated above each graph.

The N-terminal globular domain of Cek5 is sufficient to confer Lerk2 binding on the Cek9 orphan receptor

To determine whether N-terminal sequences contain the primary determinants of ligand binding in the context of an entire receptor ectodomain and whether they are sufficient to change the specificity of an orphan receptor into that of a Lerk2 receptor, we generated chimeric receptor ectodomains using sequences from the Cek9 orphan receptor (Sajjadi and Pasquale, 1993). Substitution of the N-terminal and cysteine-rich sequences of Cek5 for those of Cek9 into a Cek9–AP fusion protein or a Cek9–TrkB chimeric receptor (Brambilla *et al.*, 1995) resulted in high-affinity binding to Lerk2-expressing NIH 3T3 cells or soluble Lerk2–AP protein (Figure 3 and data not shown). Specific high-affinity binding to Lerk2 was still observed after the Cek5 contribution to the swapped ectodomain was progressively reduced from the N-terminal 331 to 232 aa (SW331–AP, SW280–AP, SW249–AP and SW232–AP) (Figure 3A). All the mutants displayed similar affinities with K_D values within 0.3 and 0.5 nM (Figure 3B). Taken together with the data from the deletion mutants, these results strongly suggest that the N-terminal globular domain is the main determinant for Lerk2 specific binding.

The N-terminal globular domain of Cek5 is sufficient to trigger Lerk2-dependent receptor signaling

To examine the ability of Cek5/9 chimeric ectodomains to trigger a functional response after Lerk2 binding, we generated Cek5/9–TrkB chimeric receptors and expressed them in NIH 3T3 cells (Figure 4A). Chimeras of Eph receptor ectodomains and TrkB kinase produce ligand-dependent transformation of NIH 3T3 cells (Brambilla *et al.*, 1995). As shown in Figure 4B, wild-type Cek5–TrkB very efficiently induces focus formation in the presence of its ligand Lerk2, whereas the orphan Cek9–TrkB chimeric receptor is completely inactive independent of the presence or absence of Lerk2. The substitution of the N-terminal globular and cysteine-rich domains of Cek5 confers transforming activity on the Cek9–TrkB chimeric receptor. Moreover, transforming activity is observed with the N-terminal globular domain of Cek5 alone (SW232–TrkB), in the context of a Cek9 ectodomain, indicating that these sequences are sufficient to bind Lerk2 and to induce receptor signaling. Transforming activity of the chimeric receptors containing swapped ectodomains was lower compared with wild-type Cek5 at low plasmid concentrations, but was only 2- to 4-fold lower at near-saturating conditions (Table I).

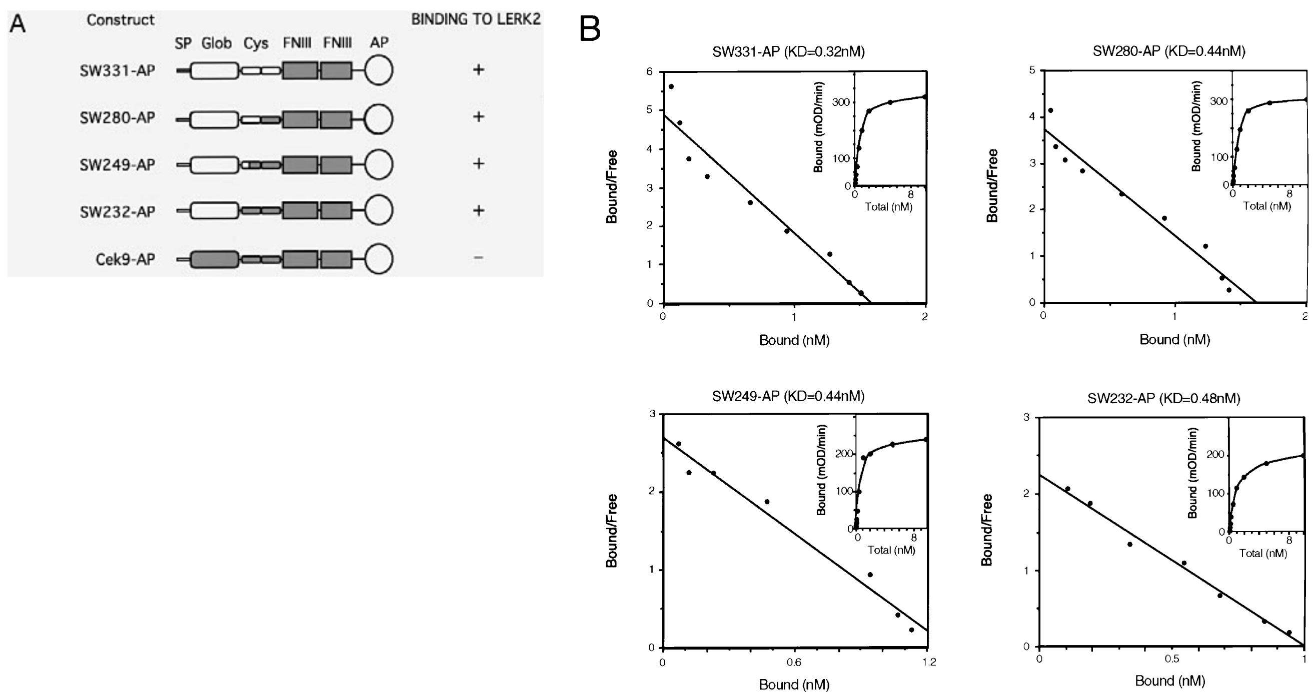

Fig. 3. The N-terminal globular domain of Cek5 confers specific Lerk2 binding on the Cek9 orphan receptor. (**A**) Schematic representation of Cek5/Cek9 chimeric mutants fused to AP. Cek5 sequences are in white, Cek9 sequences are in gray. The names of the mutants begin with SW (swapping) followed by the aa residue where the junction between Cek5 and Cek9 occurred. Abbreviations are as in Figure 1. (**B**) Scatchard analyses of the binding of Cek5/Cek9–AP chimeric mutants to membrane-bound Lerk2 expressed in NIH 3T3 cells. Dissociation constants are indicated above each graph.

The corresponding ligand-binding domain is used by other Eph receptors including those interacting with GPI-anchored ligands

We next investigated whether the same ligand-binding domain is used (i) by other receptors of the same subclass (e.g. Elk; Lhotak *et al.*, 1991) to bind transmembrane ligands and (ii) by Eph receptors, such as Cek4 (Sajjadi *et al.*, 1991), which interact with GPI-anchored ligands. We constructed and expressed deletion mutants of Elk and Cek4 as AP fusion proteins and tested their ability to bind surface-bound ligands. As shown in Figure 5A, specific Lerk2 binding was observed with the Elk deletion mutant containing only N-terminal sequences encoded by putative exon 3 (inferred from the Cek5 gene structure). Specific binding was also observed for Elf2, a second transmembrane ligand, indicating that both ligands use the same or largely overlapping binding regions in the Elk ectodomain (Figure 5B).

Cek4–AP deletion mutants were assayed for binding to the GPI-anchored ligand, Elf1 (Cheng and Flanagan, 1994). Elf1 was expressed in COS cells, since NIH 3T3 cells express endogenous Cek4-binding activity (Brambilla *et al.*, 1995). Cek4–AP deletion constructs containing the N-terminal globular domain as well as the cysteine-rich sequences (Cek4Δ331–AP and Cek4Δ280–AP) or the globular domain alone (Cek4Δ232–AP) bound to the GPI-anchored ligand Elf1, but not to untransfected COS cells (data not shown). As depicted in Figure 6, Scatchard analysis revealed that the binding affinities for the full-length Cek4 ectodomain and the Cek4–AP deletion mutants were in the subnanomolar range. These results indicate that the determinants of specific ligand binding in all Eph receptors lie in the globular domain.

The N-terminal globular domain of Cek4 is sufficient to confer Elf1 specific binding on Cek5

To analyze further whether the globular domain alone contains all elements for specific ligand binding, we generated a chimeric receptor ectodomain replacing the globular domain of Cek5 with the corresponding sequences from Cek4 into a Cek5–AP fusion protein (Cek4-GlobGek5–AP). Whereas wild-type Cek5 fails to bind Elf1 ligand, this chimeric Cek4/5 protein binds Elf1 with high affinity (K_D = 0.76 nM; Figure 7), but shows no specific binding to untransfected COS cells.

Discussion

Given the large number of Eph receptors and their surface-bound ligands and recent functional data, it seems likely that these molecules are major determinants of axon pathfinding and fasciculation events in the developing nervous system (Cheng *et al.*, 1995; Drescher *et al.*, 1995; Winslow *et al.*, 1995; Henkemeyer *et al.*, 1996; Nakamoto *et al.*, 1996; Orioli *et al.*, 1996). Moreover, Eph receptors and transmembrane ligands may be unique among receptor tyrosine kinases (RTKs) in mediating bidirectional signaling both in the receptor and ligand-expressing cells (Holland *et al.*, 1996; Brückner *et al.*, 1997). To gain insight into the structural elements of ligand–receptor interactions, we have mapped the binding site for both transmembrane and GPI-anchored ligands on Eph receptors. The specific binding activity resides in the most N-terminal 183 aa (excluding the signal peptide). According to secondary structure predictions, this portion of the ectodomain is composed of β-sheet segments interspersed with loops, a structure characteristic of extracellular globu-

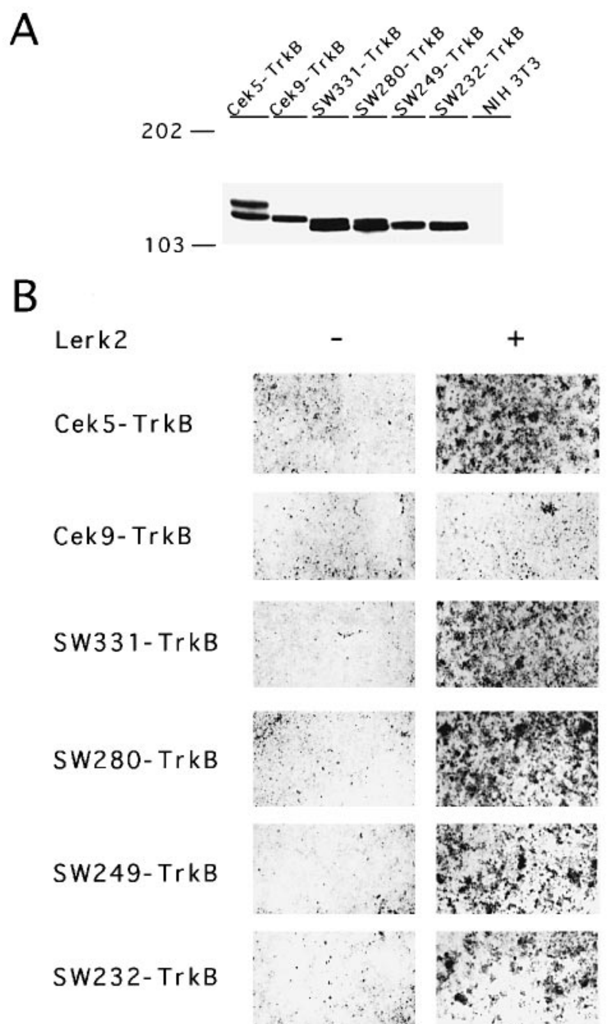

Fig. 4. The N-terminal globular domain of Cek5 is sufficient to trigger receptor signaling. (**A**) NIH 3T3 cells were transfected with pMEX-neo-derived expression plasmids containing the cDNAs of the indicated wild-type Cek5, Cek9, and Cek5/Cek9 chimeric mutants fused to the cytoplasmic domain of TrkB, selected for 1 week in G418-containing medium, lysed and immunoprecipitated with a pan-Trk specific antiserum. Immunoblotting was performed with a TrkB-specific antiserum. Double bands can be detected for some of the constructs, probably corresponding to differentially glycosylated forms of the receptor. The sizes of the molecular mass markers are indicated. (**B**) Transformation of NIH 3T3 cells by Cek5/9 chimeric receptors. NIH 3T3 cells were co-transfected with 500 ng of expression plasmids encoding Cek5, Cek9 and Cek5/Cek9 chimeric mutants fused to the TrkB cytoplasmic domain together with 100 ng of an expression plasmid encoding the membrane-bound Lerk2 ligand. Plates were stained with Giemsa 10 days later. Each photograph shows an area of ~40 cm^2.

lar domains. In addition to the globular domain (aa 27–210), the adjacent cysteine-rich domain (aa 211–331) may play a minor role in ligand binding, since we observed a 3- to 4-fold reduction in the K_D for those mutants lacking the cysteine-rich domain, in comparison to wild type Cek5. However, the globular domain of Cek5 renders the Cek9 orphan receptor competent for Lerk2-induced signaling. Likewise, the globular domain of Cek4 renders the Cek5 receptor competent to bind to the GPI-anchored Elf1. The calculated K_D values of receptor swapping mutants are all in the subnanomolar range. This suggests that the cysteine-rich region is dispensable for ligand binding and specificity, but may be involved in stabilization of the ligand–receptor complex. Whether or not the natural splice variants of other Eph receptors lacking the second cysteine-rich cluster (aa 280–331) are fully active *in vivo* remains to be analyzed.

The FN III domains are dispensable for ligand binding and receptor signaling, and may play a structural role, e.g. providing an optimal distance between interacting cells *in vivo*. FN III domains are found in ectodomains of cell adhesion molecules, RTKs and receptor protein tyrosine phosphatases (Bork *et al.*, 1996), and have been suggested

Table I. Transformation of NIH 3T3 cells by co-transfection of expression plasmids encoding Lerk2 (100 ng) and Cek5/9–TrkB chimeric mutants (indicated amounts)

Transfected DNAs		Transforming activity (foci per 1.5×10^5 cells)	
Receptor	DNA (ng)	Exp. 1	Exp. 2
Cek5–TrkB	500	102	>500
SW280–TrkB	500	>500	>500
SW249–TrkB	500	>500	306
SW232–TrkB	500	128	202
Cek5–TrkB	50	>500	>500
SW280–TrkB	50	206	194
SW249–TrkB	50	250	236
SW232–TrkB	50	150	163
Cek5–TrkB	5	122	40
SW280–TrkB	5	8	32
SW249–TrkB	5	8	38
SW232–TrkB	5	6	22
Cek5–TrkB	0.5	0	ND
SW280–TrkB	0.5	0	ND
SW249–TrkB	0.5	0	ND
SW232–TrkB	0.5	0	ND

500 ng of Cek9–TrkB co-transfected with Lerk2 failed to show any transforming activity.
ND, not done.

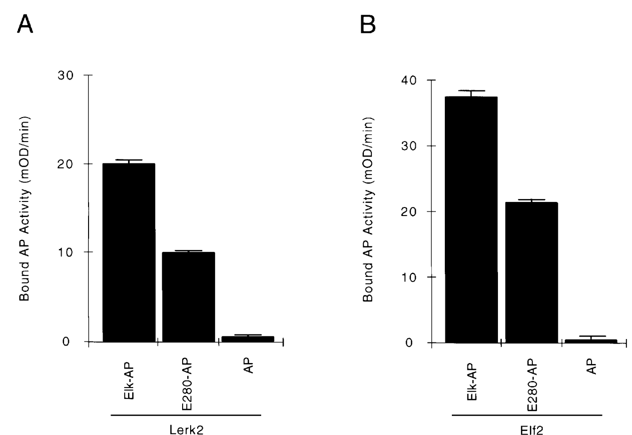

Fig. 5. The same ligand binding domain is used by Elk, another member from the same subclass as Cek5. Deletion mutants of Elk were constructed as AP fusion proteins containing either the full-length ectodomain or the sequences encoded by exon 3 (of Cek5), the N-terminal globular and part of the cysteine-rich region (Elk: E280–AP). 0.5 nM of AP activity of the indicated fusion proteins and soluble AP were assayed for binding to NIH 3T3 cells expressing Lerk2 (**A**) or Elf2 (**B**). Binding activity is expressed as the AP activity bound to a monolayer of cells in a six-well dish.

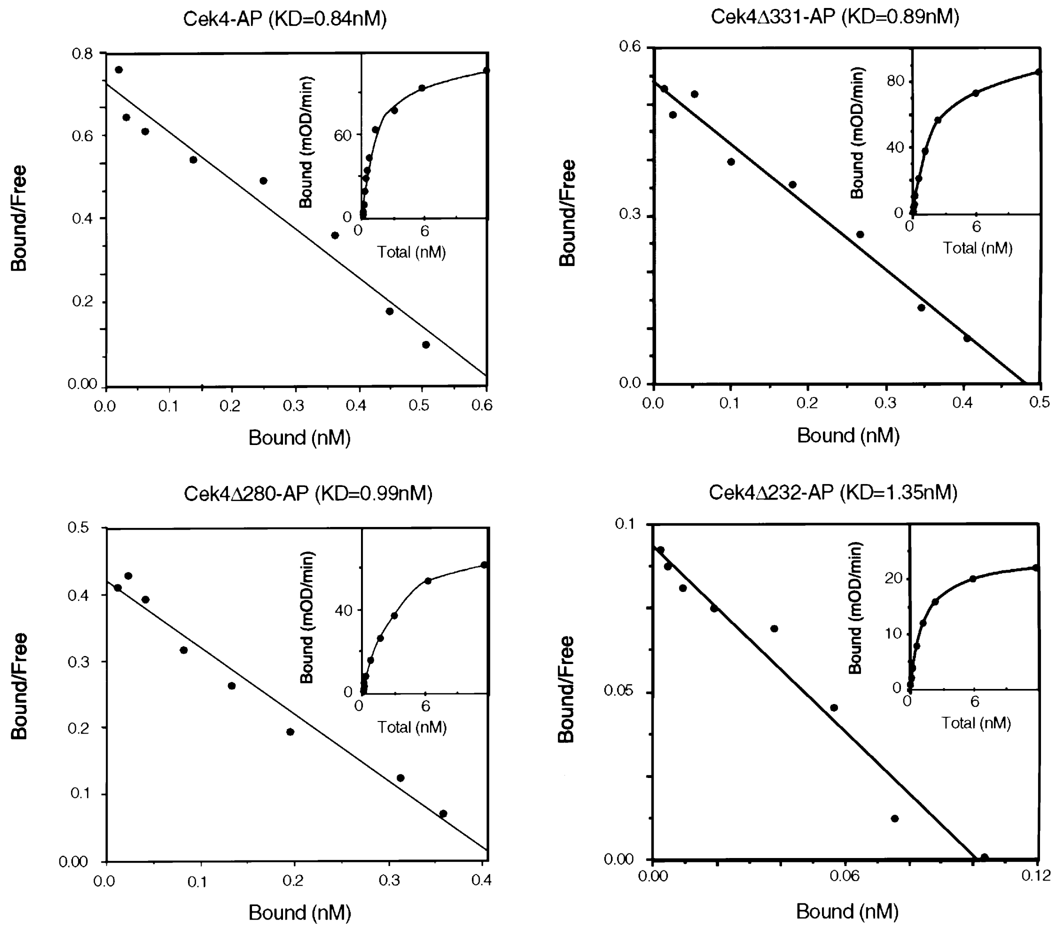

Fig. 6. The globular domain of Cek4 shows full binding activity to the GPI-anchored ligand Elf1. Binding of Cek4–AP was analyzed on wild-type COS cells and COS cells expressing Elf1. Cek4 deletion mutants were made (Cek4Δ331–AP, Cek4Δ280–AP and Cek4Δ232–AP) analogous to Cek5. Scatchard analyses were performed and dissociation constants are indicated above each graph.

to play a role in dimerization (Sommers *et al.*, 1994), similar to Ig-like domains (Blechman *et al.*, 1995).

Consistent with its important function in receptor signaling, the Cek5 ligand binding domain, when compared with other Eph receptors of the same or related species, reveals a higher degree of sequence conservation than the two FN III domains. For instance, the human Cek5 (also known as Erk; Kiyokawa *et al.*, 1994) ligand binding domain is 75.9% identical to rat Elk, 70.9% to human Cek10 (also known as Hek2; Böhme *et al.*, 1993) and 44.5% to human Eph (Hirai *et al.*, 1987). In contrast, both FN III domains of human Cek5 are 64.9% identical to Elk, 59.3% to human Cek10, and 29.1% to human Eph.

Ligand binding to single domains appears to be the exception rather than the rule among members of the superfamily of RTKs. RTKs with Ig domains in their extracellular portions, such as fibroblast growth factor receptors (with two or three Ig domains), platelet-derived growth factor receptors and c-Kit (with five Ig domains), and receptors for vascular endothelial growth factor (with seven Ig domains) all have non-contiguous ligand binding regions (Heidaran *et al.*, 1990; Lev *et al.*, 1993; Wang *et al.*, 1995; Davis-Smyth *et al.*, 1996). Whereas one Ig domain constitutes the core of the binding site, adjacent Ig domains greatly influence ligand binding, presumably by folding over the binding cleft and thereby reducing ligand dissociation.

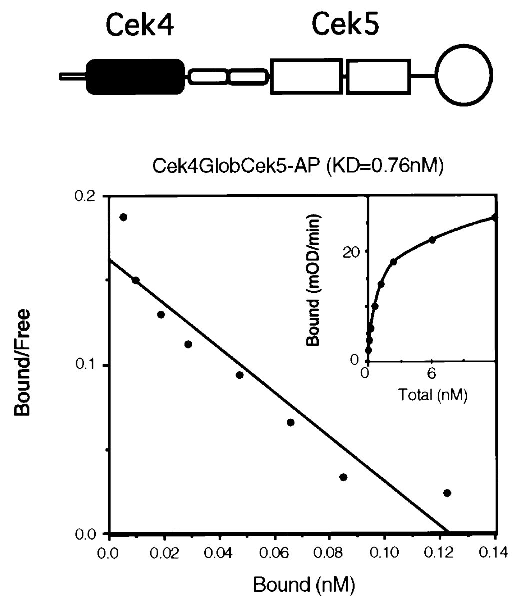

Fig. 7. The Cek4 globular domain confers specific Elf1 binding on the Cek5 receptor. The globular domain of Cek5 was replaced by the corresponding sequences of Cek4. The resulting chimeric receptor was fused to AP (Cek4GlobCek5–AP) and assayed for binding to Elf1 expressed in COS cells. The dissociation constant is indicated above the Scatchard plot.

The binding region of the Trk receptors maps to the most C-terminal Ig domain located closest to the transmembrane region of Trk receptors (Urfer *et al.*, 1995). However, based on cross-linking experiments, high-affinity binding appears also to require the adjacent N-terminal Ig domain (Perez *et al.*, 1995).

Among the RTKs that lack Ig domains, ligand binding regions were also found to be non-contiguous. EGF binding to the EGF receptor was mapped to sequences between two cysteine-rich regions with participation of the most N-terminal portion of the receptor ectodomain (Lax *et al.*, 1989, 1991). Similarly, both N-terminal and cysteine-rich sequences cooperate in high-affinity binding of insulin and insulin-like growth factor (IGF)-I to their respective receptors (Kjeldsen *et al.*, 1991; Yip *et al.*, 1991). In the case of Eph receptors, we report a ligand binding domain mapping to a single contiguous region apparently independent from the rest of the ectodomain.

It has been suggested that the N-terminal globular domain has certain features of an Ig-like structure (O'Bryan *et al.*, 1991). It is premature, however, to classify Eph receptors within the Ig superfamily of proteins (Tessier-Lavigne and Goodman, 1996). Instead, our own database searches with the minimal binding domain determined here revealed the highest similarity to the N-terminal globular domain VI of laminins (30% identity, 49% similarity over a stretch of 70 aa residues). This similarity alone is not statistically significant. However, as in laminins, and other related molecules, such as netrins/unc-6 (Serafini *et al.*, 1994), this N-terminal domain is followed by cysteine-rich EGF-like sequences (domain V of laminin). Based on the similarity in modular architecture, it is likely that rather than resembling an Ig-like domain, the Eph ectodomain is structurally similar to domains V and VI of laminin-like molecules. Considering the apparent conservation of function between netrins and Eph receptors, this structural similarity may turn out to be physiologically relevant. Experiments to determine the crystal structure of the globular domain are in progress.

In conclusion, our characterization of the ligand binding domain will allow further studies on the mechanisms of Eph function *in vivo*. Subtle changes in the binding spectrum of Eph receptors may influence the behavior of growing axons. Fine mapping of the residues directly interacting with the ligand will allow us to produce gain-of-function molecules that will interact efficiently across subclass boundaries. Such receptor mutants will be extremely useful for genetic studies, in addition to loss-of-function mutations.

Materials and methods

Production of secreted alkaline phosphatase fused ectodomains

Deletion mutants and Cek5/9 chimeric ectodomains were generated with the technique of gene splicing by overlap extension described previously (Horton *et al.*, 1989), with some modifications. Briefly, the mutant ectodomains are generated in two PCRs. In the first one, two fragments to be recombined are synthesized leaving complementary sequences in the ends to be fused. For the deletion mutants, the two fragments are the regions flanking the deletion. In Cek5/9 chimeric mutants, one fragment comes from Cek5 and the other from Cek9. The two fragments are mixed and annealed one to the other by their complementary sequence. One becomes the primer of the other in a second PCR that gives the designed deletion or chimeric molecule. Pfu polymerase was used in 7–10 cycle reactions to generate all the mutant ectodomains. A segment of the amino acid sequence at the junction region is listed below for each mutant.

Cek5 deletions:			
331–AP:	MPCT/TSVQ	I 332 to T543 deletion	(pRB73)
2FN–AP:	LGWM/SAPQ	V 45 to S334 deletion	(pRB90)
Δ280–331–AP:	VCRG/IPSA	C 278 to T 331 deletion	(pRB70)
280–AP:	VCRG/TSVQ	C 278 to T 543 deletion	(pRB86)
232–AP:	AARG/TSVQ	T 232 to T 543 deletion	(pRB87)
Cek5ΔGlobAP	IPNV/TCIS	V111 to T 233 deletion	(pJP79)
210–AP:	PRVI/TSVQ	Q 210 to T 543 deletion	(pRB89)
200–AP:	AVRV/TSVQ	F 200 to T 543 deletion	(pRB88)

Cek5/9 chimeras:		
SW331–AP:	MPCT/*GIPS*	(pRB68)
SW280–AP:	VCRG/*CPIG*	(pRB84)
SW249–AP:	KILC/*NGQG*	(pRB83)
SW232–AP:	AARG/*TCVA*	(pRB82)

The numbers refer to the aa number in the Cek5 sequence where the deletion has been introduced or, in the case of Cek5/9 chimeras, where Cek5 has been fused to Cek9. The Cek9 sequence is in italics and the plasmid names are in parentheses. All the ectodomains were synthesized by introducing an artificial *Hin*dIII site at the 5′ end (nucleotide 304 of the published sequence) and an artificial *Bgl*II site at the end of the extracellular domain (nucleotide 1006 of the published sequence), and they were cloned into *Hin*dIII/*Bgl*II-digested pAPtag-2 (Cheng *et al.*, 1995) to produce AP fusion proteins, as previously described (Brambilla *et al.*, 1995). Elk and Cek4 deletions were made in the same way, and the aa sequence at the junction region is as follows:

E280–AP:	CKAC/RSSG	deletion from C270	(pJP46)
Cek4Δ331–AP	TRPP/RSSG	deletion from P318	(pJP59)
Cek4Δ280–AP	CQAC/RSSG	deletion from C272	(pJP55)
Cek4Δ232–AP	EVRG/RSSG	deletion from G226	(pJP81)

To construct Cek4–AP (pJP47), an artificial *Bgl*II site was introduced in the sequence at the 5′ end and another at the end of the extracellular domain in order to clone it in pAPtag-2. Cek4GlobCek5–AP (pJP82) was produced in the same way as the Cek5/Cek9 chimeras, introducing an artificial *Hin*dIII site upstream of the start codon and a *Bgl*II site at the end of Cek5 ectodomain in order to fuse it with the AP from pAPtag-2. The sequence of the junction is VERG/*SSGG*. The Cek5 sequence is in italics.

Cek5–AP and Elk–AP have already been described (Brambilla *et al.*, 1995). The production of the AP fusion proteins was monitored by assaying the supernatant for heat-stable AP, as described previously (Cheng and Flanagan, 1994).

Binding assays

Binding assays were performed as described previously (Cheng and Flanagan, 1994). Briefly, NIH 3T3 and NIH 3T3-derived cell lines expressing either Lerk2 or Elf2 were seeded into six-well plates and used when confluent. For Cek4 constructs, COS cells were transfected with an Elf1 expression vector (Cheng and Flanagan, 1994).

Generation of TrkB fusion receptors

The Cek5/9 chimeric ectodomains, SW331 (pJP9), SW280 (pJP33), SW249 (pJP32) and SW232 (pJP31), were synthesized as described above including an artificial *Bam*HI site 5′ of the ATG start codon. They were cloned into *Bam*HI/*Spe*I-digested pAS13 (Brambilla *et al.*, 1995), taking advantage of a natural *Spe*I site in the Cek9 sequence.

Cell culture and gene transfer assays

COS cells (Gluzman, 1981), NIH 3T3 cells (Jainchill *et al.*, 1969) and NIH 3T3-derived cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Gene transfer assays in NIH 3T3 cells were performed by the calcium phosphate precipitation technique (Graham and van der Eb, 1973). Gene transfer assays in COS cells were carried out by using Lipofectamine (GibcoBRL), following the manufacturer's instructions.

Immunoprecipitation assays

Cell extracts from different NIH 3T3-derived cell lines were immunoprecipitated with a rabbit polyclonal antiserum raised against a peptide corresponding to the 14 C-terminal aa residues of human $gp140^{TrkA}$ (Martin-Zanca *et al.*, 1989). The resulting immunoprecipitates were fractionated by 7.5% PAGE, transferred onto nitrocellulose filters, and incubated with an antiserum raised against the mouse TrkB tyrosine

kinase domain expressed in bacteria (Klein *et al.*, 1990). Further incubations were performed using rabbit anti-mouse horseradish peroxidase (HRP)-linked antibodies or HRP-linked protein A. Specific signals were revealed using the ECL detection system (Amersham).

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Note added in proof

After this paper was submitted, the community agreed on a new nomenclature for Eph receptors and their ligands [Tessier-Lavigne,M., Flanagan,J., Gale,N., Hunter,T. and Pasquale,E.B. (1997) A unified nomenclature for Eph receptors and their ligands. *Cell*, in press]. Following this nomenclature, the new names for receptors and ligands used in this study are indicated in parentheses: Elk (EphB1), Cek5 (EphB2), Cek9 (EphB5), Cek4 (EphA3), Lerk2 (ephrin-B1), ElJ (ephrin-B2), ElJ1 (ephrin-A2).