Membrane-bound LERK2 ligand can signal through three different Eph-related receptor tyrosine kinases

Riccardo Brambilla, Andreas Schnapp¹, Franca Casagrande, Juan Pablo Labrador, Andrew D.Bergemann², John G.Flanagan², Elena B.Pasquale³ and Rüdiger Klein⁴

European Molecular Biology Laboratory, Differentiation Programme, 69012 Heidelberg, Germany, ²Department of Cell Biology, Harvard Medical School, Boston, MA 02115, and ³La Jolla Cancer Research Foundation, La Jolla, CA 92037, USA

¹Present address: Karl Thomae GmbH, Onkologie, 88397 Biberach, Germany

⁴Corresponding author

R.Brambilla, A.Schnapp and F.Casagrande contributed equally to this study

The Eph-related family of receptor tyrosine kinases consists of at least 13 members, several of which display distinctive expression patterns in the developing and adult nervous system. Recently, a small family of ligands, structurally related to the B61 protein, was identified. Binding of these ligands to Eph-related receptors did not, however, elicit measurable biological signals in cultured cells. In order to study functional interactions between B61-related ligands and Eph-related receptors, we constructed chimeric receptors, containing an Eph-related ectodomain and the cytoplasmic domain of the TrkB neurotrophin receptor. Expression and activation of such chimeric receptors in NIH 3T3 cells induced transformation in focus formation assays. Membrane-bound LERK2 ligand is shown to signal through three different Eph-related receptors, namely Cek5, Cek10 and Elk. LERK2, however, fails to interact functionally with the Cek9 receptor. Quantitative analysis including binding assays indicates that Cek10 is the preferred LERK2 receptor. Preliminary mutagenesis of the LERK2 protein suggests a negative regulatory role for its cytoplasmic domain in LERK2 signaling.

Key words: B61-related ligands/Elk/Eph-related receptors/LERK2/tyrosine kinase

Introduction

Neuronal differentiation and survival are controlled by external signals which are often transduced by transmembrane receptors with intrinsic tyrosine kinase activity. Prime examples are the nerve growth factor-related neurotrophins, which transmit their signals through the Trk family of tyrosine kinase receptors (Barbacid, 1993). The family of Eph-related receptors constitutes the largest known family of tyrosine kinase receptors. Several members of this family display developmentally regulated, and nervous system-specific expression patterns suggesting a role in neuronal differentiation and survival. The Eph-

related receptor family can be subdivided into several subgroups based on amino acid sequence homology (Sajjadi and Pasquale, 1993; Becker *et al.*, 1994; Tuzi and Gullick, 1994). We have concentrated our analysis on the four members of the Elk receptor subgroup, including Cek5, Cek9, Cek10 and Elk. The chicken Cek5 protein (Pasquale, 1991) was shown to be expressed predominantly in the developing and adult avian CNS, including cerebellar granule cells, retinal and hippocampal neurons (Pasquale *et al.*, 1992, 1994). The mouse Nuk/Sek-3 homolog of the chicken Cek5 receptor was shown to be expressed in specific segments, so-called rhombomeres, of the developing hindbrain (Becker *et al.*, 1994; Henkemeyer *et al.*, 1994) and other morphological structures of the midbrain and forebrain. The expression pattern of the chicken Cek9 receptor has not been analyzed in much detail. High levels of Cek9 mRNA were detected in embryonic avian brain, lower levels in several adult tissues including the brain and retina (Sajjadi and Pasquale, 1993). The mammalian homologs of Cek9 have not yet been identified. mRNA transcripts of chicken Cek10 (Sajjadi and Pasquale, 1993) and its human homolog HEK2 (Böhme *et al.*, 1993) were detected in several adult avian and human tissues, respectively, including brain, placenta, lung, kidney and pancreas. Most interestingly, the expression of the mouse homolog of Cek10, designated Sek-4, partially overlaps with the expression of Nuk/Sek-3, the mouse homolog of Cek5, during embryonic nervous system development, particularly in hindbrain rhombomeres (Becker *et al.*, 1994; Henkemeyer *et al.*, 1994). The chicken homolog of the rat Elk receptor cDNA (Lhotak *et al.*, 1991) was cloned as an N-terminally incomplete cDNA and called Cek6 (Sajjadi and Pasquale, 1993). Elk protein expression was shown to be highest in adult brain, and much lower in testis. For comparative analysis, we included in our studies a member of another subgroup within the Eph family, designated Cek4 or Mek4, for the chicken or mouse homologs, respectively (Sajjadi *et al.*, 1991). Cek4/Mek4 mRNA transcripts are most abundant in the developing and adult nervous system (Sajjadi *et al.*, 1991; Sajjadi and Pasquale, 1993; Cheng and Flanagan, 1994).

Recently, the first ligands for the Eph-related receptors were cloned and shown to be structurally related to a previously identified factor, called B61 (Holzman *et al.*, 1990). B61 binds to the Eck receptor (Bartley *et al.*, 1994). A second member of this ligand family, LERK2, was cloned as an Elk and Cek5 receptor binding protein (Beckmann *et al.*, 1994; Shao *et al.*, 1994), whereas a third member, ELF-1, binds Mek4, Sek1 and Cek7 receptors (Cheng and Flanagan, 1994; Shao *et al.*, 1995). Finally, a fourth ligand, EFL-2, was shown to activate EHK-1 and ECK receptors, but only when attached to the cell membrane (Davis *et al.*, 1994). The study of these

ligands has, however, proved difficult. To reach half-maximal activation of Eck tyrosine phosphorylation, several hundred ng/ml of B61 were required (Bartley *et al.*, 1994). This caused the authors to speculate that the functional B61 protein is a membrane-bound form. However, initial studies by Beckman and co-workers using membrane-bound B61 and LERK2 failed to induce tyrosine phosphorylation of the Elk receptor. An even greater problem has been that these ligands have so far failed to elicit measurable biological responses in cell lines expressing their receptors. Therefore, it could not be concluded with certainty that these proteins are the functional ligands of Eph-related receptors. We have now studied the interaction between LERK2 and several members of the Eph receptor family in NIH 3T3 focus formation assays. These assays were carried out using chimeric receptors with the Eph-related receptor ectodomains joined to the cytoplasmic domain of TrkB. We demonstrate that LERK2 is able to signal through three members of the Elk subgroup, namely Cek5, Cek10 and Elk.

Results

The cytoplasmic domain of Mek4 is not able to induce DNA synthesis or transformation of NIH 3T3 fibroblasts

In order to establish a biological assay to study functional ligand/receptor interactions, we first investigated the signaling capabilities of the Mek4 receptor in NIH 3T3 cells. Previous attempts to study Eph-related receptor signaling have resulted in contradictory results. Overexpression of the normal Eph receptor using a retroviral LTR promoter induced transformation of mouse fibroblasts and tumors in nude mice (Maru *et al.*, 1990). In contrast, a chimeric receptor containing the ectodomain of epidermal growth factor receptor (EGFR) and the cytoplasmic domain of the Elk receptor failed to induce transformation and only weakly stimulated DNA synthesis of mouse fibroblasts in the presence of EGF (Lhotak and Pawson, 1993).

We constructed chimeric receptors consisting of the ectodomain of the EGFR and the cytoplasmic domains of either Mek4 or TrkB (Figure 1A). As shown in Figure 1B, short-term stimulation with EGF of cells expressing chimeric receptors induced tyrosine phosphorylation of the Mek4 and TrkB tyrosine kinases. Parental NIH 3T3 cells lacking endogenous EGFRs did not give any positive signal (Figure 1B). Ligand stimulation of EGFR-Mek4, however, did not induce NIH 3T3 cells to enter S phase, as demonstrated by BrdU incorporation (Figure 1C). Moreover, this chimeric receptor failed to induce focus formation of NIH 3T3 cells in the presence of EGF (not shown). Control cells expressing similar levels of EGFR-TrkB receptors synthesized DNA (Figure 1C) and effectively transformed NIH 3T3 cells in the presence of EGF (not shown). Similar results were obtained with a chimeric receptor containing the ectodomain of TrkB and the cytoplasmic domain of Mek4. Co-transfection of this TrkB-Mek4 chimeric receptor with BDNF, the high-affinity TrkB ligand, did not cause focus formation of NIH 3T3 cells (data not shown). This failure to induce a mitogenic response in fibroblasts indicates that the critical substrates for the Eph-related kinases may be missing in

Fig. 1. Signaling properties of the Mek4 cytoplasmic domain. (A) Schematic representation of wild-type EGF receptor (EGFR) and two chimeric receptors containing the ectodomain and transmembrane domain of EGFR and the cytoplasmic domain of either Mek4 (EGFR-Mek4) or TrkB (EGFR-TrkB). The most C-terminal amino acid of EGFR (670) at the junction with TrkB is indicated. SP, signal peptide; ED, ectodomain; TM, transmembrane domain; TK, tyrosine kinase domain. (B) EGF induces tyrosine phosphorylation of chimeric receptors containing the ectodomain of EGFR. Wild-type NIH 3T3 cells lacking endogenous EGFR (A), EGFR-Mek4 chimeric receptor expressing F58-10-3 cells (B), and EGFR-TrkB chimeric receptor expressing F72-2-9 cells (C) were incubated for 10 min at 37°C either in the absence (–) or presence (+) of 100 ng/ml EGF. Cells were lysed and incubated with monoclonal antibodies against the ectodomain of EGFR. The resulting immunoprecipitates were analyzed by 7.5% SDS-PAGE, transferred to nitrocellulose filters, and blotted with anti-phosphotyrosine monoclonal antibody 4G10 as described in Materials and methods. The migration of EGFR-Mek4 and EGFR-TrkB chimeric receptors is indicated by arrows. Co-electrophoresed molecular weight markers include myosin (200K), phosphorylase B (97K) and albumin (68K). (C) The EGFR-Mek4 chimeric receptor fails to mediate a mitogenic response in NIH 3T3 fibroblasts. Quiescent NIH 3T3 cells lacking endogenous EGFR (A), EGFR-Mek4 expressing F58-10-3 cells (B), and EGFR-TrkB expressing F72-2-9 cells (C) were incubated in the presence of 100 µM BrdU either in the absence (–) or presence (+) of 100 ng/ml EGF, or 20% calf serum (CS). Cells were fixed after 24 h and analyzed for immunofluorescence using a mouse anti-BrdU monoclonal antibody as described in Materials and methods. At least 300 cells were counted in each case. Results are expressed as the percentage of cells showing positive nuclear fluorescence over the total number of cells counted.

Fig. 2. Construction and expression of Eph-related receptors containing the cytoplasmic domain of TrkB. **(A)** Schematic representation of chimeric receptors containing the cytoplasmic domain of TrkB and the ectodomains of either Cek5, Cek9, Cek10, Elk or Cek4. In the case of Cek4, two different constructs were made containing either the transmembrane domain of Cek4 (Cek4-TrkB¹) or of TrkB (Cek4-TrkB²). The Cek9 cDNA clone used appeared to lack a proper hydrophobic signal sequence at the N-terminus (E.Pasquale, unpublished data). We therefore attached the Cek5 signal sequence (amino acids 1–26) to the Cek9 cDNA to ensure proper cell-surface expression of the Cek9 chimeric receptor (see Materials and methods for details). The most C-terminal amino acid of each Eph-related receptor at the junction with TrkB is indicated. For abbreviations, see Figure 1. **(B)** NIH 3T3 cells were transfected with pMEXneo-derived expression plasmids containing the cDNAs of the indicated chimeric receptors, selected for one week in G418-containing medium, and then lysed and incubated with a rabbit antiserum raised against a peptide corresponding to the 14 C-terminal residues of human gp140^{TrkA}. The resulting immunoprecipitates were separated by 7.5% SDS-PAGE, transferred to nitrocellulose filters, and blotted with either a polyclonal rabbit antiserum against gp145^{TrkB} or **(C)** with mouse monoclonal anti-phosphotyrosine 4G10. Migration of the different Eph-related chimeric receptors is indicated by brackets.

fibroblasts. Alternatively, the Eph-related receptors may use entirely different mechanisms for kinase activation, that cannot be effectively studied by a chimeric receptor approach.

Expression of Eph-related receptor ectodomains fused to the TrkB tyrosine kinase domain

We decided to construct a series of chimeric receptors to study ligand–receptor interactions of Eph-related recep-

Fig. 3. Interaction of Mek4 with an endogenous ligand expressed in NIH 3T3 cells. Unfused AP tag (AP) or Mek4–AP (1 nM, 2124 mOD/min of AP activity) were assayed for binding to wild-type NIH 3T3 cells. Binding activity is expressed as the AP activity bound to 5×10^5 cells.

tors. For these chimeric receptors, we used the cytoplasmic domain of the TrkB neurotrophin receptor, which was shown to effectively transform NIH 3T3 cells after ligand activation (Klein *et al.*, 1992). For the ectodomains, we used the avian receptors, since nearly all chicken homologs of the Elk subgroup have been cloned as full-length cDNAs. These include the Cek9 receptor, for which no mammalian homolog has yet been identified. In the case of the Elk receptor, we used the mammalian homolog, since the chicken Cek6 cDNA lacks the extreme N-terminus (Sajjadi and Pasquale, 1993). In addition to the Elk subgroup, we also constructed chimeric receptors containing the ectodomain of the chicken homolog of Mek4, Cek4, and the cytoplasmic domain of TrkB. Since transmembrane domains may have an influence on receptor activation (Cao *et al.*, 1992), we constructed two different types of Cek4–TrkB chimeric receptors, containing either the transmembrane domain of Cek4 (Cek4–TrkB¹) or that of TrkB (Cek4–TrkB²). All chimeric receptors were expressed at similar levels in NIH 3T3 cells (Figure 2B). Moreover, all chimeric receptors were tyrosine phosphorylated to varying degrees in the absence of ligand (Figure 2C). To our surprise, both Cek4–TrkB constructs caused focus formation of NIH 3T3 cells in a dose-dependent manner in the absence of exogenous ligand (Table I). Transformation was clearly dependent on the TrkB cytoplasmic domain, since wild-type Cek4 did not cause transformation, even when expressed at high levels (data not shown). These results suggested that the Cek4 ectodomain was sufficient to dimerize the receptor and to thereby activate the TrkB tyrosine kinase domain. Binding studies on NIH 3T3 cells using a soluble Mek4 ectodomain fused to a placental alkaline phosphatase (AP) tag (Cheng and Flanagan, 1994) showed efficient binding of Mek4–AP to NIH 3T3 cells (Figure 3), indicating that a Mek4/Cek4 ligand was expressed at the cell surface of NIH 3T3 cells. The presence of this endogenous Mek4/Cek4 ligand most likely caused transformation through an autocrine loop. In contrast to Cek4–TrkB, expression of the four

Table I. Transformation of NIH 3T3 cells by transfection of chimeric receptors containing the TrkB cytoplasmic domain

Transfected DNAs		Transforming activity (foci per 1.5×10^5 cells)	
Receptor	DNA (ng)	Exp.1	Exp.2
Cek4-TrkB ¹	30	>500	>500
Cek4-TrkB ¹	10	>500	>500
Cek4-TrkB ¹	3	172	160
Cek4-TrkB ¹	1	64	76
Cek4-TrkB ²	30	>500	>500
Cek4-TrkB ²	10	>500	>500
Cek4-TrkB ²	3	136	140
Cek4-TrkB ²	1	45	64
Cek5-TrkB 500	0	0	
Cek9-TrkB	500	0	0
Cek10-TrkB	500	5	7
Elk-TrkB	500	32	24
TrkB	1000	1	0
BDNF	10	0	0
TrkB + BDNF	1000 + 10	224	188

members of the Elk subgroup as TrkB chimeric receptors did not cause a significant number of foci in the absence of exogenous ligand (Table I). These results indicated that focus formation in NIH 3T3 cells could be utilized as a biological assay to study functional ligand–receptor interactions using Eph-related/TrkB chimeric receptors, provided that the ligands were not expressed by the host cells.

Membrane-bound LERK2 ligand signals through Cek5, Cek10 and Elk, but not Cek9 chimeric receptors

We next determined whether or not LERK2 could signal through any of the four chimeric receptors of the Elk subgroup. As shown in Figure 4 and Table II, co-transfection of LERK2 with either Cek5–TrkB, Cek10–TrkB or Elk–TrkB expression plasmids, caused dose-dependent transformation of NIH 3T3 cells. No transformation was observed when we co-expressed Cek9 chimeric receptors with LERK2, indicating that the more distantly related Cek9 receptor interacts with a ligand distinct from LERK2. Interestingly, maximum transforming activity mediated by Cek5 and Elk chimeric receptors was observed at a 5:1 ratio of receptor to ligand DNA, e.g. at 500 ng and 100 ng, respectively. Higher amounts of ligand DNA was either saturating or inhibitory for Cek5 and Elk mediated transformation. Inhibition of Cek10–TrkB-induced transformation was observed with 5 µg of LERK2 expression plasmid DNA (data not shown). We observed a difference in the morphology of foci generated by Cek10–TrkB, compared with Cek5–TrkB and Elk–TrkB. Whereas the foci generated by Cek5 and Elk chimeric receptors were very compact, the morphology of Cek10–TrkB foci was more heterogeneous, with some compact and some diffuse (Figure 4). This heterogeneity is likely to be due to the expression of low levels of endogenous LERK2 or another, as yet unknown, ligand for Cek10. Binding assays using a soluble Cek10–AP fusion protein on wild-type NIH 3T3 cells consistently showed low levels of Cek10 binding

activity, which was not observed with either Cek5–AP or Elk–AP proteins (see Figure 8). Moreover, we confirmed the presence of LERK2 transcripts in wild-type NIH 3T3 by RT-PCR and subsequent cloning of mouse LERK2 (data not shown). In summary, these results indicated that LERK2 can interact functionally with the three most closely related members of the Elk subgroup, Cek5, Cek10 and Elk receptors. Of the three receptors, Cek10 seemed to interact most efficiently with LERK2.

Membrane-bound LERK2 induces phosphorylation of Cek10 chimeric receptors

We next examined whether the observed functional interaction between LERK2 and Cek10 was accompanied by tyrosine phosphorylation of the chimeric receptor. For this purpose, we isolated NIH 3T3-derived cell lines expressing either Cek10–TrkB alone or after co-transfection with an expression plasmid encoding LERK2. Western blot analysis using a TrkB-specific antiserum demonstrated equal levels of receptor protein in both cell lines (Figure 5A). Co-transfection with LERK2 ligand caused a ~5-fold increase in tyrosine phosphorylation of the receptor (Figure 5B). In addition, a minor, slower migrating phosphorylated protein could be observed in cells co-expressing LERK2 (Figure 5B). Since this minor protein was specifically immunoprecipitated with anti-Trk antiserum, it could either be a hyperphosphorylated form of the chimeric receptor, or a co-precipitating receptor substrate. Tyrosine phosphorylation of the Cek10 chimeric receptor was also observed after co-culturing Cek10–TrkB expressing NIH 3T3 cells with LERK2-expressing COS cells (Figure 5D). Receptor levels did not change during the incubation period with COS cells (Figure 5C). These results clearly indicate that membrane-bound LERK2 induced tyrosine phosphorylation of Cek10–TrkB receptors either in an autocrine mechanism or via cell–cell contact.

Autocrine stimulation has previously been shown to take place after co-expression of Trk receptors with their soluble ligands. As a consequence, cell proliferation was less dependent on serum concentration in the culture medium (Lamballe *et al.*, 1993). Consistent with these findings, we observed a 3-fold increase in growth rate of cell populations co-transfected with chimeric receptors and LERK2, compared with those expressing chimeric receptors alone, when grown in 1% calf serum. Up to a 10-fold increase in growth rates was observed in clonal cell lines derived from foci after co-transfection of LERK2 with either Cek10–TrkB or Cek5–TrkB (data not shown).

LERK2 binds to Cek5, Cek10 and Elk receptors

To reinforce the data obtained from the focus formation assays, LERK2 was tested for direct binding to Eph receptors. We constructed a soluble LERK2–AP fusion protein, containing the entire LERK2 ectodomain fused to an AP tag, and determined its binding to the NIH 3T3-derived cell lines expressing Cek5, Cek9, Cek10 and Elk chimeric receptors. Consistent with the focus-forming activity of LERK2, LERK2–AP specifically bound Cek5, Cek10 and Elk receptors (Figure 6A). Binding to cells expressing Cek9 receptors was comparable with wild-type NIH 3T3 cells. No specific binding was observed with the unfused AP tag or with Elf1–AP fusion protein. Since LERK2 is a transmembrane protein, we also carried out

Fig. 4. Transformation of NIH 3T3 cells by co-transfection of expression plasmids encoding Cek5, Cek9, Cek10 and Elk chimeric receptors and membrane-bound LERK2 ligand. NIH 3T3 cells were co-transfected with the indicated amounts of expression plasmid DNA using the calcium phosphate precipitation technique. Titration of ligand expression plasmid DNA reveals that maximal transforming activity is achieved at a 5:1 ratio of receptor to ligand (500 ng and 100 ng, respectively). Plates were stained with Giemsa 14 days later. Each photograph shows an area of $\sim 40 \text{ cm}^2$.

the reverse experiment. A NIH 3T3-derived cell line expressing membrane-bound LERK2 was tested for binding of soluble receptor ectodomain-AP fusion proteins. Similar to the previous experiment, LERK2-expressing cells specifically bound Cek5-AP, Cek10-AP and Elk-AP proteins (Figure 6B). No specific binding was observed with Cek9-AP or the AP tag alone. For Cek5, Cek10 and Elk, the three receptors showing LERK2-specific binding, we performed Scatchard analyses. Correlating with the results obtained by focus formation assays, Cek10 bound LERK2 with highest affinity with a dissociation constant (K_D) of $2.7 \times 10^{-10} \text{ M}$. The affinity of Cek5 was intermediate with a K_D of $4.2 \times 10^{-10} \text{ M}$. Elk-AP binding to LERK2 was biphasic with high affinity (K_D of $7.2 \times 10^{-10} \text{ M}$) and low affinity binding sites (K_D of $4.8 \times 10^{-9} \text{ M}$) (Figure 6C-E). This binding data, together with the focus-forming activity of chimeric receptors, suggests that Cek10 is the preferred LERK2 receptor. In addition, LERK2 is able to signal through Cek5 and Elk receptors.

LERK2 signaling activity is reduced by its own cytoplasmic domain

LERK2 is characterized by a transmembrane and a large, 83 amino acid long cytoplasmic domain. Other members of this family appear to be attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. To address the role of transmembrane and cytoplasmic domains in signaling, we constructed C-terminal deletion mutants of LERK2 and tested them in the NIH 3T3 focus formation

assay. As shown in Figure 7, LERK2 containing its transmembrane domain, but lacking the entire cytoplasmic domain (LERK2 Δ C) effectively transformed NIH 3T3 cells expressing Cek5-TrkB, Cek10-TrkB and Elk-TrkB. Interestingly, LERK2 Δ C produced foci that were consistently much larger than those observed with wild-type LERK2 (Figure 7). Titration of LERK2 expression plasmids indicated that the number of foci were also slightly higher with LERK2 Δ C compared with wild-type LERK2 (data not shown). In contrast, two different deletion mutants (designated S1 and S2) producing soluble forms of LERK2 lacking both transmembrane and cytoplasmic domains were completely inactive at all concentrations tested (from 10 ng to 5 μg ; data not shown). To rule out that the increased transforming activity of LERK2 Δ C was caused by higher expression levels of LERK2 Δ C, we determined the levels of cell surface expression of both ligands. NIH 3T3 cells were transfected with either wild-type LERK2 or LERK2 Δ C, incubated in selection medium for 7 days and the entire pool of cells assayed for binding to Cek10-AP. As depicted in Figure 8, Cek10-AP bound equally well to cell surface-bound wild-type LERK2 and LERK2 Δ C, demonstrating equal levels of active ligand on the cell surface of NIH 3T3 cells. In summary, these results indicated that the cytoplasmic domain was not necessary for LERK2 activity, at least in fibroblasts. To the contrary, the qualitative increase in focus size suggests a negative regulatory role for the LERK2 cytoplasmic domain in LERK2 signaling.

Table II. Transformation of NIH 3T3 cells by co-transfection of LERK2 ligand and Cek5, Cek10 and Elk chimeric receptors

Co-transfected DNAs		Transforming activity (foci per 1.5×10^5 cells)	
Receptor DNA (500 ng)	LERK2 ligand DNA (ng)	Exp.1	Exp.2
–	1000	1	0
–	100	0	0
Cek5–TrkB	–	2	7
Cek5–TrkB	1000	36	26
Cek5–TrkB	100	>500	>500
Cek5–TrkB	10	180	188
Cek5–TrkB	1	6	18
Cek9–TrkB	–	0	0
Cek9–TrkB	1000	0	0
Cek9–TrkB	100	1	2
Cek9–TrkB	10	0	0
Cek9–TrkB	1	0	1
Cek10–TrkB	–	4	0
Cek10–TrkB	1000	>500	>500
Cek10–TrkB	100	>500	>500
Cek10–TrkB	10	128 ^a	ND
Cek10–TrkB	1	43 ^a	ND
Elk–TrkB	–	38	38
Elk–TrkB	1000	26	12
Elk–TrkB	100	420	438
Elk–TrkB	10	142	128
Elk–TrkB	1	24	34

^aFoci blend in with monolayer of non-transformed NIH 3T3 cells. The number may therefore be underestimated. ND, not done.

Discussion

The recent cloning of a family of B61-related ligands has provided the tools to uncover the biological functions of the signaling pathways mediated by the Eph-related receptor family. Difficulties in demonstrating ligand-induced receptor activation (Beckmann *et al.*, 1994) and the lack of measurable biological responses in cells expressing the receptors have, however, considerably hampered progress. Chimeric receptors similar to those described in this report, have been used in the past to study cytoplasmic signaling capabilities independent from ligand–receptor interactions. It was shown that the ectodomain determines ligand binding specificity, whereas the cytoplasmic domain determines kinase specificity, mitogenic and transforming potential, and receptor routing (Riedel *et al.*, 1989). The chimeric receptor approach has been particularly successful in the case of orphan receptors, allowing the study of the signaling capabilities of the orphan receptor catalytic domain when fused to a heterologous ectodomain (Ellis *et al.*, 1987; Lee *et al.*, 1989; Lehvaslaiho *et al.*, 1989; Riedel *et al.*, 1989; DiFiore *et al.*, 1990; Lev *et al.*, 1991). We have used a variation of this technique and replaced a non-transforming cytoplasmic domain with a powerful kinase domain, in order to study ligand–receptor interactions. We demonstrated that membrane-bound LERK2 was capable of binding and activating the three most closely related Cek5, Cek10 and Elk receptors and induced transformation of cells expressing these receptors, when fused to the TrkB cytoplasmic domain. LERK2 bound most efficiently to Cek10 and induced more foci

Fig. 5. Membrane-bound LERK2 induces tyrosine phosphorylation of Cek10 chimeric receptors. NIH 3T3 cells expressing either Cek10–TrkB receptors alone (a) or after co-transfection with a LERK2-encoding expression plasmid (b) were lysed and immunoprecipitated with a polyclonal rabbit antiserum raised against a peptide encoding the 14 C-terminal amino acid residues of human gp140^{TrkA}. Immunoprecipitates were separated by 7.5% SDS–PAGE, transferred to nitrocellulose filters, and blotted with either a polyclonal rabbit antiserum raised against bacterially expressed TrkB tyrosine kinase domain (A), or monoclonal antibodies against phosphotyrosine (B). NIH 3T3 cells expressing Cek10–TrkB receptors were cultured for 48 h at 37°C either in the absence (d) or presence (e) of COS cells transiently transfected with expression plasmids encoding membrane-bound LERK2 ligand. The same COS cells were also incubated alone in the absence of Cek10–TrkB-expressing cells (c). Cell lysates were analyzed by immunoprecipitation and Western blot as described above. The anti-TrkB Western blot is shown in (C), the anti-phosphotyrosine Western blot in (D).

in the presence of Cek10–TrkB, than in the presence of Cek5–TrkB or Elk–TrkB. This suggests that Cek10 is the preferred LERK2 receptor. We have recently cloned a second transmembrane Eph ligand, called Elf-2, which is structurally closely related to LERK2 and which also binds to the same three receptors (A.D.Bergemann, H.-J.Cheng, R.Brambilla, R.Klein and J.G.Flanagan, submitted). Comparative binding and functional studies between LERK2 and Elf-2 are in progress and will reveal their relative efficiencies to activate the Elk receptor subgroup. Interestingly, the closely related Cek9 receptor, which does not interact with LERK2, also does not interact with Elf-2. This is quite remarkable, since the ectodomains of Cek9 and Cek5 are 57% identical. For comparison, the ectodomains of Cek5, Cek10 and Elk are 63–68% identical. In summary, B61-related ligands can be subdivided into two subfamilies based on whether they possess a transmembrane domain or whether they are membrane bound via a GPI anchor. The present data suggests that transmembrane ligands show preference for the Elk subgroup of Eph receptors, whereas GPI-anchored ligands bind the more distantly related Eck subgroup of Eph receptors (Figure 9).

Our findings that Cek5, Cek10 and Elk receptors can respond to the same ligands, albeit with different efficiencies, have some important implications for the study of these receptors in neural development. The mouse homologs of Cek5 and Cek10, Nuk and Sek4, have many common domains of expression during mouse development, including rhombomeres 3 and 5 in the hindbrain, and other areas of midbrain, forebrain, spinal cord and neural crest derivatives. The fact that Nuk and Sek4 can recognize the same ligand suggests that they exhibit similar levels of activation in those structures.

Fig. 6. Binding of LERK2 to Eph-related receptors. Binding is indicated as the AP activity bound to cells in a 3.5-cm tissue culture dish. (A) Binding of LERK2-AP or Elf1-AP ligand fusion proteins or unfused AP control, to receptors on NIH 3T3 cells stably expressing Cek5, Cek9, Cek10 or Elk chimeric receptors, or to non-transfected NIH 3T3 cells (NIH). (B) Binding of soluble Cek5-AP, Cek9-AP, Cek10-AP or Elk-AP fusion proteins, or unfused AP control, to wild-type NIH 3T3 cells or NIH 3T3 expressing membrane-bound LERK2. (C–E) Scatchard analyses of the binding of Cek10-AP, Cek5-AP and Elk-AP, respectively, to membrane-bound LERK2 expressed in NIH 3T3 cells. Binding characteristics calculated for the experiments shown are as follows: for Cek10, 1.5×10^5 sites per cell with $K_D = 2.7 \times 10^{-10}$ M; for Cek5, 6×10^4 sites per cell with $K_D = 4.2 \times 10^{-10}$ M; for Elk, 3.9×10^4 and 1.5×10^5 sites per cell with K_D s of 7.2×10^{-10} M and 4.8×10^{-9} M, respectively.

Double Nuk/Sek4 knockout mice are being generated to determine whether these receptors have redundant functions in the structures in which they are both expressed (D.Orioli, M.Henkemeyer, G.Lemke, T.Pawson and

R.Klein, unpublished results). The related Sek1 receptor was also shown to be expressed in rhombomeres 3 and 5 in the hindbrain, although slightly earlier in embryogenesis (Becker *et al.*, 1994). Sek1, together with Mek4, was

Fig. 7. Deletion of the entire cytoplasmic domain increases the transforming activity of LERK2. NIH 3T3 cells were co-transfected with 100 ng of expression plasmids encoding either wild-type LERK2 or a deletion mutant of LERK2 lacking the entire cytoplasmic domain (LERK2 Δ C) and with 500 ng of expression plasmids encoding Cek5-TrkB, Cek10-TrkB or Elk-TrkB chimeric receptors. Note the large size of foci generated by LERK2 Δ C compared with wild-type LERK2. Plates were stained with Giemsa 14 days later. Each photograph shows an area of ~ 40 cm².

recently shown to bind ELF-1, a related but distinct ligand of the B61 family (Cheng and Flanagan, 1994). An intriguing hypothesis is that different ligand-receptor pairs of the same family may be used in a sequential fashion to regulate, in a co-operative manner, the segmentation of brain structures.

Our preliminary structure-function analysis of LERK2 suggests that the LERK2 protein needs membrane attachment to interact functionally with its receptors. Soluble forms of LERK2 failed to transform NIH 3T3 cells. During the preparation of this manuscript, a study was published (Davis *et al.*, 1994), demonstrating that B61-related ligands require membrane attachment or clustering for receptor activation. Those results are in agreement with ours. It was suggested that membrane attachment of the ligands may facilitate their dimerization or aggregation, leading to enhanced receptor activation. Our studies suggest that in addition to cell-cell contact, receptor activation may also occur in an autocrine fashion. Autocrine mechanisms have been previously observed in other signaling pathways, e.g. with neurotrophins and Trk receptors (Korsching, 1993), and may therefore also involve membrane-bound ligands and their Eph-related receptors.

We have found that the cytoplasmic domain of LERK2 is not necessary for its signaling activity in fibroblasts. Surprisingly, the observed transformation of NIH 3T3 cells with the LERK2 Δ C deletion mutant was stronger than with wild-type LERK2. Binding assays with soluble Cek10-AP fusion proteins on cells expressing either wild-type LERK2 or LERK2 Δ C show similar binding, implying that both proteins are equally well expressed on the cell

Fig. 8. Cell surface expression of LERK2 Δ C. NIH 3T3 cells were transfected in duplicate with 1 μ g of expression plasmid encoding either wild-type LERK2 or LERK2 Δ C, incubated in selection medium for 7 days and the entire pool of selected cells assayed in 3.5 cm tissue culture plates for binding to either unfused AP control (black bars) or Cek10-AP (hatched bars) (5 nM). Non-transfected NIH 3T3 cells (NIH) were used as control.

surface. Therefore, it is likely that the cytoplasmic domain plays a negative regulatory role in LERK2 signaling. The cytoplasmic domain of LERK2 may be the target of cytoplasmic factors, such as kinases, that can introduce modifications which may negatively influence its aggregating behaviour. In this regard, it is worth noting that 19 out of 83 amino acids of the cytoplasmic domain are either serine, threonine or tyrosine residues. Another possibility could be that factors that regulate ligand-receptor internalization act on the cytoplasmic domain of LERK2. It has been shown that the Boss ligand for the sevenless (sev) tyrosine kinase receptor, is a seven-membrane spanning protein that is internalized by the R7 photoreceptor cell after sev activation (Cagan *et al.*, 1992). Modifications of the cytoplasmic domain of LERK2 may have an influence on possible internalization events by the receptor-bearing cells. Whatever the modifications of the cytoplasmic domain may be, they are likely to be conserved between LERK2 and Elf-2. The cytoplasmic domains of LERK2 and Elf-2 are remarkably well conserved, including a carboxy terminal stretch of 33 identical amino acids. A more detailed structure-function analysis of LERK2 protein is in progress, making use of the quantitative NIH 3T3 focus formation assay.

In summary, the discovery of functional ligand-receptor interactions involving B61-related ligands and Eph-related receptors and the simultaneous analysis of germline-targeted mice deficient in several members of this receptor family will rapidly increase our knowledge about the function of this signaling pathway in mammalian development.

Materials and methods

Cloning of LERK2

The complete open reading frame of the published human LERK2 cDNA (Beckmann *et al.*, 1994) was obtained by RT-PCR using as a template

Fig. 9. Schematic representation of the known interactions between the B61-related family of ligands with the Eph-related family of receptors. The names of the chicken, mouse, rat and human homologs are indicated where appropriate. Arrows indicate physical, biochemical or biological interactions. Thicker arrows indicate preferred ligand-receptor interactions. The figure contains the data from the present study and from Bartley *et al.* (1994); Beckmann *et al.* (1994); Cheng and Flanagan (1994); Davis *et al.* (1994); Shao *et al.* (1994, 1995); and A.D.Bergemann, H.-J.Cheng, R.Brambilla, R.Klein and J.G.Flanagan, submitted.

RNA derived from two different human carcinoma cell lines, A-431 and MCF-7. Briefly, 25 µg of total RNA were reverse transcribed into cDNA using oligo(dT) primers and Superscript Reverse Transcriptase (BRL) and amplified using the following 5' and 3' amplimers: 5'-CGGGATCCGGGAAGATGGCTCGGCCTGGGCAGCG-3' (upstream primer, nucleotides 304–328 of the published sequence) and 5'-CGGAATTCGGGCACTCAGACCTGTAGTAGATGTTCCGCC-3' (downstream primer, nucleotides 1354–1324). The amplified fragment was then subcloned into the mammalian expression vector pMEXneo (Martin-Zanca *et al.*, 1989) using *Bam*HI and *Eco*RI restriction enzyme sites (underlined in the primer sequences). The resulting plasmid, pRB29, was entirely sequenced to confirm the correct sequence. The three different deletion mutants described in the text were generated by PCR using the same 5' amplimer as indicated above and one of the following 3' primers: 5'-CGGAATTCAGGAGTTGAAGAAGCCATCAGGGTCCCCGC-3' (S1 soluble form); 5'-CGGAATTCAGCTGCTGCCCCCACTTGCACCTGG-3' (S2 soluble form); 5'-CGGAATTCAGGCAGCCGCCGCTGCTGTGTG-3' (ΔC transmembrane form). All the amplification products were then digested with *Bam*HI and *Eco*RI and subcloned in pMEXneo vector. For COS cell transfections, the same *Bam*HI-*Eco*RI fragment of pRB29 was subcloned in pMT2* expression vector (Ponzetto *et al.*, 1994).

Construction of chimeric receptors

All chimeric receptors with the exception of EGFR-Mek4, Cek4-TrkB² and Elk-TrkB were constructed by fusing the extracellular and transmembrane domains of the relevant receptors with the intracellular portion of the mouse TrkB cDNA. This was carried out by creating a *Nar*I site at position 1873–1878 of the published TrkB cDNA sequence (Klein *et al.*, 1989) by PCR amplification of a 184 bp long fragment, using an existing *Bst*XI site (nucleotides 2051–2062) for subcloning. In the human EGFR cDNA a corresponding *Nar*I site already exists immediately downstream of the transmembrane domain (nucleotides 2195–2200) (Ullrich *et al.*, 1984). This site was used to generate the EGFR-TrkB chimera in the expression vector pMEXneo (pFRK62). All the other chimeras were created using the same *Nar*I strategy. The EGFR-Mek4 chimera was constructed by first introducing by PCR a silent *Nar*I site in the Mek4 cDNA (nucleotides 1726–1731) (Sajjadi *et al.*, 1991) at the same position previously used for TrkB in pFRK62, and then by fusing the entire Mek4 intracellular domain to the EGFR cDNA at the same *Nar*I site. The Cek4-TrkB² construct was obtained by introducing a *Hind*III site in Cek4 (nucleotides 1649–1654) (Sajjadi *et al.*, 1991) and in TrkB (nucleotides 1796–1801) (Klein *et al.*, 1989) to allow an in-frame fusion of the two sequences. Finally the extracellular portion of mouse Elk cDNA was obtained by RT-PCR amplification from P10 mouse brain total RNA using primers based on the published rat sequence (Lhotak *et al.*, 1991). The following primers were used:

5'-CGCGGATCCGATGGCCCTGGATTGCCTGC-3' (nucleotides 1–29, *Bam*HI site underlined); 5'-TCAGGGGAAGCTTCTCTCTCAGCTCCG-3' (nucleotides 1629–1603, *Hind*III site underlined). The amplified fragment was subcloned in pMEXneo vector together with the *Hind*III-*Eco*RI TrkB fragment also used for the Cek4-TrkB² chimera. The resulting in frame construct was entirely sequenced.

A segment of the amino acid sequence at the junction region is listed below for each chimeric receptor. The junction point is indicated with /. The name of the corresponding expression plasmid is shown in brackets.

EGFR-TrkB	(pFRK62)	LFMR / <i>RHSKF</i>
EGFR-MEK4	(pAS26)	LFMR / <i>RFCGY</i>
Cek5-TrkB	(pRB3)	VCNRR / <i>RHSKF</i>
Cek9-TrkB	(pAS13)	FKSKR / <i>RHSKF</i>
Cek10-TrkB	(pAS18)	IVCFR / <i>RHSKF</i>
Cek4-TrkB ¹	(pAS6)	VLIGR / <i>RHSKF</i>
Cek4-TrkB ²	(pAS15)	SENSK / <i>LSVYA</i>
Elk-TrkB	(pAS42)	ELREK / <i>LSVYA</i>

Since the most complete Cek9 cDNA clone available at the time, was lacking the hydrophobic signal peptide, we fused to its ectodomain the sequence corresponding to the published Cek5 leader peptide (nucleotides 1–87) (Pasquale, 1991). The same Cek9 construct was used as a Cek9-AP fusion protein (see below) and was released into the culture medium showing proper transport to the cell surface. The cDNA encoding the extracellular domain of Cek9 will be published elsewhere (C.Soans and E.B.Pasquale, in preparation), but the nucleotide sequence is being deposited in the GeneEMBL database. The complete Cek10 sequence including the unpublished 5' end is available at GenBank/GeneEMBL database under the accession number Z19061.

Cell culture and gene transfer assays

COS cells (Gluzman, 1981), NIH 3T3 cells (Jainchill *et al.*, 1969) and NIH 3T3-derived cell lines, including a subclone lacking endogenous EGF receptors, 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 done by the DEAE-dextran/chloroquin method (Seed and Aruffo, 1987).

Phosphorylation and immunoprecipitation assays

To induce tyrosine phosphorylation of the Cek10-TrkB chimera, 1×10^6 NIH 3T3 cells expressing Cek10-TrkB were seeded together with the same number of COS cells transiently transfected with a LERK2 expression plasmid. Cells were co-cultured for 48 h at 37°C and subsequently lysed in the presence of orthovanadate as previously described (Klein *et al.*, 1991). Cell extracts were immunoprecipitated

with rabbit polyclonal antisera raised against a peptide corresponding to the 14 C-terminal amino acid 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 either with the mouse anti-phosphotyrosine monoclonal antibody 4G10 (UBI) or with an antiserum raised against the mouse TrkB tyrosine kinase domain expressed in bacteria (Klein *et al.*, 1990). The following incubations were then performed using rabbit anti-mouse HRP-linked antibodies or protein A HRP-linked, respectively. Specific signals were revealed using the ECL detection system (Amersham). For EGF-induced tyrosine phosphorylation of EGFR-Mek4 and EGFR-TrkB receptors, cells were stimulated for 10 min at 37°C with 100 ng/ml EGF (Boehringer). Cell lysates were incubated with mouse monoclonal antibody against the ectodomain of EGFR (gift of A.Ullrich and also purchased from UBI). Immunoprecipitation and Western blot analysis were performed as above.

DNA synthesis assay

Cells grown on glass coverslips at a density of 5×10^5 per well were made quiescent by incubating them in DMEM containing 0.5% calf serum (CS) for 48 h. Cells were stimulated with EGF (100 ng/ml) or CS (20%) for 18 h, followed by an additional 6 h incubation in the presence of 100 μ M bromodeoxyuridine (BrdU). BrdU incorporation was measured by immunofluorescence essentially as described (Cordon-Cardo *et al.*, 1991). Briefly, cells were fixed with cold methanol for 10 min on ice, denatured for 30 min in 1.5 M HCl in PBS, and incubated for 30 min with a mouse anti-BrdU monoclonal antibody coupled to fluorescein (Boehringer). Finally, the cells were incubated for 10 min with Hoechst dye to visualize the nuclei. Coverslips were mounted onto glass slides using Mowiol (Calbiochem) and analyzed under a fluorescence microscope.

Binding assays

To test for ligand binding to Eph receptors expressed on NIH 3T3 cells, the extracellular domain of LERK2 was fused to a placental alkaline phosphatase (AP) tag. The LERK2 extracellular domain was amplified by PCR, introducing an artificial *HindIII* site at the 5' end (nucleotide 304 of the published sequence) and an artificial *BglII* site at the end of the extracellular domain (nucleotide 1006 of the published sequence). This fragment was then inserted into the plasmid pAptag-2 (A.D. Bergemann, H.-J.Cheng, R.Brambilla, R.Klein and J.G.Flanagan, submitted), to produce pLERK2-AP encoding a fusion protein with Ser236 of LERK2 linked to AP through a four amino acid linker (Arg-Ser-Ser-Gly). To test for binding of soluble receptor ectodomains to membrane-bound LERK2, we constructed receptor-AP fusions using the same strategy. The extracellular domains of Cek5, Cek10 and Elk were amplified by PCR introducing an artificial *HindIII* site at the 5' end (immediately upstream of the ATG) and an artificial *BamHI* site at the end of the extracellular domain. Cek9 was amplified as a *BamHI*-*BamHI* fragment. A segment of the amino acid sequence at the junction region is listed below for each chimeric receptor. The junction is indicated with /:

pCek5-AP	SVQE / GSSG
pCek9-AP	MAQD / GSSG
pCek10-AP	TFQE / GSSG
pElk-AP	ELRE / GSSG

COS cells were transfected using Lipofectamine (Gibco-BRL), and the production of the AP fusion proteins was monitored by assaying the supernatant for heat-stable alkaline phosphatase. Quantitative studies of receptor-ligand interactions using the binding of soluble AP fusion proteins to cell surfaces were performed as described previously (Flanagan and Leder, 1990; Cheng and Flanagan, 1994).

Acknowledgements

We wish to thank A.Ullrich for anti EGFR monoclonal antibodies, Hwai-Jong Cheng for the Mek4-AP and Elf1-AP proteins, and Katja Brückner for testing the NIH 3T3 cell line expressing LERK2. We are thankful to S.Courtneidge, F.Maina and K.Lloyd for critically reading the manuscript. This work was supported by postdoctoral fellowships from the Human Frontier Science Program Organization (R.B.), from the European Molecular Biology Organization (A.S.) and the American Cancer Society (A.D.B.) and by NIH grants DK45580 and HD29417 (J.G.F.) and CA56721 and HD26351 (E.B.P.) and by grant KI 948/1-1 from the Deutsche Forschungsgemeinschaft (R.K.).

References

- Barbacid,M. (1993) Nerve growth factor: a tale of two receptors. *Oncogene*, **8**, 2033-2042.
- Bartley,T.D. *et al.* (1994) B61 is a ligand for the ECK receptor protein-tyrosine kinase. *Nature*, **368**, 558-560.
- Becker,N., Seitanidou,T., Murphy,P., Mattei,M.-G., Topilko,P., Nieto, M.A., Wilkinson,D.G., Charnay,P. and Gilardi-Hebenstreit,P. (1994) Several receptor tyrosine kinase genes of the *Eph* family are segmentally expressed in the developing hindbrain. *Mech. Dev.*, **47**, 3-17.
- Beckmann,M.P. *et al.* (1994) Molecular characterization of a family of ligands for eph-related tyrosine kinase receptors. *EMBO J.*, **13**, 3757-3762.
- Böhme,B., Holtrich,U., Wolf,G., Luzius,H., Grzeschik,K.-H., Strebhardt,K. and Rübsamen-Waigmann,H. (1993) PCR mediated detection of a new human receptor-tyrosine-kinase, HEK 2. *Oncogene*, **8**, 2857-2862.
- Cagan,R., Kramer,H., Hart,A. and Zipurski,S. (1992) The bride of sevenless and sevenless interaction: internalization of a transmembrane ligand. *Cell*, **69**, 393-399.
- Cao,H., Bangalore,L., Bormann,B. and Stern,D. (1992) A subdomain in the transmembrane domain is necessary for p185neu activation. *EMBO J.*, **11**, 923-932.
- Cheng,H.-J. and Flanagan,J.G. (1994) Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. *Cell*, **79**, 157-168.
- Cordon-Cardo,C., Tapley,P., Jing,S.Q., Nanduri,V., O'Rourke,E., Lamballe,F., Kovary,K., Klein,R., Jones,K.R., Reichardt,L.F. and Barbacid,M. (1991) The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. *Cell*, **66**, 173-183.
- Davis,S., Gale,N.W., Aldrich,T.H., Maisonpierre,P.C., Lhotak,V., Pawson,T., Goldfarb,M. and Yancopoulos,G.D. (1994) Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science*, **266**, 816-819.
- DiFiore,P., Segato,O., Taylor,W., Aaronson,S. and Pierce,J. (1990) EGF receptor and erbB-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. *Science*, **248**, 79-83.
- Ellis,L., Morgan,D., Jong,S., Wang,L., Roth,R. and Rutter,W. (1987) Heterologous transmembrane signaling by a human insulin receptor-v-rs hybrid in Chinese hamster ovary cells. *Proc. Natl Acad. Sci. USA*, **84**, 5101-5105.
- Flanagan,J. and Leder,P. (1990) The kit ligand: a cell surface molecule altered in Steel mutant fibroblasts. *Cell*, **63**, 185-194.
- Gluzman,Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. *Cell*, **23**, 175-182.
- Graham,F. and van der Eb,A. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*, **52**, 456-467.
- Henkemeyer,M., Marengere,L.E.M., McGlade,J., Olivier,J.P., Conlon, R.A., Holmyard,D.P., Letwin,K. and Pawson,T. (1994) Immunolocalization of the Nuk receptor tyrosine kinase suggests roles in segmental patterning of the brain and axonogenesis. *Oncogene*, **9**, 1001-1014.
- Holzman,L., Marks,R. and Dixit,V. (1990) A novel immediate-early response gene of endothelium is induced by cytokines and encodes a secreted protein. *Mol. Cell. Biol.*, **10**, 5830-5838.
- Jainchill,J., Aaronson,S. and Todaro,G. (1969) Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. *J. Virol.*, **4**, 549-553.
- Klein,R., Parada,L.F., Coulier,F. and Barbacid,M. (1989) trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. *EMBO J.*, **8**, 3701-3709.
- Klein,R., Conway,D., Parada,L.F. and Barbacid,M. (1990) The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell*, **61**, 647-656.
- Klein,R., Jing,S.Q., Nanduri,V., O'Rourke,E. and Barbacid,M. (1991) The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell*, **65**, 189-197.
- Klein,R., Lamballe,F., Bryant,S. and Barbacid,M. (1992) The trkB tyrosine protein kinase is a receptor for neurotrophin-4. *Neuron*, **8**, 947-956.
- Korsching,S. (1993) The neurotrophic factor concept: a re-examination. *J. Neurosci.*, **13**, 2739-2748.
- Lamballe,F., Tapley,P. and Barbacid,M. (1993) trkC encodes multiple neurotrophin-3 receptors with distinct biological properties and substrate specificities. *EMBO J.*, **12**, 3083-3094.

- Lee,J., Dull,T., Lax,I., Schlessinger,J. and Ullrich,A. (1989) HER cytoplasmic domain generates normal mitogenic and transforming signals in a chimeric receptor. *EMBO J.*, **8**, 167–173.
- Lehvaslaiho,H., Lehtola,L., Sistonsen,L. and Alitalo,K. (1989) A chimeric EGF-R-neu proto-oncogene allows EGF to regulate neu tyrosine kinase and cell transformation. *EMBO J.*, **8**, 159–166.
- Lev,S., Givol,D. and Yarden,Y. (1991) A specific combination of substrates is involved in signal transduction by the kit-encoded receptor. *EMBO J.*, **10**, 647–654.
- Lhotak,V. and Pawson,T. (1993) Biological and biochemical activities of a chimeric epidermal growth factor-Elk receptor tyrosine kinase. *Mol. Cell. Biol.*, **13**, 7071–7079.
- Lhotak,V., Greer,P., Letwin,K. and Pawson,T. (1991) Characterization of Elk, a brain-specific receptor tyrosine kinase. *Mol. Cell. Biol.*, **11**, 2496–2502.
- Martin-Zanca,D., Oskam,R., Mitra,G., Copeland,T. and Barbacid,M. (1989) Molecular and biochemical characterization of the human trk proto-oncogene. *Mol. Cell. Biol.*, **9**, 24–33.
- Maru,Y., Hirai,H. and Takaku,F. (1990) Overexpression confers an oncogenic potential upon the eph gene. *Oncogene*, **5**, 445–447.
- Pasquale,E.B. (1991) Identification of chicken embryo kinase 5, a developmentally regulated receptor-type tyrosine kinase of the Eph family. *Cell Reg.*, **2**, 523–534.
- Pasquale,E.B., Deerinck,T.J., Singer,S.J. and Ellisman,M.H. (1992) Cek5, a membrane receptor-type tyrosine kinase, is in neurons of the embryonic and postnatal avian brain. *J. Neurosci.*, **12**, 3956–3967.
- Pasquale,E.B., Connor,R.J., Rocholl,D., Schnürch,H. and Risau,W. (1994) Cek5, a tyrosine kinase of the Eph subclass, is activated during neural retina differentiation. *Dev. Biol.*, **163**, 491–502.
- Ponzetto,C., Bardelli,A., Zhen,Z., Maina,F., dalla Zonca,P., Giordano,S., Graziani,A., Panayotou,G. and Comoglio,P. (1994) A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell*, **77**, 261–271.
- Riedel,H., Dull,T., Honegger,A., Schlessinger,J. and Ullrich,A. (1989) Cytoplasmic domains determine signal specificity, cellular routing characteristics and influence ligand binding of epidermal growth factor and insulin receptors. *EMBO J.*, **8**, 2943–2954.
- Sajjadi,F.G. and Pasquale,E.B. (1993) Five novel avian Eph-related tyrosine kinases are differentially expressed. *Oncogene*, **8**, 1807–1813.
- Sajjadi,F.G., Pasquale,E.B. and Subramani,S. (1991) Identification of a new eph-related receptor tyrosine kinase gene from mouse and chicken that is developmentally regulated and encodes at least two forms of the receptor. *New Biol.*, **3**, 769–778.
- Seed,B. and Aruffo,A. (1987) Molecular cloning of the CD2 antigen, the T cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl Acad. Sci. USA*, **84**, 3365–3369.
- Shao,H., Lou,L., Pandey,A., Pasquale,E.B. and Dixit,V.M. (1994) cDNA cloning and characterization of a ligand for the Cek5 receptor protein-tyrosine kinase. *J. Biol. Chem.*, **269**, 26606–26609.
- Shao,H., Lou,L., Pandey,A., Verderame,M., Siever,D. and Dixit,V. (1995) cDNA cloning and characterization of a Cek7 receptor protein-tyrosine kinase ligand that is identical to the ligand (ELF-1) for the Mek4 and Sek receptor protein-tyrosine kinases. *J. Biol. Chem.*, **270**, 3467–3470.
- Tuzi,N.L. and Gullick,W.J. (1994) eph, the largest known family of putative growth factor receptors. *Br. J. Cancer*, **69**, 417–421.
- Ullrich,A. et al. (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature*, **309**, 418–425.

Received on January 5, 1995; revised on March 22, 1995