Oncogene (1998) 17, 255–260

http://www.stockton-press.co.uk/onc

SHORT REPORT

Tyrosine-614, the major autophosphorylation site of the receptor tyrosine kinase HEK2, functions as multi-docking site for SH2-domain mediated interactions

Björn Hock[1], Beatrix Böhme[1], Thomas Karn[1], Stephan Feller[2], Helga Rübsamen-Waigmann[3] and Klaus Strebhardt[1]

[1]Chemotherapeutisches Forschungsinstitut, Georg-Speyer-Haus, Paul-Ehrlich-Str. 42-44, 60596 Frankfurt, the [2]Labor für Molekulare Onkologie, Medizinisches Institut für Strahlenkunde & Zellforschung (MSZ), Versbacherstr. 5, 97078 Würzburg and the [3]Bayer AG, Institut für Virologie, 42096 Wuppertal, Germany

HEK2 belongs to the family of EPH-related receptor tyrosine kinases (RTK) which are involved in axonal pathfinding and the formation of the embryonic body plan. The knowledge about intracellular pathways of signal transduction mediated by EPH-related receptors is still limited. Many of the known key players of cellular signalling contain Src homology 2 (SH2) domains, which recognize phosphotyrosine motifs in RTKs. Thus, we examined the interactions of various SH2-containing molecules like PLC-γ1, rasGAP, p85 subunit of PI3-kinase, Src, Fyn, Crk, Nck, Grb2 and Shc with HEK2 using *in vitro* binding assays, immunoprecipitations and yeast Two-Hybrid assays. We found that rasGAP, Crk and Fyn bind in a SH2-dependent manner to autophosphorylated HEK2. rasGAP, which contains two SH2- and one SH3-domain, was shown to associate with its N-terminal SH2-domain to HEK2. Furthermore, we demonstrated that a single amino acid substitution (Y614F) clearly reduces the phosphotyrosine content of HEK2 and abrogates its ability to bind rasGAP, Crk and Fyn indicating that this residue functions as major phosphorylation and multi-docking site. The conservation of this predicted binding site among various EPH-related RTKs provides evidence that Fyn, Crk and rasGAP are key players in signal transduction of at least a subset of these receptors.

Keywords: Eph-related kinases; HEK2; SH2-domains; signal transduction

Eph-related receptors comprise the largest family of receptor tyrosine kinases (RTKs). At least 14 distinct members have been identified so far in vertebrates (Tuzi and Gullick, 1994; Brambilla and Klein, 1995). Up to date eight ligands, named LERKs (ligands for EPH-related kinases) have been described, which are either glycosylphosphatidyl-inositol (GPI)-anchored or transmembrane molecules. The family of EPH-related RTKs can be divided into two major subclasses based on sequence similarity and on their binding preference for either transmembrane or GPI-anchored LERKs (Brambilla and Klein, 1995; Böhme *et al*., 1996; Gale *et al*., 1996). Binding studies using soluble antibody fusion proteins revealed that receptors and ligands within each subclass identify similar expression patterns (Gale *et al*., 1996). Moreover, the composite distributions from a given subclass demonstrate that the developing embryo consists of domains defined by reciprocal and apparently exclusive expression of a receptor subclass and its corresponding ligands. Eph-related RTKs are widely expressed in the nervous system and have been implicated in axon fasciculation and guidance, as well as in segmentation of the embryonic hindbrain and forebrain (reviewed in Brambilla and Klein, 1995).

Recently, we isolated two EPH-related RTKs, named HEK2 (Böhme *et al*., 1993) and HEK5 (Fox *et al*., 1995, Böhme *et al*., unpublished data). Both receptors, which show a widespread expression in adult tissues, are members of the subclass of EPH-related receptors that bind specifically to the transmembrane subgroup of LERKs (Böhme *et al*., 1996; Gale *et al*., 1996). The genetic analysis of NUK and SEK4 (the mouse orthologues of HEK5 and HEK2) revealed a physiological requirement of both receptors for correct pathfinding of specific commissural axons in the central nervous system showing that they have partially overlapping functions (Henkemeyer *et al*., 1996; Orioli *et al*., 1996). Interestingly, mice deficient in SEK4 and NUK die immediately after birth, primarily due to a cleft palate, suggesting that these two receptors have also essential and cooperative functions outside the nervous system.

Up to date, the information about signalling molecules mediating EPH receptor specific responses is still limited to a few family members. After ligand binding the ECK receptor interacts with the p85 subunit of PI3-kinase and elevates its enzymatic activity in endothelial cells (Pandey *et al*., 1995). In addition, activated ECK was shown to bind SLAP, a novel c-Src related adapter protein (Pandey *et al*., 1994). Furthermore, Stein *et al*. (1996) identified an interaction of autophosphorylated ELK with Grb2 and Grb10. The SEK receptor was shown to associate with Fyn by means of a specific interaction between the SH2-domain of Fyn and a phosphorylated tyrosine residue in the juxtamembrane domain of the SEK receptor, which serves as the major autophosphorylation site (Ellis *et al*., 1996).

To characterize the HEK2 gene product, we inserted the complete HEK2 coding sequence into the pRc/

Correspondence: K Strebhardt
The first two authors made equal contributions to this work
Received 3 October 1997; revised 13 February 1998; accepted 13 February 1998

CMV expression plasmid placing the cDNA under the transcriptional control of the cytomegalovirus enhancer-promoter. A second vector containing a kinase-deficient mutant (K665R) of HEK2 was generated by site-directed mutagenesis (Böhme *et al*., 1996). Recently, Ellis *et al*. (1996) reported that Y602 of SEK functions as major phosphorylation site of this receptor. Since this residue is conserved among various EPH-related RTKs, we investigated whether the corresponding residue Y614 in HEK2 serves also as major phosphorylation site. Therefore, we substituted this tyrosine residue by phenylalanine using site-directed mutagenesis. COS-7-transfectants expressing these different forms of HEK2 were analysed by Western blotting. Rabbit polyclonal antibodies directed against the carboxyterminus of HEK2 (aa 897–998) specifically recognized polypeptides of approximately 110 kDa in lysates of either HEK2, K665R or Y614F transfected cells, but not in lysates of untransfected COS-7 cells (Figure 1). To determine the autophosphorylation activity of the receptor in COS-7 cells, we applied anti-phosphotyrosine antibodies: In absence of the HEK2-specific ligand, LERK2, the wildtype receptor showed a considerable level of autophosphorylation (Figure 1). This observation confirms our previous data, which demonstrate that overexpression of HEK2 under the transcriptional control of the cytomegalovirus enhancer-promoter causes a substantial level of receptor phosphorylation (Böhme *et al*., 1996). In comparison to the wildtype receptor, the phosphorylation of the Y614F mutant is below the level of detection, suggesting Y614 to be the major autophosphorylation site of the HEK2 receptor. The kinase-inactive mutant did not show any detectable level of tyrosine phosphorylation. Incubation of COS-7 cells expressing either HEK2 or Y614F with LERK2-Fc fusion proteins (Böhme *et al*., 1996) did not alter the level of receptor-phosphorylation, indicating that no additional tyrosine residues become phosphorylated upon ligand stimulation (data not shown).

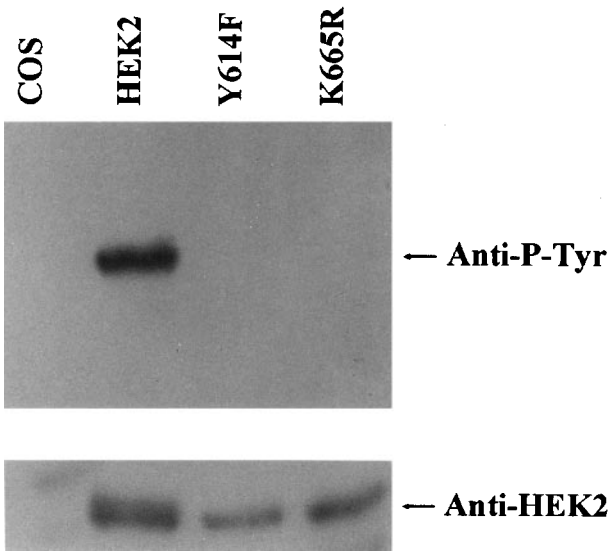
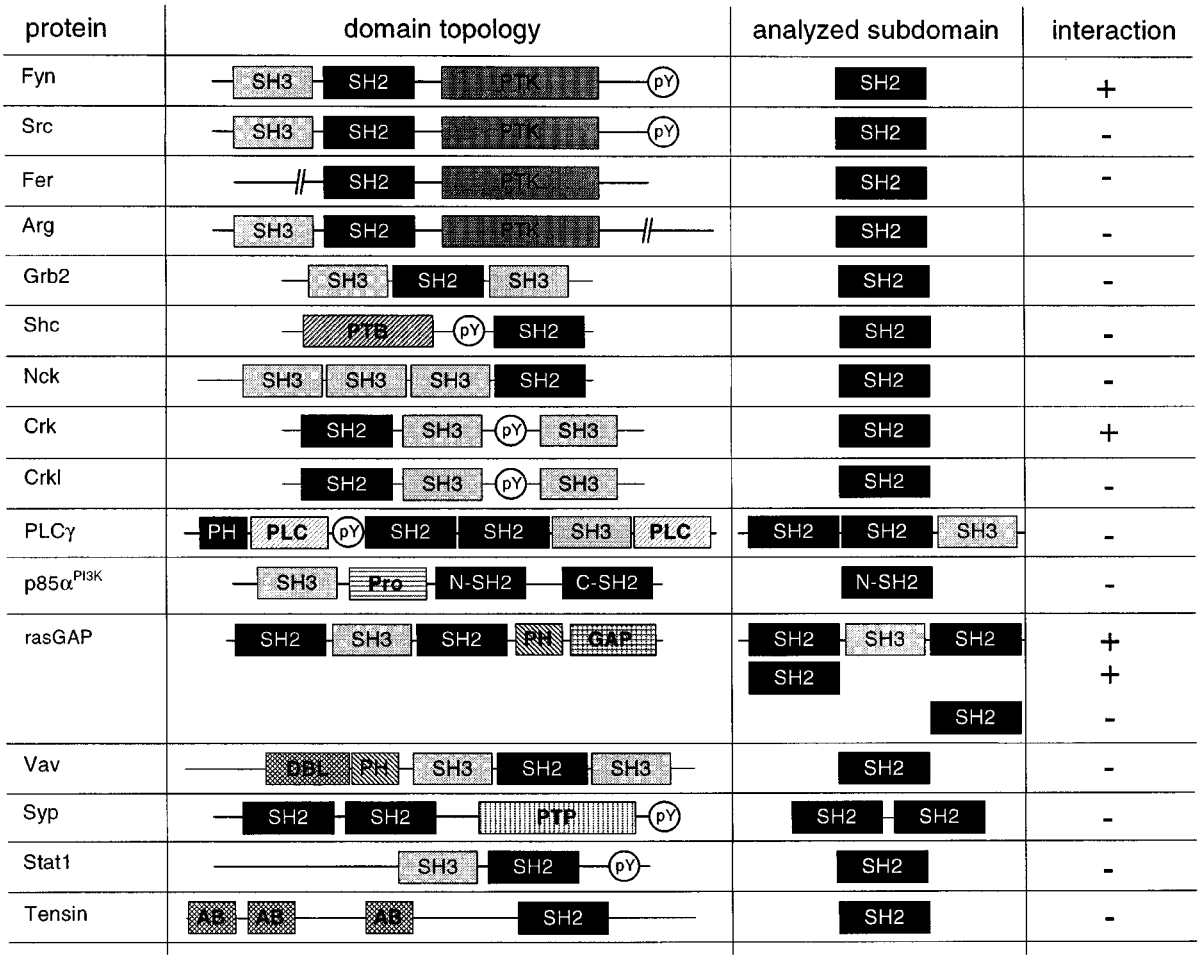


Figure 1 Expression and phosphorylation of HEK2 in COS-7 cells. The HEK2 cDNA containing the complete open reading frame (nt 4-3086, Böhme *et al*., 1993) was inserted into the *Hind*III/*Xba*I site of the mammalian expression vector pRc/CMV. A K665R kinase-deficient HEK2 receptor as well as a Y614F mutant were generated by site-directed mutagenesis. COS-7 cells were grown in DMEM (BioWhittaker) containing 10% FCS, 2 mM L-Glutamine, 100 μg/ml Streptomycine and 100 iU/ml Penicillin. Transfections were performed using the lipofectamine method (BRL) according to the manufacturers instructions. Forty-eight hours post transfection cells were washed twice in phosphate-buffered saline (PBS) and lysed in ice cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% Glycerol, 1% Triton-X-100, 1.5 mM $MgCl_2$, 1 mM EGTA), freshly supplemented with 10 μg/ml leupeptin and aprotinin, 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM Na_3VO_4. Cell lysates were incubated on ice for 15 min with occassional vortexing and then clarified by centrifugation for 10 min at 12000 *g*. The protein concentration was determined using the BCA-method (Pierce) according to the manufacturers instructions. Lysates from COS-7 cells expressing HEK2, HEK2Y614F or HEK2K665R were subjected to 7.5% SDS–PAGE and Western blotting. An equal amount of lysate from untransfected COS-7 cells was used as negative control. Blots were probed with a HEK2 antiserum or monoclonal anti-phosphotyrosine antibodies (PY20, Santa Cruz Biotechnology)

To further investigate the signalling potential of HEK2, we attempted to identify HEK2 interacting proteins by generating and testing multiple known key components of signal transduction which include at least one SH2-domain as classical binding motif. To this end, we analysed the ability of different recombinant GST-SH2-fusion-proteins including Fyn, Src, Shc, Grb2, Nck, Crk, Crkl, Syp, rasGAP, PI3-kinase, PLC-γ1, Vav, Stat1, Fer, Tensin and Arg to associate with HEK2 (Figure 2). In these experiments equal amounts of GST-SH2 fusion proteins bound to glutathione-sepharose beads were mixed with cell lysates prepared of HEK2-expressing COS-7 cells. In a Western blot analysis levels of precipitated HEK2 were evaluated by using specific polyclonal antibodies. These *in vitro* binding studies clearly demonstrated HEK2 association with GST-Fyn, GST-rasGAP and GST-Crk, whereas no HEK2 precipitated with all other proteins analysed (Figure 2). Since the GST-rasGAP fusion protein used in this study encompasses two SH2- and one SH3-domain, we tested different subfragments, GST-rasGAP/NSH2 and GST-rasGAP/CSH2, to specify the region required for HEK2 interaction. Only the GST-fusion-protein containing the aminoterminal SH2-domain of rasGAP (aa 175–274) had the capacity to bind HEK2 (Figure 3).

Recent data suggest, that autophosphorylation on Y602 in the juxtamembrane region of the EPH-related RTK SEK is the high affinity binding site for the SH2-domain of the cytoplasmic protein tyrosine kinase Fyn. This tyrosine residue is embedded in a YxxP motif and represents a major autophosphorylation site of this receptor (Ellis *et al*., 1996). A sequence alignment of HEK2 and SEK shows that the corresponding juxtamembrane region of SEK at Y602 is also conserved at Y614 in HEK2. Recognition of the YxxP motif in PDGFR and p190 has been described for the aminoterminal SH2-domain of rasGAP (Kashishian *et al*., 1992; Hu and Settleman, 1997). In addition, the SH2-domain of the adapter protein Crk recognizes YxxP motifs *in vitro* (Songyang *et al*., 1993). Based on these observations, we wondered whether this motif is also essential for HEK2 interactions. Therefore, we expressed the Y614F receptor mutant in COS-7 cells and used lysates for *in vitro* binding assays. Lysates from COS-7 cells expressing the kinase dead mutant K665R of the

receptor were used to demonstrate that the SH2-mediated interactions depend on autophosphorylation of HEK2. As shown in Figure 4, the substitution of either Lys-665 (K665R) or Tyr-614 (Y614F) completely abolished HEK2 binding to the GST-Fyn/SH2, the GST-Crk/SH2 and the GST-rasGAP/NSH2



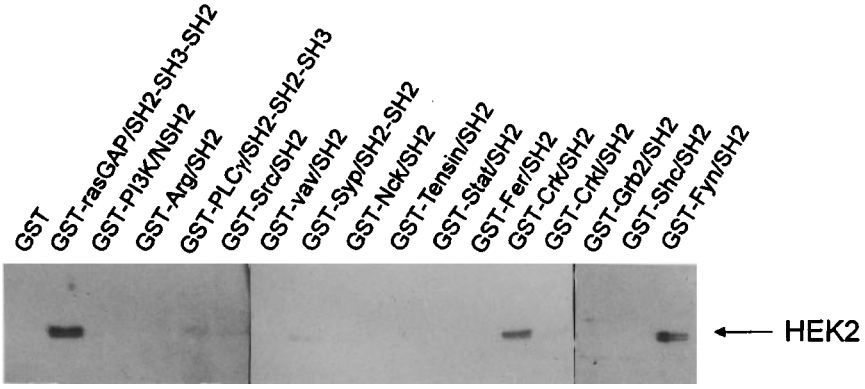


Figure 2 *In vitro* analysis of SH2-containing proteins for their interaction with HEK2. Subdomains of human rasGAP (aa 171–448), human PLC-γ1 (aa 548–760) and subunit p85 of human PI3-kinase (aa 333–430) were purchased as GST-fusion-proteins from Santa Cruz Biotechnology. The cDNA encoding the SH2-domain of human Arg (aa 163–274) was amplified by PCR and inserted into the vector pGEX-2T. SH2 fusion proteins of Crk, Crkl, Nck and Fer have been described previously (Feller *et al*., 1994, 1995). The following pGEX-vectors were generous gifts: Vav/SH2 (S Katzav), Stat1/SH2 (JE Darnell Jr.), Syp/SH2 (N+C) (T Tauchi and H Broxmeyer), Tensin/SH2 (LB Chen), Fyn/SH2 (C Rudd). The SH2-domains of Src, Shc and Grb2 in pGEX-2T were provided by Dr M Tavassoli. Proteins were expressed in *E. coli* BL21. Cells were lysed in buffer containing 50 mM Tris/HCl pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM Dithiothreitol (DTT) and 1 mM PMSF by mild sonification. Cellular debris was removed by centrifugation and the fusion proteins were immobilized onto glutathione-sepharose beads (Pharmacia) following the suppliers recommendations. GST-fusion-proteins coupled to glutathione-sepharose beads were incubated for 1 h at 4°C with lysates from HEK2-expressing COS-7 cells. After washing thoroughly with ice cold lysis buffer samples were analysed by SDS–PAGE and Western blotting with anti-HEK2 antibodies. AB, actin binding site; DBL, DBL homology domain; GAP, GTPase activating domain; PH, pleckstrin homology domain; PLC, phospholipase catalytic domain; Pro, proline-rich SH3 binding site; PTB, phosphotyrosine binding domain; PTK, protein tyrosine kinase catalytic domain; PTP, protein tyrosine phosphatase catalytic domain; pY, phosphotyrosine containing SH2 binding site; SH2, Src homology domain 2; SH3, Src homology domain 3

matrix. Taken together, these results demonstrate that autophosphorylation of Y614 in the HEK2 receptor plays a crucial role for stable association with the described signalling molecules.

To test the interaction of HEK2 with full length rasGAP, both proteins were coexpressed in COS-7 cells. Anti-rasGAP immunoprecipitates were subjected to immunoblotting with specific HEK2 antibodies to detect coprecipitated receptor. In contrast to the wildtype receptor the kinase-deficient mutant did not show any detectable binding to rasGAP (Figure 5). rasGAP specific signals could not be detected in untransfected COS-7 cells. Thus, rasGAP seems to bind HEK2 in a phosphorylation dependent manner.

Interestingly, using anti-phosphotyrosine antibodies phosphorylation of rasGAP could not be observed in lysates of COS-7 cells coexpressing rasGAP and HEK2 (data not shown). In contrast, HEK2 shows strong autophosphorylation in this assay.

We next investigated whether the interaction of HEK2 with rasGAP and Fyn can also be observed in living cells using the yeast Two-Hybrid system. Therefore, the intracellular domain of HEK2 was expressed as a LexA fusion protein in *Saccharomyces cerevisiae* L40 strain (Vojtek *et al*., 1993, Hollenberg *et al*., 1995). The coexpression of VP16 fusion proteins of the aminoterminal SH2-domain of rasGAP or the Fyn/SH2-domain with the HEK2 bait construct strongly induced reporter gene activity allowing growth on media lacking histidine. In contrast, several control strains expressing unrelated VP16 fusions such as the carboxyterminal SH2-domain of PI3-kinase did not result in reporter gene activation. Moreover, other LexA DNA-binding domain fusions like the intracellular domain of the RTK TKT (Karn *et al*., 1993) did not show association with rasGAP and Fyn in this assay (data not shown). These data suggest that the SH2-domain mediated interaction of HEK2 with rasGAP and Fyn is specific and occurs also in living cells.

To demonstrate that the interaction of HEK2 with rasGAP/NSH2 and Fyn/SH2 also depends on autophosphorylation of the receptor in yeast, we used a LexA fusion of the kinase-dead intracellular domain of HEK2 in a Two-Hybrid assay: Coexpression with VP16 fusions of the SH2-domains of rasGAP and Fyn did not result in reporter gene activity (data not

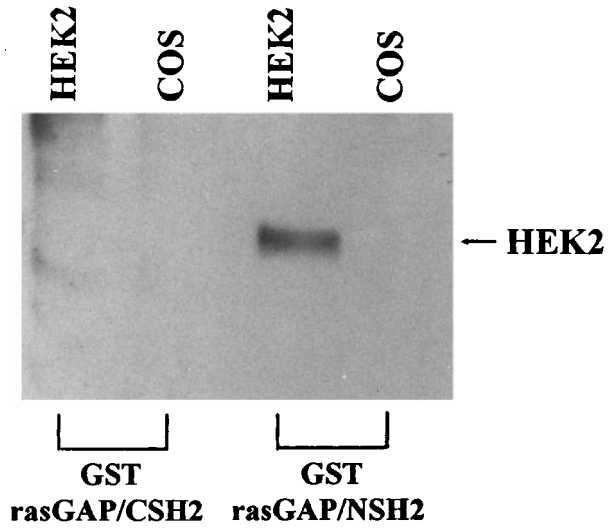


Figure 3 *In vitro* binding assay of rasGAP subfragments with HEK2. COS-7 cells or COS-7 cells expressing HEK2 were lysed and incubated with GST-fusions of the C-terminal or N-terminal rasGAP/SH2-domain coupled to glutathione-sepharose beads (Santa Cruz Biotechnology). Bound material was washed thoroughly and analysed by Western blotting using anti-HEK2 antisera. Quantification of HEK2 in the lysates of transfected COS-7 cells is shown in Figure 1 (lower panel)

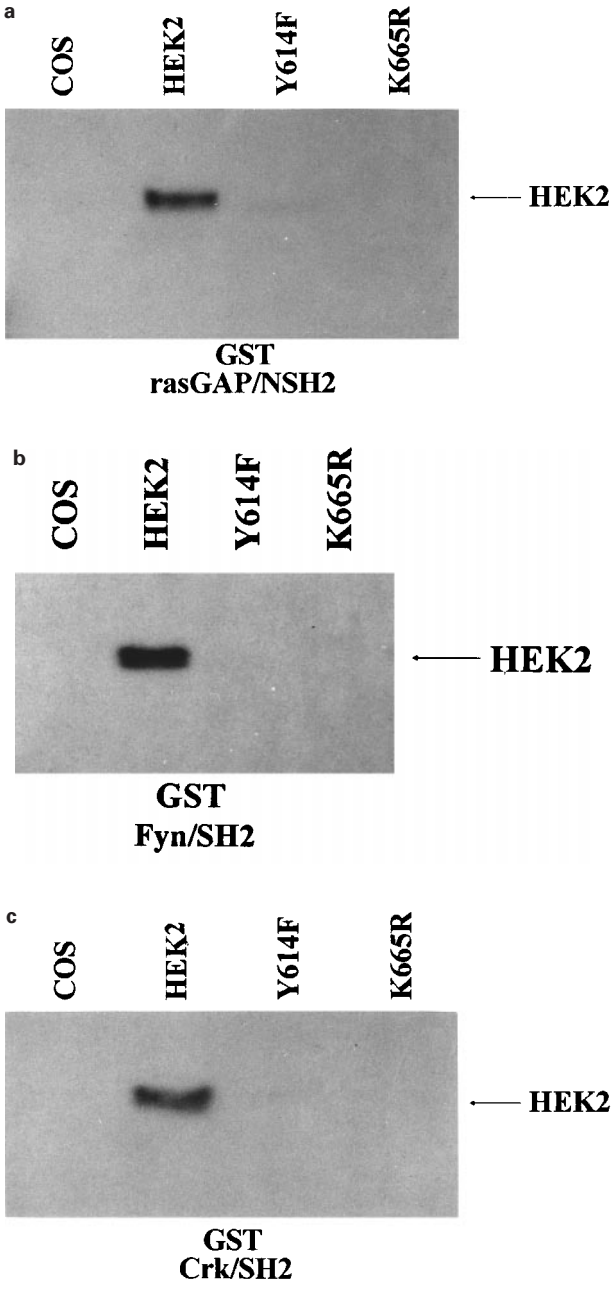


Figure 4 *In vitro* binding assay of the SH2-domains of Fyn, rasGAP and Crk with wildtype and mutated HEK2. COS-7 cells were transfected with an expression vector containing either the wildtype HEK2 or point mutated forms of HEK2 including the Y614F and the kinase-deficient K665R mutant. Cell lysates were incubated with (**a**) GST-rasGAP/NSH2, (**b**) GST-fyn/SH2 or (**c**) GST-Crk/SH2. An equal amount of lysate from untransfected cells was used as a negative control. Proteins that associated stably with the SH2-domains were separated by SDS–PAGE and probed on a Western blot with anti-HEK2 antisera. Quantification of HEK2 in the lysates of transfected COS-7 cells is shown in Figure 1 (lower panel)

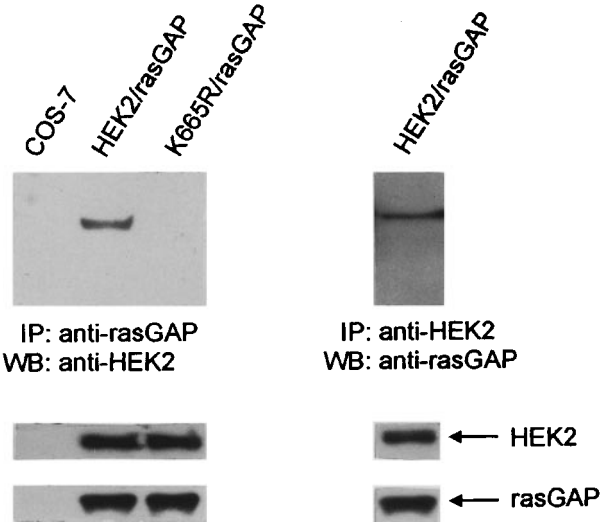


Figure 5 Coimmunoprecipitation of rasGAP and HEK2 in COS-7 cells.The expression vector pCDL3/rasGAP, a generous gift from K Kaibuchi, was used to express the complete coding sequence of bovine rasGAP. Lysates COS-7 cells coexpressing HEK2 or kinase-deficient K665R receptor and full length rasGAP were incubated with monoclonal anti-rasGAP antibodies (B4F8, Upstate Biotechnology) on ice for 1 h. Subsequently protein-A-sepharose was added and the incubation was continued for 1 h. Beads were recovered by low speed centrifugation and washed extensively in ice cold lysis buffer. Proteins were released from sepharose beads by boiling 2 min in SDS-sample buffer and probed with antibodies specific for HEK2 in a Western blot. Lower panels show the quantification of HEK2 and rasGAP in whole cell lysates as judged by Western blot with specific antibodies. A reciprocal experiment is shown on the right

Table 1 Alignment of putative rasGAP binding sites

protein	rasGAP-binding site
PDGFR-β	S N M M A P Y D N Y V
p190 (1)	S D Y A E P M D A V V
p190 (2)	N I Y S V P H D S T Q
p190B (1)	D N Y A E P I D T I F
p190B (2)	E I Y V V P D D S Q N
IGFR	P E Y F S A A D V Y V
HEK2, HEK5, ELK, HTK	F T Y E D P N E A V R
CEK9	S T Y E D P N E A I R
SEK	F T Y E D P N Q A V R
ECK	H T Y E D P N Q A V L
HEK4, HEK7	H T Y E D P T Q A V H
HEK11	E T Y E D P N R A V H

Different putative rasGAP binding domains are aligned in relation to the juxtamembrane regions of EPH-related kinases. Amino acids are given in single letter code, conserved residues are shaded. Note that SEK does not associate with rasGAP

shown). In addition to the *in vitro* and *in vivo* binding assays these data suggest that the autophosphorylation of HEK2 at tyrosine seems to be essential for the interaction with these two proteins as predicted by the phosphotyrosine-binding function of SH2-domains.

Previous observations gave rise to the notion that HEK2 and HEK5 have overlapping functions in the murine system (Henkemeyer *et al*., 1996; Orioli *et al*., 1996). Moreover, the intracellular domains of both receptors are homologues and the sequence of the predicted binding site for rasGAP/NSH2 and Fyn/SH2 in HEK2 is conserved in HEK5. Thus, we asked the question whether both receptors have comparable signalling properties. To this end, we used LexA fusions of the intracellular domain of the wildtype HEK5 receptor and a kinase-dead mutant (HEK5V619E) in a Two-Hybrid assay. Coexpression of the wildtype receptor fusion in yeast with VP16 fusions of the SH2-domains of Fyn or rasGAP resulted in strong reporter gene activity, indicating that both SH2-domains interact also with HEK5 in living cells. In contrast, no interaction was detectable with the kinase-dead mutant of the receptor (data not shown). These data provide evidence, that the SH2-domains of rasGAP and Fyn interact also with HEK5 in a receptor-autophosphorylation dependent manner.

As described above Y614 is the major autophosphorylation site of HEK2 and seems to be sufficient for the association with rasGAP, Fyn and Crk, suggesting this residue to function as a multi-docking site. Y614 is embedded in a TYEDP motif, which is highly conserved among various EPH-related RTKs. Thus, this residue could have a similar function in many other EPH-related RTKs as well. To test this hypothesis we used a Two-Hybrid assay and demonstrated that in addition to the HEK2 receptor the intracellular portion of HEK5 also interacts with Fyn/SH2 and rasGAP/NSH2 in a phosphorylation dependent manner. Although the corresponding tyrosine residue Y586 in the HEK5 receptor has not been substituted by site-directed mutagenesis, it might be possible that autophosphorylation of this residue mediates also the interaction with both signalling molecules. Moreover, Ellis *et al*. have recently reported that autophosphorylation of Y602 in the juxtamembrane region of the SEK receptor, which corresponds to Y614 in HEK2, mediates association with Fyn/SH2 (Ellis *et al*., 1996). Taken together, the studies on HEK2, HEK5 and SEK provide evidence that the juxtamembrane TYEDP motif plays a crucial role for signalling of EPH-related receptors.

The alignment of consensus binding sequences of *in vivo* binding sites of numerous SH2-domain-containing proteins gave rise to the notion that residues carboxyterminal of the phosphotyrosine provide considerable selectivity (Songyang *et al*., 1993). Interestingly, recognition of tyrosine phosphorylated YxxP motifs in PDGFR and p190^{rhoGAP} has been described for the SH2-domains of rasGAP and the adapter protein Crk (Kashishian *et al*., 1992; Hu and Settleman, 1997). The comparison of rasGAP binding regions in different proteins such as rhoGAP, PDGFR and IGFR revealed that the amino acids at positions +3 (proline) and +5 (aspartic acid) relative to phosphotyrosine might be crucial for the interaction (Table 1). Notably, this sequence motif (YxxPxD) is conserved in the juxtamembrane region of HEK2 and HEK5: It shows a conservative substitution at position +5 with glutamic acid (E619 in HEK2) instead of aspartic acid. In contrast, the SEK receptor which does not bind rasGAP (Ellis *et al*., 1996) contains a nonconservative substitution at position +5 (glutamine) relative to the corresponding phosphotyrosine residue Y602. Therefore, we assume that an acidic amino acid at position +5 determines the binding affinity to the aminoterminal SH2-domain of rasGAP. The sequence divergence at position +5 relative to Y614 might be crucial for the

different rasGAP binding properties of HEK2 and HEK5 compared to SEK. The nonconservative substitution of E619 in HEK2 could provide interesting hints for further evaluation of the role of this residue in rasGAP binding: Interestingly, sequence comparison revealed that EPH-related receptors such as HEK2, HEK5, ELK and HTK, which show high affinity binding to transmembrane LERKs display the motif YxxPxE relative to Y614 in HEK2. In contrast, members such as SEK, HEK4 and ECK, which prefer GPI-anchored ligands, have a nonconservative substitution at this position. In summary, these data suggest that the subdivision of EPH-related RTKs into two major groups based on sequence homology and ligand binding preferences (Gale *et al*., 1996) might also be important for certain intracellular signalling properties.

The binding of rasGAP and Fyn to various EPH-related RTKs indicate that some of these receptors have overlapping functions in intracellular signalling. However, our data provide evidence that signalling of HEK2 differs from other EPH-related RTKs: In contrast to the ECK receptor (Pandey *et al*., 1994) HEK2 does not bind to the p85 subunit of the PI3-kinase *in vitro*. Also, binding of Grb2, which associates with the ELK RTK (Stein *et al*., 1996), could not be demonstrated. Further investigations on the binding properties of other EPH-related RTKs to a broad spectrum of SH2-containing intracellular signalling molecules would be of interest for the understanding of overlapping and distinct signalling features of EPH-related RTKs.

Taken together, our data provide evidence that rasGAP, Fyn and Crk associate with the HEK2 RTK. These interactions are mediated in a SH2-dependent manner by a single multi-docking site. In order to understand the role of certain HEK2-interacting partners identified in this work the respective binding-affinities to the multi-docking site should be taken into account. Moreover, the selection of substrates may be dependent on the dynamic temporal and spatial expression profiles of these proteins. Since multi-docking sites have also been described for c-*met* and the Ufo-receptor (Ponzetto *et al*., 1994; Braunger *et al*., 1997), signalling of different SH2-domains through multi-docking sites might be a common mechanism for signal transduction of RTKs.

Note added in proof:
According to the unified nomenclature for Eph-family receptors HEK2 has been renamed to EphB3, HEK5 to EphB2 and SEK to EphA4 (Eph Nomenclature Committee 1997, *Cell*, **90,** 403–404).

Abbreviations
The abbreviations used are: RTK, receptor tyrosine kinase; PLC-γ1, phospholipase C-γ1; rasGAP, ras-GTPase-activating protein; PI3-kinase, p85 α subunit of phosphatidylinositol 3-kinase; aa, amino acid; nt, nucleotide position; PCR, polymerase chain reaction; HEK, human embryonic kinase; LERK, ligand for EPH-related kinases; TKT, tyrosine kinase related to TRK; IP, immunoprecipitation.

Acknowledgements
We are grateful to Stanley Fields and Paul Bartel for providing the plasmid pBTM116. The plasmid pVP16 was a generous gift from Stanley Hollenberg. Yeast strain L40 was kindly provided by Rolf Sternglanz. The Georg-Speyer-Haus is supported by the Bundesgesundheitsministerium and the Hessisches Ministerium für Wissenschaft und Kunst. This work was further supported by grants from the Georg und Franziska Speyer'sche Hochschulstiftung, the Hessischer Verein zur Förderung der Jugendgesundheitspflege e.V. (RE), the Deutsche Forschungsgemeinschaft (RU 242/11-1, STR 336/5-1), the Deutsche Krebshilfe (W 102/93/Rü 2) and the SFB 474.

References

Böhme B, Holtrich U, Wolf G, Luzius H, Grzeschik K-H, Strebhardt K and Rübsamen-Waigmann H. (1993). *Oncogene,* **8,** 2857–2862.
Böhme B, VandenBos T, Cerretti DP, Park LS, Holtrich U, Rübsamen-Waigmann H and Strebhardt K. (1996). *J. Biol. Chem.,* **271,** 24747–24752.
Brambilla R and Klein R. (1995). *Mol. Cell. Neurosci.,* **6,** 487–495.
Braunger J, Schleithoff L, Schulz AS, Kessler H, Lammers R, Ullrich A, Bartram CR and Janssen JWG. (1997). *Oncogene,* **14,** 2619–2631.
Ellis C, Kasmi F, Ganju P, Walls E, Panayotou G and Reith AD. (1996). *Oncogene,* **12,** 1727–1736.
Feller SM, Knudsen B and Hanafusa H. (1994). *EMBO J.,* **13,** 2341–2351.
Feller SM, Knudsen B and Hanafusa H. (1995). *Oncogene,* **10,** 1465–1473.
Fox GM, Holst PL, Chute HT, Lindberg RA, Janssen AM, Basu R and Welcher AW. (1995). *Oncogene,* **10,** 897–905.
Gale NW, Holland SJ, Valenzuela DM, Flenniken A, Pan L, Ryan TE, Henkemeyer M, Strebhardt K, Hirai H, Wilkinson DG, Pawson T, Davis S and Yancopoulos GD. (1996). *Neuron,* **17,** 9–19.
Henkemeyer M, Orioli D, Henderson JT, Saxton TM, Roder J, Pawson T and Klein R. (1996). *Cell,* **86,** 35–46.
Hollenberg SM, Sternglanz R, Cheng PF and Weintraub H. (1995). *Mol. Cell. Biol.,* **15,** 3813–3822.
Hu K-Q and Settleman J. (1997). *EMBO J.,* **16,** 473–483.
Karn T, Holtrich U, Bräuninger A, Böhme B, Wolf G, Rübsamen-Waigmann H and Strebhardt K. (1993). *Oncogene,* **8,** 3433–3440.
Kashishian A, Kazlauskas A and Cooper J. (1992). *EMBO J.,* **11,** 1373–1382.
Orioli D, Henkemeyer M, Lemke G, Klein R and Pawson T. (1996). *EMBO J.,* **15,** 6035–6049.
Pandey A, Lazar DF, Saltiel AR and Dixit VM. (1994). *J. Biol. Chem.,* **269,** 30154–30157.
Pandey A, Duan H and Dixit VM. (1995). *J. Biol. Chem.,* **270,** 19201–19204.
Ponzetto C, Bardelli A, Zhen Z, Maina F, dalla Zonca P, Giordano S, Graziani A, Panayotou G and Comoglio PM. (1994). *Cell,* **77,** 261–271.
Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, Neel BG, Birge RB, Fajardo JE, Chou MM, Hanafusa H, Schaffhausen B and Cantley LC. (1993). *Cell,* **72,** 767–778.
Stein E, Cerretti DP and Daniel TO. (1996). *J. Biol. Chem.,* **271,** 23588–23593.
Tuzi NL and Gullick WJ. (1994). *Br. J. Cancer,* **69,** 417–421.
Vojtek AB, Hollenberg SM and Cooper JA. (1993). *Cell,* **74,** 205–214.