Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 2523–2527, March 1996
Biochemistry

Purification of a ligand for the EPH-like receptor HEK using a biosensor-based affinity detection approach

MARTIN LACKMANN*†, TAMARA BUCCI‡, RICHARD J. MANN§, LUCY A. KRAVETS*, ELIZABETH VINEY‡, FIONA SMITH‡, ROBERT L. MORITZ¶, WENDY CARTER‡, RICHARD J. SIMPSON¶, NICOS A. NICOLA§, KAREN MACKWELL‡, EDOUARD C. NICE‖, ANDREW F. WILKS*, AND ANDREW W. BOYD‡

Cooperative Research Centre for Cellular Growth Factors: *Growth Regulation Laboratory, ‖Epithelial Laboratory, and ¶Joint Protein Structure Laboratory, Ludwig Institute for Cancer Research (Melbourne Branch), and the ‡Lions Cancer Research Laboratory, §Cancer Research Unit, The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia

Communicated by G. J. V. Nossal, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia, November 21, 1995 (received for review September 8, 1995)

ABSTRACT **Advances in screening technologies allowing the identification of growth factor receptors solely by virtue of DNA or protein sequence comparison call for novel methods to isolate corresponding ligand growth factors. The EPH-like receptor tyrosine kinase (RTK) HEK (human EPH-like kinase) was identified previously as a membrane antigen on the LK63 human pre-B-cell line and overexpression in leukemic specimens and cell lines suggested a role in oncogenesis. We developed a biosensor-based approach using the immobilized HEK receptor exodomain to detect and monitor purification of the HEK ligand. A protein purification protocol, which included HEK affinity chromatography, achieved a 1.8 × 10^6-fold purification of an ≈23-kDa protein from human placental conditioned medium. Analysis of specific sHEK (soluble extracellular domain of HEK) ligand interactions in the first and final purification steps suggested a ligand concentration of 40 pM in the source material and a K_d of 2–3 nM. Since the purified ligand was N-terminally blocked, we generated tryptic peptides and N-terminal amino acid sequence analysis of 7 tryptic fragments of the S-pyridylethylated protein unequivocally matched the sequence for AL-1, a recently reported ligand for the related EPH-like RTK REK7 (Winslow, J. W., Moran, P., Valverde, J., Shih, A., Yuan, J. Q., Wong, S. C., Tsai, S. P., Goddard, A., Henzel, W. J., Hefti, F., Beck, K. D. & Caras, I. W. (1995) *Neuron* 14, 973–981). Our findings demonstrate the application of biosensor technology in ligand purification and show that AL-1, as has been found for other ligands of the EPH-like RTK family, binds more than one receptor.**

The pivotal role of receptor tyrosine kinases (RTKs) in regulation of cellular growth and differentiation has triggered considerable interest in the identification of novel members of this ubiquitous protein family. Screening techniques, which were not dependent on function, brought about the isolation of numerous novel RTKs for which the ligands were not known. Many were EPH-like RTKs, which comprise the largest RTK family known to date (1–19).

In contrast to the PCR-based approaches used for most other EPH-like RTKs, human EPH-like kinase (HEK) was identified on the cell surface of a human pre-B-cell line with monoclonal antibody (mAb) IIIA4 (2). HEK protein was affinity purified on a mAb IIIA4 column (2), and its amino acid sequence revealed homology with EPH and the predicted amino acid sequences of other EPH-like RTKs [ELK (3), ECK (4), and ERK (5)]. The sequences of the mouse (MEK4) and chicken (CEK4) homologues of HEK have also been reported (6, 7). To date, at least 28 members of the EPH subfamily have been identified in diverse vertebrate species including zebrafish (8), frog (9, 10), chicken (6, 11, 12), mouse (6, 13–18), rat (3, 5), and human (1, 2, 4, 19, 20), and their expression patterns suggest distinct roles in developmental processes. Structural features of EPH-like RTKs include an extracellular N-terminal domain of 10–20 highly conserved cysteine residues followed by two fibronectin III repeats, a hydrophobic transmembrane segment, and a cytoplasmic domain with characteristic features of protein tyrosine kinase catalytic domain. Overexpression of HEK in leukemic cell lines and some leukemic specimens (2) and of EPH and ERK in some carcinomas (1, 21) and ECK in melanoma (22) suggests a role for these RTKs in oncogenesis.

The BIAcore (Pharmacia) biosensor has been shown to be a sensitive tool for monitoring receptor–ligand interactions. This paper provides evidence that BIAcore technology could be applied to identification and purification of the ligand for HEK from a complex protein mixture. In this case, the BIAcore technology had the sensitivity to replace a bioassay and provided a specific monitor within a classical protein purification scheme, yielding a 1.8 × 10^6-fold purification of the HEK ligand from human placental conditioned medium (HPCM). We show that the amino acid sequence identified this protein as a member of the emerging ligand of EPH-like receptor tyrosine kinase (LERK) family.

MATERIALS AND METHODS

Conditioned Media. Cell lines and cell suspensions from tissues were cultured in either RPMI 1640 or Iscove's-modified Dulbecco's modified Eagle's medium, with the minimum serum required for optimal growth, to produce conditioned medium. Supernatant fluid was recovered from confluent cultures, filtered, and stored in aliquots for BIAcore analysis.

HPCM was prepared from normal placentas obtained from the Royal Womens Hospital, Melbourne, as approved by their Institutional Review Board. Each placenta was dissected into 0.5- to 1-cm cubes, washed with phosphate-buffered saline (PBS), and cultured in 75-cm^2 tissue culture flasks containing 60 ml of RPMI 1640 medium supplemented with 5% newborn calf serum (HyClone). After 4 days of incubation, supernatant fluid was harvested, centrifuged to remove cells and debris, and frozen at −20°C.

Production of Soluble HEK (sHEK) Protein. The extracellular region of HEK was generated from HEK cDNA (7) by PCR using primers based on the 5′ untranslated region (5′-AGATATGCTCCTCTCAC-3′) and the end of the extracellular domain (5′-TTGGCTACTTTCACCAG-3′) with a

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HEK, human EPH-like kinase; sHEK, soluble extracellular domain of HEK; RTK, receptor tyrosine kinase; RU, relative response unit(s); SE, size exclusion; mAb, monoclonal antibody; RP, reversed-phase.

†To whom reprint requests should be addressed.

terminal in-frame stop codon in the antisense oligonucleotide. The PCR fragment was cloned into the pEF-BOS vector (23) and clones were analyzed by DNA sequencing. The sHEK-pEF-BOS DNA and pSV2neo DNA were cotransfected into Chinese hamster ovary (CHO) cells and transfectant clones were selected with G418. High producer clones were selected by screening on a IIIA4 mAb-derivatized BIAcore sensor chip. sHEK was extracted from sHEK-CHO cell-derived conditioned medium on IIIA4 Trisacryl beads (2) and eluted with 3 M $MgCl_2$/25% ethylene glycol/0.075 M Hepes/0.1% Triton X-100, pH 7.2. The eluate was purified to homogeneity by Mono Q HPLC (5 × 50 mm; Pharmacia) using a linear NaCl gradient (0–600 mM in 20 mM Tris/0.02% Tween 20, pH 8.5). The identity and concentration of the purified sHEK in the final preparation were confirmed by N-terminal amino acid sequence analysis and amino acid analysis.

Biosensor Measurements. All measurements were performed on the BIAcore biosensor equipped with CM5 sensor chips and immobilization reagents provided by the manufacturer (Pharmacia). Immobilization of mAb IIIA4 or sHEK to the sensor chip surface was carried out essentially as described (24, 25). Parallel channels of the sensor chip were derivatized under identical conditions either with nonrelevant proteins or in the absence of protein and were used as sham-derivatized sensor surfaces in control experiments. The conformational stability of immobilized sHEK was routinely monitored by measuring the BIAcore response to the mAb IIIA4, which did not react with the denatured protein (M.L., K.M., R.J.M., L.A.K., and A.W.B., unpublished observations). Denaturation of the immobilized receptor was achieved *in situ* by injection of 0.5% dithiothreitol in 6 M guanidine hydrochloride/5 mM EDTA/50 mM Tris, pH 8.0, over 20 min. Screening of cell culture supernatants and column fractions for a putative HEK ligand was performed by injecting aliquots (35 μl) of 1- to 10-fold concentrated samples onto the sHEK-derivatized sensor chip. The relative BIAcore response expressed in relative response units (RU) was determined as the response 30 s after sample injection relative to the response 10 s before injection. Receptor binding was monitored as the relative response of a sample minus the relative response of the same sample incubated with 10 μg of sHEK per ml for >1 hr before analysis.

Purification of HEK Ligand from HPCM. The sHEK-specific binding activity in batches (0.9–1.0 liter) of 10-fold concentrated (YM-10 Spiral ultrafiltration cartridge, Amicon) HPCM was subjected to sequential 30–55% ammonium sulfate and 4 M sodium chloride precipitation prior to application to a phenyl-Sepharose column (16 × 5 cm) in 4 M NaCl/20 mM Tris/0.02% Tween 20, pH 8.5. The column was eluted at 7 ml/min with 20 mM Tris/0.02% Tween 20, pH 8.5, and the NaCl concentration in the eluate was determined from the conductivity in individual fractions. Active material was concentrated and dialyzed (YM 10 membrane) to 50 mM NaCl and further purified by Q-Sepharose (6 × 5 cm) chromatography at 5 ml/min using a 40-min gradient of 0–600 mM NaCl in 20 mM Tris/0.02% Tween 20, pH 8.5, at 23°C. Active fractions were passed through 10 ml of protein G-Sepharose FF (Pharmacia) before extraction on sHEK Sepharose (1 mg of sHEK coupled to 0.5 ml of packed CnBr-activated Sepharose). After a 1-hr incubation at room temperature (end-over-end rotator), the sHEK affinity resin was washed with 9 column vol of PBS/0.02% Tween 20 and eluted with 3 ml of 50 mM diethylamine/0.02% Tween 20, pH 12.2. The column eluate was neutralized immediately by addition of 1 M Hepes. A homogeneous HEK ligand preparation was obtained by sequential fractionation of sHEK-binding activity on a Superose-12 size exclusion (SE) HPLC column (300 × 10 mm; Pharmacia) at 0.25 ml/min (50 mM $NaHPO_4$/0.5 M NaCl/0.02% Tween 20, pH 7.4) and a μ-Mono Q column (50 × 1.5 mm; Pharmacia), which was eluted with a 40-min gradient of 0–600 mM NaCl in 20 mM Tris/0.02% Tween 20, pH 8.5, at a flow rate of 100 μl/min.

Peptide Mapping and Amino Acid Sequence Analysis. Before proteolytic fragmentation, the μ-Mono Q-purified HEK ligand was rechromatographed on a narrow bore RP300 reversed-phase (RP) HPLC column (1 × 50 mm; Brownlee Lab) to confirm the homogeneity of the material. After reduction (dithiothreitol) and S-pyridylethylation the RP-HPLC purified protein was desalted from the reaction mixture on the same RP-HPLC column and digested with trypsin before multidimensional chromatography as described (26). N-terminal amino acid sequence analysis of purified tryptic peptides was performed on a Hewlett-Packard model G1005A protein sequencer operated with the routine 3 sequencer program (27).

RESULTS

Screening of Conditioned Media for a Putative Ligand Source. In an initial search for a potential source of HEK ligand, the relative BIAcore responses of concentrated cell supernatants were evaluated on a chip bearing immobilized sHEK. Using this approach, high nonspecific responses became apparent when selected samples were analyzed in parallel either on a sham-derivatized channel of the sensor chip or on channels decorated with denatured sHEK or irrelevant protein at comparable densities. To increase the specificity of the assay system, it was necessary to monitor the reduction of the BIAcore response (i.e., the HEK specific component of the total response) in samples supplemented with a competing concentration of sHEK. In a survey of some 150 samples of cell and organ conditioned media, supernatants from human placental tissue cultured in the presence of supplemented newborn calf serum gave small but consistent sHEK-competable responses (see Fig. 3*B Left*). These initial results were confirmed by analyzing a number of partially purified extracts (ammonium sulfate precipitation, HEK-affinity extraction, and SE-HPLC) of concentrated HPCM. These procedures resulted in an increase in the competable response, and size fractionation of crude samples suggested active material in fractions within an apparent molecular size range defined by the standard proteins bovine serum albumin (66 kDa) and lysozyme (17 kDa).

Purification of the HEK Ligand from HPCM. The purification of the protein responsible for the HEK-specific biosensor response in HPCM was achieved by a protocol that was aimed at reducing protein complexity, and associated nonspecific binding, before receptor affinity chromatography. Sequential precipitation of proteins with ammonium sulfate and sodium chloride reduced the total protein level ≈3.2-fold and some 54% of the initial activity was recovered in the 4 M NaCl supernatant (Table 1). Subsequent fractionation on preparative hydrophobic interaction and ion-exchange columns yielded a 13-fold purification of the starting material (Fig. 1). To deplete immunoglobulins that coeluted with the active material in most purification steps, active fractions from Q-Sepharose were passed through a protein G-Sepharose column before sHEK affinity extraction. Although enrichment of the specific response was achieved (Table 1), both the activity and protein profiles (Fig. 2 *Left Inset* and *Right Inset*, lane ld) of the sHEK affinity eluate indicated persisting heterogeneity. Additional fractionation on SE-HPLC and μ-Mono Q columns yielded an apparently homogeneous HEK ligand with apparent masses of 28 (SE-HPLC; Fig. 2) and 23 (SDS/PAGE; Fig. 3*A Inset*) kDa and a specific binding response to sHEK (Fig. 3*B Right*, sensorgram C) of 7.7×10^5 RU/mg. A comparison of the specific activity with the HEK-specific BIAcore response of crude HPCM (Fig. 3*B Left*, sensorgram C) revealed an $\approx 1.8 \times 10^6$-fold purification. Approximately 9% of the total HEK-binding activity in the

Table 1. Analysis of purification of HEK ligand from HPCM

Purification step	Total protein, mg*	Total response, RU†	Specific response, RU/mg	Yield, %	Purification, -fold
HPCM concentrate	29,780	13,020	0.44	100	1.0
AS/NaCl precipitate	9,200	6,975	0.76	53.6	1.7
Ph-Sepharose	2,872	3,380	1.18	26	2.7
Q-Sepharose	483	2,769	5.7	21.3	13.1
sHEK agarose	0.156	2,400	1.54×10^4	18.4	3.5×10^4
SE-HPLC	0.0045	2,340	5.2×10^5	18.0	1.2×10^6
μ-MonoQ	0.0015	1,150	7.7×10^5	9.0	1.8×10^6

HEK ligand was purified from batches of 10-fold concentrated HPCM by sequential ammonium sulfate/NaCl (AS/NaCl) precipitation, Phenyl (Ph) and Q-Sepharose LC, sHEK agarose LC, SE-HPLC, and μ-Mono-Q HPLC.

*Protein concentration in eluates from SE-HPLC and μ-Mono-Q HPLC was estimated by comparison with the absorbance (peak area) of a standard protein.

†BIAcore responses were expressed as HEK-competable RU per ml of undiluted sample, and these were used to estimate total response of the preparation.

starting material (29.8 g of total protein) was recovered as 1.5 μg of pure ligand (Table 1).

Amino Acid Sequence Analysis of the HEK Ligand. N-terminal amino acid sequence analysis of the ligand after the RP-HPLC step (Fig. 4) yielded blank sequencing cycles indicative of an N-terminally blocked protein. A total of ≈4 μg of homogeneous ligand was therefore purified from three batches of HPCM and used to generate peptides for internal sequence analysis. Sequence analysis of the indicated tryptic peptides (Fig. 4 *Inset*) from ≈3.5 μg of reduced and S-pyridylethylated ligand yielded sequences that could be unambiguously assigned (Fig. 5) to the recently published sequence of AL1, a ligand for another EPH-like RTK, REK7 (28).

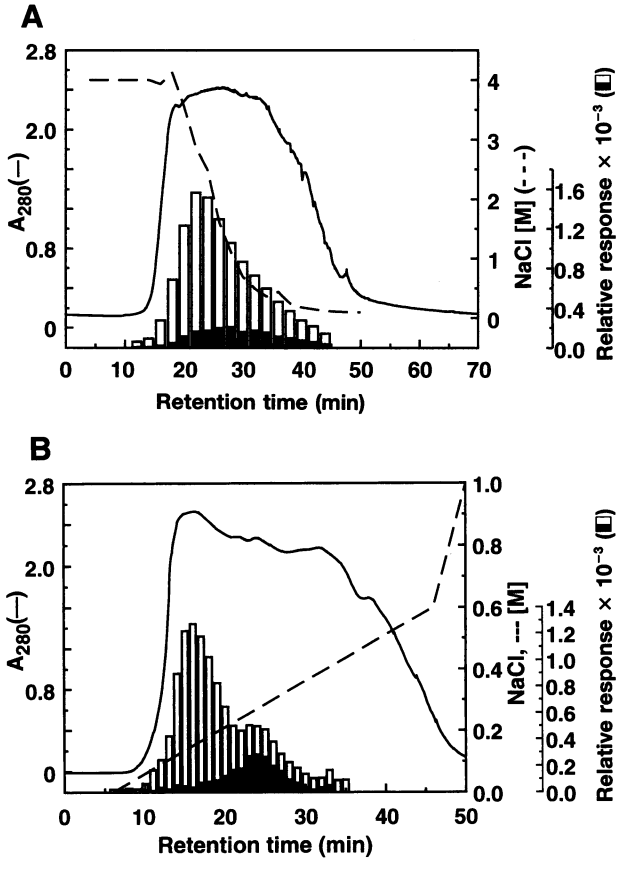

FIG. 1. Preparative hydrophobic interaction and ion-exchange LC of a crude HEK ligand preparation. (*A*) Phenyl-Sepharose LC of the 30–55% ammonium sulfate precipitate of 1 liter of 10× HPCM, adjusted to 4 M NaCl and cleared by centrifugation (10,000 × *g*). The column was eluted with 20 mM Tris·HCl (pH 8.5) containing 0.02% Tween 20 and the decreasing NaCl concentration (– – –) was estimated from the conductivity of individual 2-min fractions. (*B*) Q-Sepharose LC of the active fractions (20–40 min) from *A* dialyzed into 50 mM NaCl/20 mM Tris/0.02% Tween 20, pH 8.5. The column was eluted with a 0–600 mM NaCl gradient (– – –) and 2-min fractions were collected. Elution of proteins was monitored at 280 nm (——) and HEK binding in samples preincubated with or without 10 μg of sHEK per ml was determined on the BIAcore (□, total response; ■, sHEK competable response).

DISCUSSION

The development of novel screening techniques, which allow identification of growth factor receptors solely by virtue of their DNA or protein sequence, has created a demand for methods for isolation of the corresponding ligand growth factors. Where the biological activity is unknown, thus precluding the use of bioassays, direct measurement of the binding between candidate ligand and cell surface receptor may be the most appropriate test system. Here we report the use of an optical biosensor as an affinity detector, both in the search for and in the screening of fractions during purification to homogeneity of the HEK ligand.

One hundred and fifty biological samples were screened to identify a protein that would bind specifically to native, but not

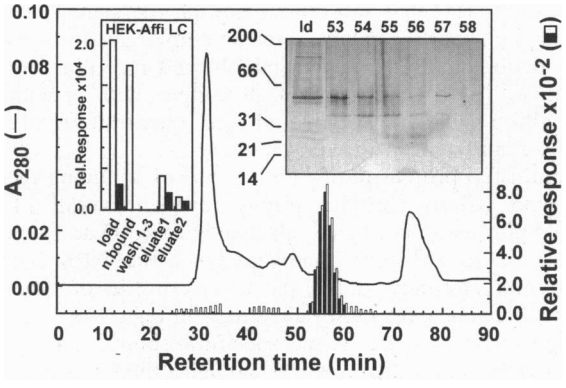

FIG. 2. HEK affinity chromatography, SE-HPLC, and SDS/PAGE of the HEK ligand. (*Left Inset*) The eluate of the sHEK receptor affinity resin was fractionated on a Superose-12 SE-HPLC column, which had been calibrated with commercial molecular weight marker proteins of the indicated sizes. Eluting proteins (——) were collected every minute and the sHEK binding response in fractions was monitored at 1:40 dilution on the BIAcore with or without sHEK competition. (□, total response; ■, sHEK competable response). (*Right Inset*) Aliquots of the column load (lane ld) and of active fractions were analyzed by SDS/PAGE with silver staining.

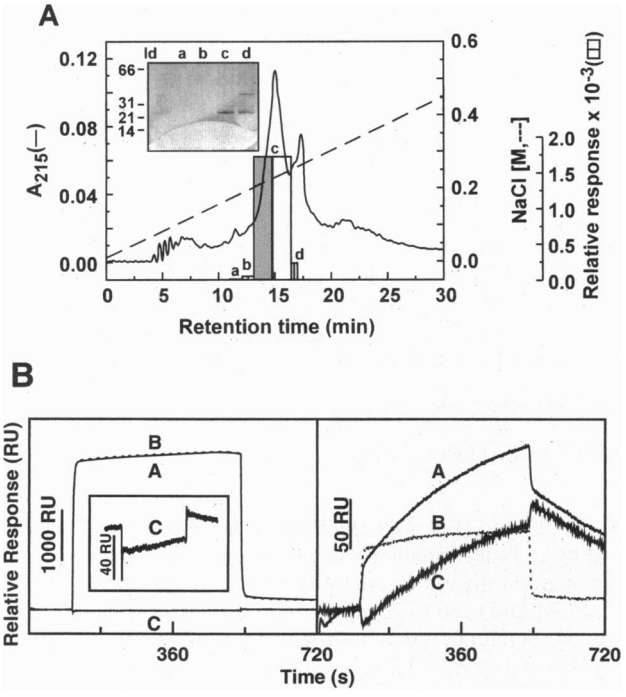

FIG. 3. Micropreparative ion-exchange chromatography of the HEK ligand and specificity of the BIAcore response. (*A*) Combined active peak fractions recovered from the SE-HPLC column were adjusted to 50 mM NaCl and fractionated on a μ-Mono-Q column with a 40-min gradient (- - -) of 0–600 mM NaCl in 20 mM Tris·HCl (pH 8.5) at a flow rate of 100 μl/min. Elution of proteins was monitored at 215 nm (——) and HEK binding with or without competing sHEK was measured on the BIAcore at 1:50 dilution (□, total response; ▨, sHEK competable response). (*Inset*) Proteins in the column load (lane ld) and in selected fractions were analyzed by SDS/PAGE with silver staining. (*B*) Sensorgrams illustrating the response to immobilized sHEK in crude HPCM (*Left*) or in the μ-Mono-Q-derived ligand preparation (*Right*). Response in the presence of competing sHEK (- -B- -) is subtracted from total response (—A—) to yield the difference sensorgram representing the specific response (—C—). (*Left Inset*) For clarity, the difference sensorgram of the crude sample is shown at increased sensitivity (—C—).

to denatured, sHEK. HPCM was identified as a candidate source for the putative HEK receptor ligand. However, analysis of BIAcore responses to crude and partially purified ligand preparations revealed substantial nonspecific signals, which depended on both the extent of sensor chip derivatization and the structural integrity of the immobilized receptor protein over time. The routine assay of all samples with or without competition with free sHEK allowed quantitation of the specific component of the sensor signal.

A multistep protein purification protocol including receptor/ligand affinity chromatography gave an overall 1.8 × 10^6-fold purification of this weak signal (Table 1) and yielded purification to apparent homogeneity of the HEK ligand. Furthermore, a comparison of the activity profiles of total and competable sHEK responses illustrated in Figs. 1–3 indicates a proportional increase of the specific response concurrent with progressive purification and highlights the importance of the competition-based assay system. Even after SE-HPLC of the affinity eluate, a proportion of the total BIAcore response was found to be due to nonspecific binding of contaminating proteins (Fig. 2 *Inset*, lanes 53–58) and only the final purification step yielded a preparation characterized by a fully HEK-competable BIAcore response (Fig. 3).

In contrast to our observations, an unambiguous identification of the ECK ligand in a BIAcore approach, lacking these precautions and using a single-step receptor affinity purification of B61 as ECK ligand, was reported during the progress of our work (29). This suggests that the specificity of the BIAcore response of a receptor/ligand interaction in crude samples may depend on the relative abundance of the ligand and the type of contaminating proteins present. In accord with this notion, the BIAcore response of a Tyro 3-derivatized sensor chip to 10% fetal bovine serum, an abundant source for the ligand of the RTK Tyro 3 (the reported serum concentration of protein S, the proposed ligand for Tyro 3, is ≈10 μg/ml), was largely abrogated by premixing the sample with 10 μg of the soluble receptor protein per ml (30). In comparison, only 5–7% (14–20 RU) of the total BIAcore response of crude HPCM (280–300 RU) to immobilized sHEK was inhibited with 10 μg of sHEK per ml, suggesting a ligand concentration of ≈1 ng/ml in the starting material (Table 1). Only a second dimension of the screening procedure employing some basic fractionation steps substantiated the initial signal. This protein concentration of a 23- to 25-kDa protein corresponds to a concentration of 40 pM, well within the concentration range detected in conventional biological assays.

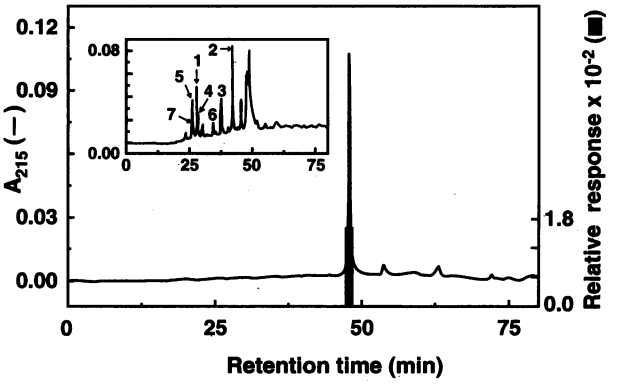

FIG. 4. Micropreparative RP-HPLC of the HEK ligand and derived tryptic fragments. Approximately 0.6 μg of μ-Mono-Q-purified HEK ligand was applied to a 1-mm i.d. RP300 HPLC column and eluted with a 0–60% CH_3CN gradient in 0.09% trifluoroacetic acid at a flow rate of 100 μl/min. Eluting protein was collected manually according to absorbance at 215 nm (——) and tested for binding to sHEK at a 1:50 dilution on the BIAcore. Binding was fully competable with 10 μg of sHEK per ml. (*Inset*) Tryptic map of the reduced and alkylated HEK ligand was produced on the same narrow bore RP-HPLC column. Indicated peaks were loaded directly or after repurification using alternative chromatography conditions (26) onto the protein sequencer.

Unambiguous amino acid sequence determination of tryptic peptide fragments of the purified HEK ligand revealed complete identity with AL-1 (Fig. 5), a recently identified member

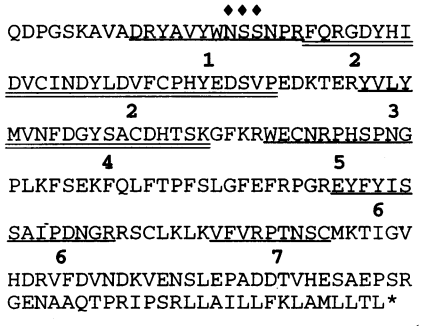

FIG. 5. Tryptic peptide sequences of the HEK ligand corresponding to the AL-1 amino acid sequences. Amino acid sequence of AL-1 (28) is shown together with the sequences obtained for tryptic peptides **1–7** (see Fig. 4) of the HEK ligand underlined. Peptide **4** is a fragment of peptide **3**. Usage of the potential glycosylation site (♦) was verified by amino acid sequence analysis, which gave a blank on cycle 8 (N) for peptide 1.

of the LERK family of ligands suggested to be involved in axon bundling (28). As with other ligands for this RTK family, AL-1 is encoded as a membrane-anchored protein. The soluble form isolated in this report is reminiscent of the soluble form of B61 originally identified as the ECK ligand (29). Although AL-1 was identified as a ligand for REK7, a splicing variant of EHK-1 lacking the first fibronectin type III repeat, the ability of LERKs to bind to several EPH-like RTKs (29) would not preclude the possibility of it also being the HEK ligand. This is also supported by the high degree of sequence identity of AL-1 with ELF-1, the ligand for the murine HEK homologue MEK4 (28, 31). Preliminary deconvolution of the binding kinetics (BIAEVALUATION software, ver. 2.1) between μ-Mono-Q purified AL-1 and sensor chip-immobilized sHEK (four experiments) suggest an apparent dissociation rate constant of 3.1×10^{-3} s^{-1} (± 0.6) and an apparent equilibrium dissociation constant K_d between 2 and 3 nM. This is well within the range reported for the interaction between AL-1 and REK7/IgG (28), since the apparent affinity of this reaction would be increased by the bivalency of the REK7/IgG bivalency of the REK7/IgG construct. It is also notable that the affinity is significantly higher than that of two previously described HEK ligands, LERK3 and LERK4 (32).

Current reports on the activation mechanisms of EPH family receptors give conflicting views. While in some studies receptor activation was found only with the membrane-bound forms of the ligands (28, 33, 34), in other cases the same ligand induced receptor transphosphorylation and a physiological effect as a soluble protein (22, 29). Detailed studies of the kinetics of the HEK/AL-1 interaction and evaluation of its action on receptor-expressing cells remain to be done.

Taken together, our findings demonstrate that in the context of a traditional growth factor purification scheme, the use of the BIAcore as a receptor/ligand affinity detector can replace a conventional biological assay provided that appropriate specific controls are used. They also further demonstrate that LERKs can bind to multiple EPH-like kinases and that this may underlie their multiple effects in important biological processes, including development and oncogenesis.

We would like to thank Janna Stickland for preparation of figures. This work was supported by the Anti-Cancer Council of Victoria, National Health & Medical Research Council, Australian Medical Research and Development Corporation, Melbourne, and the Australian Government Cooperative Research Centres scheme.

1. Hirai, H., Maru, Y., Hagiwara, K., Nishida, J. & Takaku, F. (1987) *Science* **238,** 1717–1720.
2. Boyd, A. W., Ward, L. D., Wicks, I. P., Simpson, R. J., Salvaris, E., Wilks, A., Welch, K., Loudovaris, M., Rockman, S. & Busmanis, I. (1992) *J. Biol. Chem.* **267,** 3262–3267.
3. Letwin, K., Yee, S. P. & Pawson, T. (1988) *Oncogene* **3,** 621–627.
4. Lindberg, R. A. & Hunter, T. (1990) *Mol. Cell. Biol.* **10,** 6316–6324.
5. Chan, J. & Watt, V. M. (1991) *Oncogene* **6,** 1057–1061.
6. Sajjadi, F. G., Pasquale, E. B. & Subramani, S. (1991) *New Biol.* **3,** 769–778.
7. Wicks, I. P., Wilkinson, D., Salvaris, E. & Boyd, A. W. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1611–1615.
8. Xu, Q., Holder, N., Patient, R. & Wilson, S. W. (1994) *Development (Cambridge, U.K.)* **120,** 287–299.
9. Winning, R. S. & Sargent, T. D. (1994) *Mech. Dev.* **46,** 219–229.
10. Jones, T. L., Karavanova, I., Maeno, M., Ong, R. C., Kung, H. F. & Daar, I. O. (1995) *Oncogene* **10,** 1111–1117.
11. Pasquale, E. B. (1991) *Cell Regul.* **2,** 523–534.
12. Sajjadi, F. G. & Pasquale, E. B. (1993) *Oncogene* **8,** 1807–1813.
13. Gilardi, H. P., Nieto, M. A., Frain, M., Mattei, M. G., Chestier, A., Wilkinson, D. G. & Charnay, P. (1992) *Oncogene* **7,** 2499–2506.
14. Maisonpierre, P. C., Barrezueta, N. X. & Yancopoulos, G. D. (1993) *Oncogene* **8,** 3277–3288.
15. Henkemeyer, M., Marengere, L. E., McGlade, J., Olivier, J. P., Conlon, R. A., Holmyard, D. P., Letwin, K. & Pawson, T. (1994) *Oncogene* **9,** 1001–1014.
16. Zhou, R., Copeland, T. D., Kromer, L. F. & Schulz, N. T. (1994) *J. Neurosci. Res.* **37,** 129–143.
17. Andres, A. C., Reid, H. H., Zurcher, G., Blaschke, R. J., Albrecht, D. & Ziemiecki, A. (1994) *Oncogene* **9,** 1461–1467.
18. Ganju, P., Shigemoto, K., Brennan, J., Entwistle, A. & Reith, A. D. (1994) *Oncogene* **9,** 1613–1624.
19. Bohme, B., Holtrich, U., Wolf, G., Luzius, H., Grzeschik, K. H., Strebhardt, K. & Rubsamen, W. H. (1993) *Oncogene* **8,** 2857–2862.
20. Bennett, B. D., Wang, Z., Kuang, W. J., Wang, A., Groopman, J. E., Goeddel, D. V. & Scadden, D. T. (1994) *J. Biol. Chem.* **269,** 14211–14218.
21. Kiyokawa, E., Takai, S., Tanaka, M., Iwase, T., Suzuki, M., Xiang, Y. Y., Naito, Y., Yamada, K., Sugimura, H. & Kino, I. (1994) *Cancer Res.* **54,** 3645–3650.
22. Easty, D. J., Guthrie, B. A., Maung, K., Farr, C. J., Lindberg, R. A., Toso, R. J., Herlyn, M. & Bennett, D. C. (1995) *Cancer Res.* **55,** 2528–2533.
23. Mizushima, S. & Nagata, S. (1990) *Nucleic Acids Res.* **18,** 5322.
24. Ward, L. D., Shi, P. T. & Simpson, R. J. (1992) *Biochem. Int.* **26,** 559–565.
25. Nice, E. C., Lackmann, M., Smyth, F., Fabri, L. & Burgess, A. W. (1994) *J. Chromatogr.* **660,** 169–185.
26. Simpson, R. J., Moritz, R. L., Rubira, M. R. & Van Snick, J. (1988) *Eur. J. Biochem.* **176,** 187–197.
27. Reid, G. E., Hong, J., Eddes, J. S., Moritz, R. L. & Simpson, R. J. (1995) *Electrophoresis* **16,** 1120–1130.
28. Winslow, J. W., Moran, P., Valverde, J., Shih, A., Yuan, J. Q., Wong, S. C., Tsai, S. P., Goddard, A., Henzel, W. J., Hefti, F., Beck, K. D. & Caras, I. W. (1995) *Neuron* **14,** 973–981.
29. Bartley, T. D., Hunt, R. W., Welcher, A. A., Boyle, W. J., Parker, V. P. *et al.* (1994) *Nature (London)* **368,** 558–560.
30. Stitt, T. N., Conn, G., Gore, M., Lai, C., Bruno, J., *et al.* (1995) *Cell* **180,** 661–670.
31. Cheng, H. J. & Flanagan, J. G. (1994) *Cell* **79,** 157–168.
32. Kozlosky, C. J., Maraskovsky, E., McGrew, J. T., VandenBos, T., Teepe, M., Lyman, S. D., Srinivasan, S., Fletcher, F. A., Gayle, R. B., Cerretti, D. P. & Beckmann, M. P. (1995) *Oncogene* **10,** 299–306.
33. Davis, S., Gale, N. W., Aldrich, T. H., Maisonspierre, P. C., Lhotak, V., Pawson, T., Goldfarb, M. & Yancopoulos, G. D. (1994) *Science* **266,** 816–819.
34. Pandey, A., Shao, H., Marks, R. M., Polverini, P. J. & Dixit, V. M. (1995) *Science* **268,** 567–569.