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High-Level Expression of a Full-length Eph Receptor

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Abstract

Eph receptors are the largest family of Receptor Tyrosine Kinases containing a single membrane-spanning segment. They are involved in a various developmental and cell-cell communication events. Although there is extensive structural information available on both the extra- and intracellular regions of Eph's in isolation, no structures are available for the entire receptor. To facilitate structural studies on functionally relevant Eph/ephrin complexes, we have developed an expression system for producing the full-length human EphA2 receptor. We successfully expressed milligram amounts of the receptor using baculovirus-based vector and insect cells. We were also able to extract the protein from the cell membranes and purify it to near homogeneity in two simple steps. The purified receptor was shown to retain its biological activity in terms of both binding to its functional ligands and being able to auto-phosphorylate the key tyrosine residues of the cytoplasmic kinase domain.

Keywords

Receptor Tyrosine Kinases; Eph receptors; transmembrane proteins; insect cell; overexpression

Introduction

One of the revelations coming from the publication of the human genome was that out of the approximately 30,000 genes, about a third code for membrane proteins. Yet, the Protein Structure Initiative that aims to determine the structures of all proteins, unique or homologous, has so far brought out only about 400 membrane protein structures and none of single-pass transmembrane (TM) receptors ([http://blanco.biomol.uci.edu/mpstruc/listAll/list; \[1,2\]](http://blanco.biomol.uci.edu/mpstruc/listAll/list; [1,2])). Indeed, the past years have seen a fairly steady development of methods for the production and crystallization of ion channels, membrane pores, and G-protein-coupled receptors [3,4,5], but reports for obtaining mg- amounts of purified single-pass membrane proteins have thus far been limited. In fact, the purification of an Eph receptor that we describe in this report, to the best of our knowledge, is the first description of a method for

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obtaining any receptor tyrosine kinase (RTK) in amounts suitable for crystallization trials. This is truly surprising taking into account the importance of RTK's in health and disease [6].

RTKs are involved in a large variety of vital cellular processes such as cell differentiation, communication, and growth. Consequently, several cellular abnormalities, most notably numerous cancers, are a consequence of RTK malfunction. Considerable knowledge on the biochemistry of RTK signaling has been gained over the past 30 years and several antibodies, targeting the extracellular domain, or small molecule inhibitors, targeting the cytoplasmic kinase domain (PKI's), have been evaluated in the clinic [7]. Examples include anti-HER2, Herceptin for treating breast cancer [8] and a prototype of the PKI's, Gleevec, a Bcr-Abl kinase inhibitor for treating chronic myeloid leukemia (CML; [9]). Structural data exist for isolated domains of both the intra- and extracellular parts of RTK's [10,11], but lack of atomic resolution data on any full-length single-pass transmembrane receptor has hampered the development of next-generation drugs, as well as a detailed understanding of the initiation of RTK signaling. By far the most important reason for this is the lack of efficient expression and purification methods for the single-pass transmembrane receptors.

Eph (erythropoietin-producing hepatocellular) receptors are the largest family of RTK's. There are 16 known Eph receptors that bind to nine ephrin ligands with various affinities. Both are divided in A and B subclasses, based on sequence conservation and binding specificities. In general, they bind each other promiscuously within each subclass but very seldom between subclasses [12]. Since both the receptors and the ligands are membrane-bound, their interaction at the site of cell-cell contact initiates unique bidirectional signaling [13]. There is extensive structural information available on both the extra- and intracellular regions of Eph's, but none on the entire receptor. In our efforts to study structures of functionally relevant Eph/ephrin complexes, we have developed an expression system for producing milligram amounts of the full-length human EphA2 receptor.

Materials and Methods

Cloning and production of ephrin ligands and full-length Eph in mammalian cells

The ectodomains of ephrins and the full-length EphA3 were cloned N-terminally to a thrombin cleavage site and the Fc domain of IgG in a modified pcDNA3.1 vector (Fig 1A). The ligands were expressed in a HEK293 (Human Embryonic Kidney) cell line using a CD5 signal sequence [14], purified by Protein-A Sepharose affinity chromatography (Amersham), and the Fc-tags removed by thrombin (Novagen) cleavage. Final purification was achieved on a size-exclusion Superdex 200 column (Amersham) using HBS as running buffer.

For the expression of the full-length Eph receptor in mammalian cells, LK63 and HEK cells were grown in DMEM medium (Gibco), supplemented with Fetal Bovine Serum (Sigma) and antibiotics (PenStrep), at 37°C under 5% CO₂. The expression levels were evaluated with Western Blots using appropriate antibodies (see below) after harvesting the cells and extracting the membranes with 0.5% n-Dodecyl- β -D-Maltopyranoside (Anatrace) and 0.1% Cholesteryl Hemisuccinate (Anatrace) in 50 mM HEPES (pH 7.5) and 150 mM NaCl.

Cloning and expression of EphA2FL in yeast

The gene for the human EphA2-receptor (from QGKEVVLL till SLLGLKDQ, a 944 amino acid fragment) was cloned in *Saccharomyces cerevisiae* expression vector pYES2 (Invitrogen). Six additional histidines were added to the C-terminus of the protein to facilitate protein purification and recognition. The correct clones were transformed in *S.*

cerevisiae and the EphA2-FL was produced in a yeast expression medium. After three days, the cells were harvested, washed, and resolved via SDS-PAGE. EphA2-FL was identified using a 6-His-specific antibody (5 Prime GmbH, Germany).

The same EphA2 gene was also cloned in a pPIC3.5 or pPIC9 vector (Invitrogen) for *Pichia pastoris* expression according to the manufacturer's manual. Verified clones were transformed in *P. pastoris* cells using electroporation and transformants were selected on Yeast Extract Peptone Dextrose (YPD)-plates. Cells were grown for expression in a methanol medium for three days. The sonicated cell lysates were tested with a Bio-Dot apparatus (Bio Rad) by pipetting them into the wells of a dot blot cassette and absorbing on an Immun-Blot PVDF membrane (Bio Rad) with an aspirator pump. EphA2-FL-His samples on the membrane were detected with a 6-His-specific antibody.

Cloning, expression, and purification of EphA2-FL from insect cells (Fig 1B)

The full length gene of the EphA2 receptor (EphA2-FL) was cloned into a pAcGP67-based (BD Biosciences) insect cell vector, pMA152a [15], using the BamHI and NotI sites. The A2-FL fragment starts from amino acid sequence ADLGSQGKEVVLL, where the first five amino acids come from the vector, and ends in SLLGLKDQ, Q being the last amino acid of EphA2 before the tags. Following the protein coding sequence is a thrombin cleavage site, a Myc-tag, and an Fc-tag. Another construct was composed by using a primer that adds a His-tag to the end of the EphA2, and abolishes the Myc and the Fc tags. The gene for the EphA2-His was then cloned at the BamHI site of the pMA152a vector.

Expression constructs were verified by DNA sequencing. The constructs were then transfected into insect cells according the BaculoGold baculovirus expression system protocol (BD Biosciences). Briefly, the gene construct was mixed with BaculoGold DNA, Grace's medium (Gibco, Invitrogen) and Cellfectin (Invitrogen) were added, and the mixture was incubated for 30 minutes at room temperature. The mixture was then transfected in the sf9 (Invitrogen) cells, the cells were incubated at 27°C for 5 days, centrifuged at 4000 rpm for 10 min, and the supernatant was isolated to obtain a virus stock 'P0'. P0 was used to transduce a next aliquot of sf9 cells that were incubated at 27°C for 3–4 days and centrifuged as before. The procedure was repeated until a virus stock number P3 was ready to be used for protein expression in Hi5 cells (Invitrogen). For protein production, Hi5 insect cells were cultivated in Insect-Xpress medium (Lonza), in baffled Erlenmeyer flasks with slow rotation (100 rpm) at 27°C for two to three days.

For recombinant protein isolation, Hi5 cells were harvested (4000rpm, 30min) and washed with 10mM Hepes-buffer, pH 7.3 containing 20 mM KCl and 10 mM MgCl₂. The cells were suspended in 50 mM Hepes-buffer, pH 7.3 containing 1 M KCl, 1 M NaCl, 25 mM glycine, 10 % glycerol, and protease inhibitors, incubated over-night at +4°C in a rotator shaker, and the lysed cells were centrifuged at 20,000 rpm for 1 h. The cell pellet was suspended in the same buffer, supplemented with 0.5 % dodecyl-D-maltoside (DDM), 0.2 % cholesteryl hemisuccinate (CHS; Anatrace), and protease inhibitors and incubated in a rotator shaker at 4°C over-night. After incubation, the cells were centrifuged at 20,000 rpm for 1 h. The supernatant was analyzed for the extracted membrane proteins. If necessary, the extraction procedure was repeated.

Fc-tag containing EphA2-FL was purified from the extraction solution using Protein-A affinity chromatography. A protein-A Sepharose (GE Healthcare) column was equilibrated with 20 mM Hepes-buffer, pH 7.3 containing 0.5 M NaCl, 2.5 mM MgCl₂, 0.5 % DDM, and 0.01% CHS. The extracted EphA2-FL-Fc was loaded on the column, rinsed with the equilibration buffer, and the EphA2-FL-Fc was eluted with 0.1 M glycine buffer pH 3.0

containing 0.5 M NaCl, 0.05% DDM and 0.01% CHS. After elution the samples were neutralized with 1 M Tris-HCl, pH 9.0.

The EphA2-FL-His construct was purified using Ni Sepharose™ 6 Fast Flow (GE Healthcare) column. The column was equilibrated with 30 mM imidazole-20 mM Hepes-buffer, pH 7.3 containing 0.05% DDM, 0.01% CHS, 0.5 M KCl and 2.5 mM MgCl₂. The EphA2FL-His was loaded on the column and the unbound protein rinsed out with the stabilization buffer. EphA2FL was eluted from the Ni-column with 500 mM imidazole-20 mM Hepes buffer, pH 7.3 containing 0.05% DDM, 0.01% CHS, 0.5 M KCl and MgCl₂. The purity of the eluted EphA2FL-His was analyzed by SDS-PAGE.

Human EphA2 antibody (MAB3035, R&D Systems) was used at a 1:1000 dilution to detect the EphA2-Fc protein. Penta-His HRP conjugate (5 PRIME GmbH, Germany) was used at a 1:2000 dilution to detect EphA2-His. After SDS gel electrophoresis, proteins were blotted onto Immun-Blot PVDF membrane (Bio Rad), and immunoreactive proteins were detected by a chemiluminescence technique (ECL, Pierce).

Pull-down assay

Pull-down experiments were performed essentially as described earlier [16]. In brief, 4.5-μg of the recombinant EphA2-FL was incubated with 10-μg ephrin ectodomains at room temperature for 30 min in a 500-μl binding buffer containing 20 mM HEPES (pH 8.2), 150 mM KCl, and 2 mM MgCl₂. Chelating Sepharose Fast Flow beads (Amersham) were washed with 50 mM NiSO₄ and 30 mM Imidazole in the binding buffer, added to the reaction mixture, and shaken at room temperature for 30 min. The beads were then harvested by centrifugation, washed twice with 500-μl of the binding buffer, and the bound proteins were separated on a 10–20% polyacrylamide gel.

Binding assays on Blitz

Binding kinetics were measured by Biolayer Interferometry (BLI) on a BLItz instrument (ForteBio) as described in [17]. In short, Protein-A biosensors were loaded with 50 μg/ml solutions of purified, Fc-tagged ephrin ligands. The loaded sensors were washed with HBS and the association and dissociation measurements for the EphA2-FL at concentrations from 100 nM to 2.5 μM were carried out for two minutes each. Kinetic parameters (k_{on} and k_{off}) and affinities (K_D) were calculated using the BLItzPro software.

N-terminal sequencing analysis

Automated chemical ('Edman') protein sequencing was done using a Procise 494 instrument from Applied Biosystems (AB) as described (Tempst et al., 1994). Stepwise liberated PTH-amino acids were identified using an "on-line" HPLC system (AB) equipped with a PTH C18 (2.1 × 220 mm; 5 micron particle size) column (AB).

Phosphopeptide identification by nano-Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS/MS) analysis

In situ trypsin digestion of gel resolved EphA2 was performed as described [18]. The tryptic peptides were purified using a 2-μl bed volume of Poros 50 R2 (Applied Biosystems, CA) reversed-phase beads packed in Eppendorf gel-loading tips [19]. The purified peptides were diluted to 0.1% formic acid and then subjected to nano-liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) analysis as follows. Peptide mixtures (in 20 μl) were loaded onto a trapping guard column (0.3 × 5mm Acclaim PepMap 100 C18 cartridge from LC Packings, Sunnyvale, CA) using an Eksigent nano MDLC system (Eksigent Technologies, Inc. Dublin, CA) at a flow rate of 20 μl/min. After washing, the

flow was reversed through the guard column and the peptides eluted with a 5–45% acetonitrile gradient over 85 min at a flow rate of 200 nl/min, onto and over a 75-micron $\times$ 15-cm fused silica capillary PepMap 100 C18 column (LC Packings, Sunnyvale, CA). The eluent was directed to a 75-micron (with 10-micron orifice) fused silica nano-electrospray needle (New Objective, Woburn, MA). The electrospray ionization needle was set at 1800 V. A linear ion quadrupole trap-Orbitrap hybrid analyzer (LTQ-Orbitrap, ThermoFisher, San Jose, CA) was operated in automatic, data-dependent MS/MS acquisition mode with one MS full scan (450–2000 m/z) in the Orbitrap analyzer at 60,000 mass resolution and up to ten concurrent MS/MS scans in the LTQ for the ten most intense peaks selected from each survey scan. Survey scans were acquired in profile mode and MS/MS scans were acquired in centroid mode. The collision energy was automatically adjusted in accordance with the experimental mass (m/z) value of the precursor ions selected for MS/MS. Minimum ion intensity of 2000 counts was required to trigger an MS/MS spectrum; dynamic exclusion duration was set at 60 s.

Initial protein/peptide identifications from the LC-MS/MS data were performed using the Mascot search engine (Matrix Science, version 2.3.02; www.matrixscience.com) with the human segment of Uniprot protein database (20,329 sequences; European Bioinformatics Institute, Swiss Institute of Bioinformatics and Protein Information Resource). The search parameters were as follows: (i) two missed cleavage tryptic sites were allowed; (ii) precursor ion mass tolerance = 10 ppm; (iii) fragment ion mass tolerance = 0.8Da; and (iv) variable protein modifications were allowed for methionine oxidation, cysteine acrylamide derivatization, phosphoserine, phosphothreonine, phosphotyrosine and protein N-terminal acetylation. MudPit scoring was typically applied using significance threshold score $p < 0.01$. Decoy database search was always activated and, in general, for merged LS-MS/MS analysis of a gel lane with $p < 0.01$, false discovery rate averaged around 1%.

Results and Discussion

Production of Eph-FL using various expression systems

We initially tested the production of a full-length EphA (EphA3-FL) using mammalian cell lines; lymphoblastic leukemia cells (LK63) and two Human Embryonic Kidney (HEK293) cell lines, one of which was engineered to express a biotin-tagged Eph for easier purification. The Eph receptor was pulled-down from the LK63 and the 293 cell lysates using a monoclonal antibody IIIA4 as described earlier [14,20] and from the engineered HEK lysates using an 'AP-technology' [21], based on the use of a biotin Acceptor Peptide (AP), a short peptide (AQKIEWHE) that can be ligated with biotin in a simple enzymatic reaction. The purification of the biotinylated protein is facilitated by the high affinity ($K_d \sim 10^{-15} \text{ M}^{-1}$) of biotin to (strept)avidin for which commercial affinity resins are readily available. We constructed a pcDNA-based vector [14] that contains an AP-expressing, N-terminal sequence followed by a thrombin-cleavage site and the full-length EphA3-expressing sequence. While both systems could express well-detectable EphA3 levels, the yields were between 10 and 50 μg per liter (Fig 2), too low for structural studies.

We, therefore, tested the production of the Eph-FL protein in two non-mammalian expression systems, yeast and insect cells. We cloned the full-length human EphA2 (EphA2-FL; amino acids QGKEVVLL and SLLGLKDQ) in *Saccharomyces* vector pYES2 and in *Pichia* vector pPIC3.5 or pPIC9. All expression constructs were confirmed by nucleotide sequencing. We grew *Saccharomyces* cells in SC-U medium containing 2% galactose for 3 days and *Pichia* cells in BMMY medium also for 3 days. After analyzing the cell lysate using a commercial anti-EphA2 antibody, we observed only modest expression levels, around 0.1 mg/l (not shown).

Consequently, we cloned the same fragments in a Baculoviral vector pMA152 that has earlier been successfully used for the production of the extracellular domains of Eph receptors [22] (see methods). We evaluated both Sf95 and Hi5 insect cells for their ability to express the full length receptor. Surprisingly, the expression levels were dramatically improved as compared to the yeast cell system; the construct that extends beyond the SAM domain produced a maximum amount of around 10 mg of protein from a one-liter cell culture, as evaluated after breaking the cells using a standard, detergent-containing buffer and by a direct Coomassie gel analysis, (see Methods; Fig 3). As seen in the Figure, cells before transfection do not show the EphA2-specific band as compared to the cells 48 h after transfection. We further confirmed the identity of the protein by performing a Western blot with a commercial anti-EphA2 antibody (Fig 3). Moreover, N-terminal sequencing of the expressed protein showed the anticipated sequence, starting with ADLGS that comes from the expression vector, followed by the cloned, N-terminal sequence of EphA2 (QGK...). Both Sf9 and Hi5 cells produced equal amounts of the receptor, as judged by the band intensities. However, the Hi5 cells are easier to handle and grow faster and were, therefore, used in further studies.

We next tested whether the receptor could be efficiently extracted from the Hi5 cells. We broke the cells with a lysis buffer and extracted the membrane proteins using 0.5% DDM detergent and 0.2% CHS. After centrifugation, the solubilized proteins were pulled down from the supernatant by Protein-A Sepharose beads. As seen in Fig 4, only a small fraction of the expressed protein was bound to Protein-A, suggesting a reduced efficiency of the extraction system that is commonly used for other membrane proteins. Because the pMA152 expression vector produces proteins that have an Fc-tag in their C-terminus, we wanted to see if the tag was the reason for the low recovery of the expressed protein. We therefore modified the vector to attach a His-tag, instead of the Fc-tag, in the C-terminus of the recombinant protein. Details on how the vector was engineered are given in the Methods section. We used the His-tag vector to express EphA2-FL from Hi5 cells essentially as described above for the Fc-tagged receptor. Although the expression levels did not show noticeable changes as compared to the Fc-tagged receptor, the His-tagged protein was recovered remarkably (up to an estimated 50-fold) better from the insect cells as compared to the Fc-tagged protein (Fig 4A). We could also easily elute the EphA2-FL from Chelating Sepharose beads by a buffer containing 500 mM Imidazole (see Fig 4B that shows the eluted bands after two consecutive detergent extractions). When loaded on a size-exclusion column (Superdex 200, GE Healthcare), EphA2-FL-His eluted close to the 670 kDa molecular weight marker. Since the average size of the DDM micelles is 50 kDa, this result suggests that the receptor has a tendency of forming tetramers in solution. After size-exclusion purification, EphA2-FL was over 95% pure (Fig 4B). According to the Coomassie-stained protein gel and photometric analysis, we could recover up to 4 mg of the purified EphA2-FL from a one-liter cell culture. We are currently investigating whether the low yields of the Fc-construct could be due to its compromised binding ability to Protein-A, rather than inadequate extraction from the cells.

Expression dynamics

We also evaluated the expression dynamics of recombinant EphA2-FL in insect cells. As shown in Fig. 5A, expression was barely visible after 24 hours and reached its maximum after 48 hours. Insect cells are frequently grown for three days to maximize the yields, but in our case the levels of EphA2-FL were dramatically diminished after 72 hours of cell growth (Fig 5A). To study if this was caused by the instability of the cell membranes during prolonged cell growth or the action of proteinases, we stored the protein, after elution from the Chelating beads, at +4 C. The purified receptor showed considerable stability, and only an additional minor band of around 65 kDa was visible after seven days of storage.

Furthermore, upon limited proteolysis with trypsin, the receptor was gradually cleaved in two fragments, both around 45–50 kDa (Fig 5B). Although the exact identity of these fragments is not relevant to our studies, we can speculate that they represent the intra- and extracellular fragments of EphA2-FL. This should not be a surprise, taking into consideration the architecture of single-pass membrane proteins: the multi-domain, fairly large extra- and intra-cellular parts of the receptors are expected to have increased propensity for a mechanical degradation in comparison to e.g. GPCR's or other multipass membrane proteins [23]. In addition, we know from our previous work on the extracellular domains of the Eph receptors [24] that the membrane-proximal fibronectin domain has considerable structural flexibility and can fairly easily be digested away from the rest of the Eph ectodomain. The resulting molecule would be expected to migrate around the 50-kDa-marker on SDS-PAGE.

Biological activity of purified recombinant EphA2-FL

To confirm that the purified EphA2-FL retains its biological activity, we performed several assays. First, we used a pull-down assay with ephrin-A5. EphA2-FL-His showed clear binding to ephrin-A5, since it could pull down the un-tagged ligand using Chelating Sepharose beads (not shown). Next, we used a newly-developed method, employing Biolayer Interferometry to measure the binding affinities of EphA2-FL to ephrin ligands. Fig 6 demonstrates that the purified EphA2-FL-His shows excellent ligand-binding properties, with apparent K_d values of 360 nM (k_{on} 2.4×10^4 $M^{-1}s^{-1}$; k_{off} 0.53 min^{-1}) and 340 nM (k_{on} 2.7×10^4 $M^{-1}s^{-1}$; k_{off} 0.56 min^{-1}) for ephrin-A5 and ephrin-A1 (respectively). On the other hand, it shows much weaker binding to ephrin-B1, a low affinity EphA2 ligand, with an apparent K_d value of 8,200 nM (k_{on} 5.8×10^4 $M^{-1}s^{-1}$; k_{off} 28 min^{-1}).

Finally, we performed a phospho-proteomics analysis using liquid chromatography coupled to mass spectrometry to monitor the auto-phosphorylation of the EphA2-FL. As shown in Table I, we could identify eight peptide fragments after tryptic cleavage that had a phosphorylated Tyrosine. One fragment had two phospho-Tyrosines. Most importantly, the juxtamembrane Tyr-594 and Tyr-772 that resides within the activation loop of the kinase domain, both of which are critically important for the receptor activation [25], were shown to be auto-phosphorylated. These results clearly demonstrate that our expression system produces a fully active Eph receptor, since the extracellular region retains its ligand-binding ability and the intracellular kinase domain maintains the enzymatic, auto-phosphorylation activity.

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Abbreviations

RTK	Receptor Tyrosine Kinase
TM	transmembrane
Eph	erythropoietin-producing hepatocellular
FL	full-length
DDM	n-Dodecyl- β -D-Maltoside

CHS	cholesteryl hemisuccinate
Fc	Fragment constant
His	6xHistidine purification tag
BLI	biolayer interferometry
LC	liquid chromatography
MS	mass spectrometry
GPCR	G-protein-coupled receptor

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Highlights

- We successfully expressed milligram amounts of human EphA2 receptor using baculovirus-based vector and insect cells
- EphA2 was extracted from the cell membranes using dodecyl maltoside- and hemisuccinate-containing buffer
- The receptor was purified in two simple steps
- The purified receptor was able to bind its biological ligands
- EphA2 was auto-phosphorylated in its cytoplasmic kinase domain, showing that it had retained its biological activity

Fig. 1A

Fig. 1B

Figure 1.

A. Schematic representation of the vectors used. pcDNA is for mammalian cell expression, pYES2 and pPIC9 for yeast expression, and pMA152 for insect cell expression. The gene for the Eph-receptor was cloned to contain amino acids EVNLLDSK till SIKALETQ (a 947 amino acid fragment) in mammalian cells, and amino acids from QGKEVVLL till SLLGLKDQ (a 944 amino acid fragment) in yeast and insect cells. The mammalian construct has Acceptor Protein (AP) and thrombin cleavage site N-terminally to the Eph-gene. The yeast constructs contain six Histidines at the C-terminus of Eph. The insect cell construct has either a thrombin cleavage site, a Myc-tag, and an Fc-tag following the protein coding sequence or six Histidine residues immediately after the Eph (see panel B). **B.** Purification strategy for the full-length EphA2-His from insect cells. The vector construct (shown on top) was mixed with BaculoGold DNA, transfected into Sf9 insect cells that were incubated for 5 days to obtain a virus stock 'P0'. The procedure was repeated until a virus stock number P3 was ready to be used for a three-day protein expression in Hi5 cells using Insect-Xpress medium. Hi5 cells were harvested, lysed in a buffer containing dodecyl-D-maltoside (DDM), and centrifuged to obtain the extracted membrane protein preparation. EphA2-FL-His was purified using Ni Sepharose™ 6 Fast Flow column in the presence of DDM. Final purification was performed by size-exclusion chromatography on a Superdex200 column.

Figure 2.

Western blot evaluation of the expression levels of EphA3-FL in mammalian cells, lymphoblastic leukemia cells (LK63) and two Human Embryonic Kidney (HEK) cell lines, one of which was engineered to express a biotin-tagged Eph for easier purification. Total cell lysates from 10-ml cultures were loaded on the gel and detected using EphA3-specific monoclonal antibody (III A4). Numbers on the right show the amounts of soluble EphA3 ectodomain (ECD) used as standards. The yields can be evaluated to be between 10 μ g and 50 μ g per liter.

Figure 3. Expression of EphA2-FL in Hi5 insect cells. Upper panel, Coomassie staining; Lower panel, Western blot and anti-EphA2 antibody staining. Cells before (lane 1) transfection do not show the EphA2-specific band as compared to the cells 48 h after transfection (lane 2). Arrows show the position of the full-length EphA2.

Figure 4.

Extraction of the Hi5-expressed EphA2-FL. **A.** Hi5 cells after harvesting and suspending in the sample buffer (lane 1). Fc-tagged (lane 2) and His-tagged (lane 3) protein after extracting the cells with a detergent solution and pulling the fusion proteins down with either ProtA or His-Sepharose beads. The His-tagged protein was recovered remarkably (up to an estimated 50-fold) better from the insect cells as compared to the Fc-tagged protein. **B.** Hi5 cells before (lane 1) and after (lane 2) expression. Lanes 3 and 4 show samples after the first and the second detergent extractions and purification on a Ni-Sepharose column. Further purification was achieved using size-exclusion chromatography (lane 5). After the purification, EphA2-FL was over 95% pure, according to the Coomassie-stained protein gel and photometric analysis, and we could recover up to 4 mg of the purified EphA2-FL from a one-liter cell culture. Arrows show the position of the full-length EphA2.

Figure 5.

Expression dynamics. **A.** Time-course, showing the expression of EphA2-FL after 24, 48, and 72 hours of incubation. Expression is barely visible after 24 hours, reaches its maximum after 48 hours, and is dramatically diminished after 72 hours of cell growth. **B.** Purified EphA2-FL shows only minor degradation after seven-days incubation at +4°C with an additional band around 65 kDa (lane 3). After incubating with trypsin (1:1,000 ratio, +4°C) for 1.5 h (lane 1) or 3 h (lane 2), two bands around 45–50 kDa appear that presumably represent the intra- and extracellular fragments of EphA2-FL. Arrows show the position of the full-length EphA2.

Figure 6.

Binding of EphA2-FL to ephrin-A1 as measured using Biolayer Interferometry on a BLItz instrument. EphA2 shows binding to its biological A-ligand but not to the B-class ligands. Shown is the binding of ephrin-A1 to EphA2-FL with an apparent K_d value of 340 nM (k_{on} $2.7 \times 10^4 \text{ Ms}^{-1}$; k_{off} 0.56 min^{-1}).

Table I

Phospho-proteomics analysis of the auto-phosphorylated EphA2-FL, identifying seven peptide fragments that have one phosphorylated tyrosine residue and one fragment that has two phospho-Tyrosines. The analysis shows that the juxtamembrane Tyr-594 and Tyr-772 that resides within the activation loop of the kinase domain, both of which are critically important for the receptor activation, are auto-phosphorylated. The phospho-Tyrosines are highlighted in blue.

<u>Tyr #</u>	<u>Tryptic Peptide</u>	<u>Amino Acid Span</u>
594	TYVDPHTYEDPNQAVLK	587–603
628	VIGAGEFGEVYK	618–629
772	VLEDDPEATYTTSGGKIPIR	763–782
791	WTAPEAISYR	783–792
921	MQQYTEHFMAAGYTAIEK	918–935
930	MQQYTEHFMAAGYTAIEK	918–935
921 and 930	MQQYTEHFMAAGYTAIEK	918–935
960	IAYSLGLK	958–966