



NMR resonance assignment of a ligand-binding domain of ephrin receptor A2

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Abstract

Ephrin receptors regulate intercellular communication and are thus involved in tumor development. Ephrin receptor A2 (EphA2), in particular, is overexpressed in a variety of cancers and is a proven target for anti-cancer drugs. The N-terminal ligand-binding domain of ephrin receptors is responsible for the recognition of their ligands, ephrins, and is directly involved in receptor activation. Here, we report on the complete ¹H, ¹⁵N and ¹³C NMR chemical shift assignment of EphA2 ligand binding domain that provides the basis for NMR-assisted drug design.

Keywords Ephrin receptor · EphA2 · Ephrin · NMR assignment · Ligand binding domain · Extracellular domain

Biological Context

Ephrin receptors (Eph) belong to the class of receptor tyrosine kinases, constituting their biggest subfamily, including 13 members in the case of humans. The receptors share an architecture of a type I membrane protein, with intracellular kinase and regulative SAM domains, a single-pass transmembrane domain and an extracellular part, consisting of two fibronectin type III domains, EGF-like domain, Sushi domain and a terminal ligand-binding domain (LBD) (Kullander and Klein 2002). Eph recognize their cognate ligands, ephrins, which are attached to the cell membrane either with GPI anchors (ephrins A) or by their own transmembrane domains (ephrins B) (Himanen 2012). Depending on the preferred type of the bound ligand, receptors are also classified into two groups: EphA and EphB. Eph are believed to be activated by self-clustering: binding of ephrin to the N-terminal ligand-binding domain of Eph triggers the rearrangement of the receptor cluster on the cell membrane, which, in turn, leads to the activation of their kinase activity (Singh et al. 2018; Shi et al. 2023). Eph receptors regulate

the migration and mobility of various cells (Pasquale 2008; Kania and Klein 2016) and take part in angiogenesis and metastasis formation (Pasquale 2010), which makes them important pharmacological targets. EphA2, among others, is a proven target to treat the colorectal cancer (Tröster et al. 2023b). Therefore, structural investigations and Eph-targeted structure-guided drug design campaigns are highly important.

The three-dimensional structure of EphA2 is rather well explored. There are X-ray structures of the receptor kinase domains, both in the apo state and in complex with various ligands (Nowakowski et al. 2002; Heinzlmeir et al. 2016; Tröster et al. 2023a), an NMR structure of transmembrane domains in the dimeric state (Bocharov et al. 2010), several X-ray structures of truncated and non-truncated extracellular domains (Himanen et al. 2009, 2010; Seiradake et al. 2010; Himanen 2012), including those obtained in complex with ephrin ligands. NMR spectroscopy investigation of EphA2 kinase domains was also reported (Gande et al. 2016). On the other hand, it would be beneficial to conduct the NMR studies of extracellular LBD, to run NMR-assisted ligand screenings or structure-activity relationship investigations. The general possibility of obtaining the EphA2 LBD NMR spectra was previously reported (Wu et al. 2015), however, no chemical shift assignment or further structural study was conducted. Here, we present the complete NMR chemical shift assignment of EphA2 LBD, which provides the basis for further investigation.

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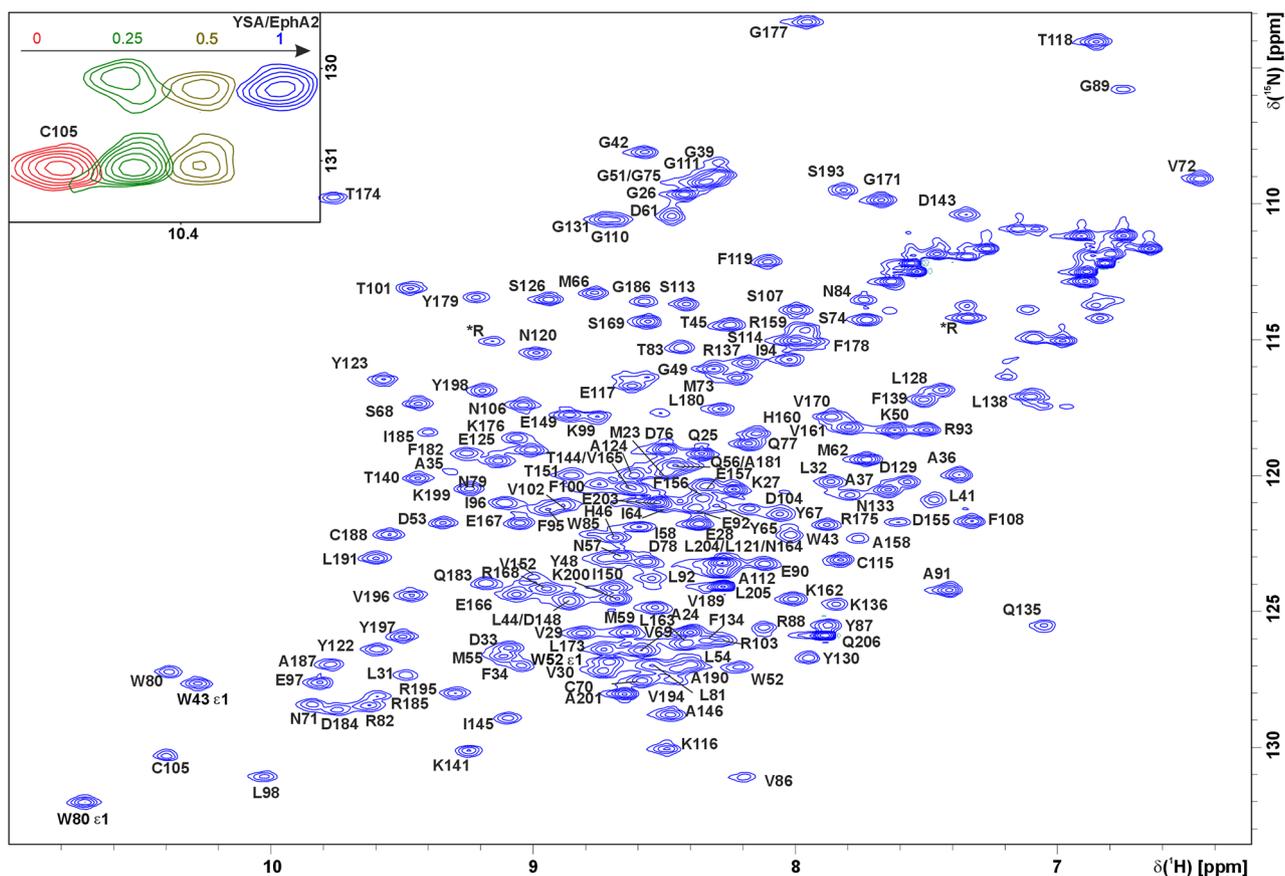


Fig. 1 NMR assignment of EphA2 LBD. ^1H , ^{15}N -HSQC spectrum of $^{13}\text{C}/^{15}\text{N}$ -labeled EphA2 LBD, recorded at 303 K and 600 MHz in 25 mM NaPi pH 6.7, 5% D_2O , 0.01% NaN_3 . Assignments of the backbone amide resonances are shown for each residue, an asterisk (*) denotes the signal of the protein side chains. Insert on top shows the overlay

of ^1H , ^{15}N -HSQC spectra (fragments, containing the C105 cross-peak), obtained after the addition of 0.25 (green), 0.5 (brown) and 1 (blue) equivalents of YSA peptide. The spectra are shifted horizontally with respect to each other for readability

Methods and experiments

Peptide synthesis

YSA peptide (H-YSAYPDSVPMMS-OH) was synthesized with free N- and C- termini using solid phase peptide synthesis on an ABI 433 A peptide synthesizer (Thermo Fisher). The resulting product was dissolved in water and further purified by reversed phase HPLC with C18 column material, followed by determination of purity and identity of the product by NMR and ESI-MS.

Expression and purification of EphA2 LBD

The EphA2 LBD construct used in this study comprises the residues A24 to Q206 of human EphA2 (UniProt ID: P29317) and bears one single-point mutation, C201A, which was introduced to remove the unpaired cysteine residue that in the full-length protein forms a disulfide within the adjacent

Sushi domain. The EPHA2 LBD gene construct, optimized for *E. coli*, was synthesized by GenScript Biotech (Netherlands) B.V. Subsequently, it was subcloned into the in-house modified pMALTEV vector, which harbors Maltose binding protein (MBP) to enhance the solubility and folding of the expressed fusion partner. pMALTEV vector is a modified version of the standard pMAL-c4x vector (NovoPro), that provides N-terminal hexahistidine residues to the MBP fusion protein and the tobacco etch virus (TEV) protease cleavage site after the 6XHis-MBP fusion tag. After the TEV cleavage, the resulting protein contained three additional amino acids at the N-terminus: (^{21}GAM) due to the cloning. The expression was conducted in Shuffle[®] T7 competent *E. coli* cells (New England BioLabs). Protein expressions were performed in 5 L Erlenmeyer flask either in either 15 N M9 (1 g/L $^{15}\text{NH}_4\text{Cl}$) or 13 C, 15 N M9 (2 g/L ^{13}C -D-Glucose, 1 g/L $^{15}\text{NH}_4\text{Cl}$) medium with 100 $\mu\text{g}/\text{ml}$ ampicillin. When the A_{600} of the cells reached 0.6–0.7, a cold shock was given to the cells by placing the flask in

an ice bath for 20 min. Followed by induction with 75 μ M IPTG concentration and overnight expression at 18 °C with 120 RPM. Cells were harvested by centrifugation at 4,000 \times g for 20 min and the cell pellets were stored at -70 °C until further processing.

The cell pellet was thawed and resuspended in 150 ml of lysis buffer or Ni-buffer A (50 mM Tris pH 8.2, 150 mM NaCl, 5 mM imidazole), supplemented with a protease inhibitor tablet (cOmplete™, Roche, Germany). Mechanical cell lysis was performed using a microfluidizer (Microfluidics M-100P) at a pressure of 15000 PSI for 4–6 cycles, while maintaining continuous cooling with ice. Eventually, the supernatant was loaded onto HisTrap HP columns (Cytiva, USA) that had been equilibrated with Ni-buffer A (following the manufacturer's recommendations). Following the loading of the cell lysate, the HisTrap HP column was washed

with 5% of Ni-buffer B (50 mM Tris pH 8.2, 150 mM NaCl, and 500 mM imidazole) with 10 column volumes and the elution of the target protein was performed with a linear gradient of Ni-buffer B from 5 to 100% in 10 column volumes. Fractions containing the target protein were pooled together and incubated with TEV-protease overnight at 4 °C, with simultaneous dialysis against Ni-buffer A. After the TEV cleavage, the dialyzed EPHA2 protein was applied once again on the HisTrap HP column to separate the EPHA2 LBD protein from the cleaved His-MBP fusion tag and TEV protease. The final purification of the EPHA2 LBD protein was achieved through HiLoad26/60 S75 size-exclusion chromatography using buffer C (50 mM Tris pH 8.2, 150 mM NaCl). The pure protein obtained from the Sect. 75 was concentrated using an Amicon concentrator with a regenerated cellulose membrane of 10 kDa cut-off, achieving

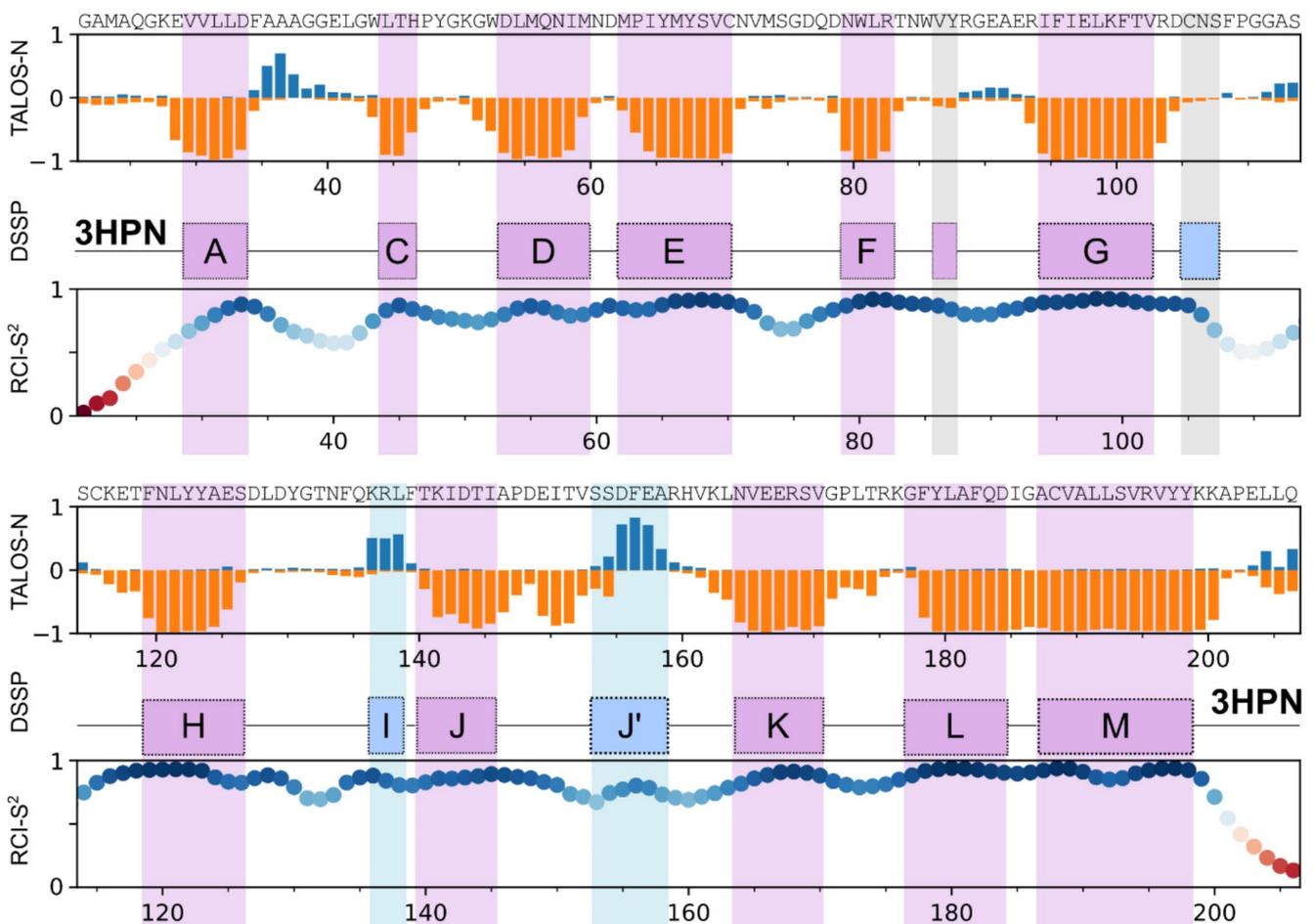


Fig. 2 Results of the NMR chemical shift analysis. The chemical shift-derived propensity of the secondary structure (TALOS-N, is orange and negative for the β -structure and blue or positive for the α -helix); the secondary structure of EphA2 LBD, according to the PDB ID 3HPN is shown for reference (DSSP, blue rectangles correspond to the helical structure and purple - to the extended conformation, grey rectangles denote the elements that are found in the structure, but are not present in solution, according to the NMR chemical shifts); and the

order parameters of backbone amide groups, calculated based on the random coil chemical shift index (RCI-S²), are shown as a function of residue number. Elements of secondary structure are named according to the work (Himanen et al. 2009). Points are colored with respect to the RCI-S² magnitude. The secondary structure shown is the consensus result of the DSSP (Kabsch and Sander 1983) and STRIDE (Heinig and Frishman 2004) packages

Table 1 List of the NMR experiments with experimental conditions

Experiments	Labeling	Time domain data size [points]			Spectral width [ppm]			ns	Delay time [s]	Mixing time, ms	NUS %	T, K
		T1	T2	T3	F1	F2	F3					
BEST-TROSY	¹³ C/ ¹⁵ N	200	2024		35 (¹⁵ N)	16.0 (¹ H)		8	0.3	-	-	303/298
^{1,13} C-HSQC		300	1024		80.0 (¹³ C)	13.0 (¹ H)		2	1.0	-	-	303/298
HNCACB		256	116	2024	64.0 (¹³ C)	31.0 (¹⁵ N)	13.6 (¹ H)	24	0.3	-	40	298
HNGO		100	116	2024	14.0 (¹³ C)	31.0 (¹⁵ N)	13.6 (¹ H)	8	0.3	-	-	298
HN(CA)CO		100	116	2024	14.0 (¹³ C)	31.0 (¹⁵ N)	13.6 (¹ H)	32	0.3	-	40	298
HNCA		128	116	2024	26.0 (¹³ C)	31.0 (¹⁵ N)	13.6 (¹ H)	8	0.3	-	-	303/298
HN(CO)CA		128	116	2024	26.0 (¹³ C)	31.0 (¹⁵ N)	13.6 (¹ H)	8	0.3	-	-	298
H(C)CH-TOCSY		136	180	2048	10 (¹ H)	42.0 (¹³ C)	13.6 (¹ H)	8	1.0	16.3	48	298
(H)CCH-TOCSY		220	128	1024	80.0 (¹³ C)	42.0 (¹³ C)	11.9 (¹ H)	8	1.0	10.9	45	303
NOESY- ¹⁵ N-HSQC		400	128	1024	11.4 (¹ H)	31.0 (¹⁵ N)	11.4 (¹ H)	8	1.0	120	47	298
NOESY- ¹³ C-HSQC		220	128	1024	(¹ H)	42.0 (¹³ C)	12.0 (¹ H)	16	1.0	120	40	303
NOESY- ¹³ Caro-HSQC		220	66	1024	(¹ H)	30.0 (¹⁵ N)	12.0 (¹ H)	32	1.0	120	42	303
(H)CCH-COSY		142	48	2048	36.0 (¹³ C)	36.0 (¹³ C)	13.6 (¹ H)	8	1.0	-	-	303
(Hb)Cb(CgCC)H		94	1024		40.0 (¹³ C)	10.9 (¹ H)		129	1.0	-	-	303

concentrations of 2–10 mg/mL (0.1–0.5 mM) with the target protein yields of around 7 mg/L for M9 media. For NMR experiments, EPHA2 LBD was re-buffered to 25 mM NaPi pH 6.7).

The appropriateness of the EphA2 LBD folding was confirmed by titrating the ¹⁵N-labeled protein (300 μM) with 10 mg/mL stock solution of the YSA peptide, the well documented EphA2 LBD binder with low-micromolar affinity (Koolpe et al. 2002). Recorded NMR spectra revealed the clear high-affinity binding, which occurred in the slow exchange regime (Fig. 1), suggesting the proper folding and ligand-binding propensity of the protein.

NMR experiments

Prior to NMR measurements, EphA2 LBD samples were supplied with 5% D₂O, 0.1% NaN₃ and 0.1 mM TSP for chemical shift referencing. The NMR samples (330 μL) were measured at either 298 or 303 K on Bruker Avance Neo 600 MHz spectrometer equipped with QCI cryoprobe. Triple resonance spectra were acquired using the BEST-TROSY pulse sequences (Favier and Brutscher 2011; Solyom et al. 2013), and non-uniform sampling of indirect dimensions was applied, if required. The backbone and side chain chemical shift assignments of EphA2 were performed using the standard triple resonance NMR experiments, with the assistance from 3D-HCCH-TOCSY and 3D NOESY-HSQC spectra. Aromatic sidechains were assigned using the 2D (Hb)Cb(CgCC)H (Löhr et al. 2007) and (H)CCH-COSY (Pervushin et al. 1998) experiments. Data were processed using TopSpin 4.4.0 software (Bruker BioSpin) and the spectra were analyzed using CARRA 1.9.2 (Keller 2004) software. Spectra recorded with non-uniform sampling were processed in qMDD software using the iterative soft thresholding algorithm with virtual echo (Mayzel et al. 2014). The list of recorded NMR spectra is provided in Table 1.

Extent of assignment and data deposition

The EphA2 LBD protein consists of 186 amino acids, including six prolines, implying that 179 cross-peaks can be in theory observed in an ¹H,¹⁵N-HSQC, while 172 amide cross-peaks were actually assigned, corresponding to 96% of maximally possible (Fig. 1B). Overall, we assigned 96.2% of the protein backbone, 97.6% of the aliphatic and 79.8% of aromatic side chain atoms. In addition, we identified the ¹H and ¹⁵N resonances of eight asparagine and glutamine sidechains, three Hε₁ cross-peaks of W43, W52 and W80, and one Hε cross-peak of arginine sidechain (R195), based on the NOESY connectivities. Cross-peaks of following amide groups were not found: A22, E40, N60, T132,

S153 and S154, due to the exchange-induced line broadening. Noteworthy, for A22, E40, N60 and T132 we assigned the signals of respective side chains.

Figure 2 shows the secondary structure analysis of EphA2 LBD, performed using the TALOS-N software (Shen and Bax 2015). As a reference, we utilized the structure of EphA2 LBD with PDB ID 3HPN, which corresponds to a shorter ^{28}E - ^{201}C construct (Himanen et al. 2009). According to the literature, EphA2 LBD has a jellyroll β -sandwich fold, comprising 11 β -strands and two short α -helices, which are traditionally named by letters from A to M. Additionally, the secondary structure analysis software, such as STRIDE (Heinig and Frishman 2004) or DSSP (Kabsch and Sander 1983), detects a short β -strand in the F-G loop and a 3–10 helix in the G-H loop. Altogether this fold is perfectly reproduced in the NMR chemical shifts data. All the major elements of secondary structure are clearly observed, except for the short strand and 3–10 helix in the loops, mentioned above. Besides, one could state that the β -strand J in solution becomes elongated, compared to the X-ray structure, which could be the consequence of either construct length or crystallization. According to the random coil chemical shift index (Berjanskii and Wishart 2005), the first eight and last six residues of EphA2 LBD construct are disordered.

In conclusion, here we report an NMR chemical shift assignment of the ligand binding domain of Ephrin receptor A2. The assignment is almost complete and is in good agreement with the spatial structure of EphA2, reported previously. The quality of the data and completeness of the assignment allow exploiting our data in future ligand screening campaigns and structural studies.

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Data availability NMR resonance assignments have been deposited to the BMRB under the accession code: 52671.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication The authors agree with the submission and

publication at the Biomolecular NMR Assignments.

Competing interests The authors declare no competing interests.

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