

Illuminating the Molecular Dance: Characterization of ALK Receptor and AUG- α Ligand Complex

Jason Fang

Mentor: Andrey Reshetneyak,

Dr. Charalampos G. Kalodimos Lab

Department of Structural Biology

St. Jude Children's Research Hospital

Abstract

Understanding the interaction between the Anaplastic Lymphoma Kinase (ALK) receptor and its ligand Augmentor- α (AUG- α) is crucial for revealing their signaling mechanisms. Dysregulation of ALK signaling has been implicated in several malignancies, such as anaplastic large cell lymphoma and neuroblastoma. The goal of the project is to characterize the stoichiometry of ALK:AUG- α complex and investigate the necessity of 2:1 or 2:2 binding ratios or both of the states for receptor activation, as the field has seen divergent observations supporting both models¹⁻³. This study focuses on the development of purification protocol of “full-length” ALK receptor and initial structural characterization of ALK:AUG- α complex. ALK receptor and AUG- α were purified using affinity purification and Size Exclusion Chromatography (SEC). ALK:AUG- α complex was purified using SEC and was further stabilized using Gradient Fixation (GraFix). Purified complex was characterized by negative staining electron microscopy.

Introduction

Anaplastic lymphoma kinase (ALK), a member of the receptor tyrosine kinase family (RTK)⁴, is encoded by the ALK gene on chromosome 2p23. The full-length ALK receptor has an approximate molecular weight of 220 kDa. ALK was initially identified as a component of the NPM-ALK oncogenic fusion protein, a result of the translocation between chromosomes 2 and 5 (t[2;5][p23;q35]), which is associated with anaplastic large cell lymphoma⁵. This specific translocation has also been observed in Hodgkin lymphoma⁶. ALK rearrangements are present in 3–7% of non-small cell lung cancer (NSCLC) patients, predominantly occurring in individuals with no or minimal smoking history, those diagnosed with adenocarcinoma, younger patients, females, and in tumors that are wild-type for EGFR and KRAS⁷⁻¹¹. Subsequent research has also linked ALK to melanoma¹², colorectal adenocarcinoma¹³, neuroblastoma¹⁴. Additionally, ALK has been implicated in the invasion of tumor cells through endothelial monolayers in glioblastoma¹⁵⁻¹⁶ and in the development of pancreatic neuroendocrine carcinomas¹⁷. Although ALK was once considered an orphan receptor tyrosine kinase, recent identifications of AUG- β and AUG- α (also known as ALKAL1 and ALKAL2 or FAM150A and FAM150B, respectively) have provided further insights into its functioning¹⁸⁻²⁰.

When exploring the relationship between ALK expression and overall survival probability using Kaplan-Meier analysis (R2: Genomics Analysis and Visualizing KaplanScanner tool)²¹ across various cancers including neuroblastoma, melanoma, and lung cancer, among others, the analysis

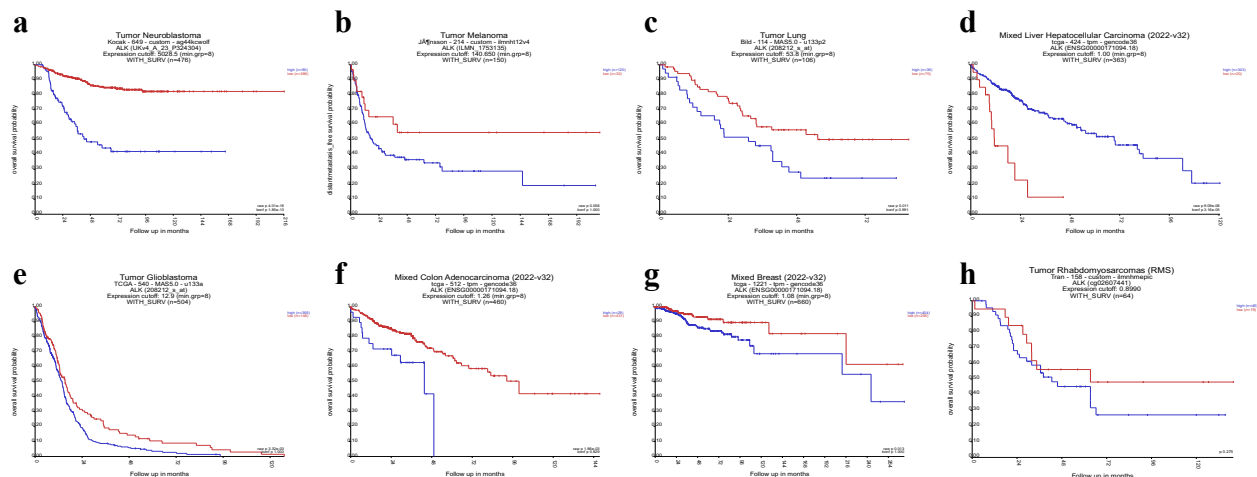


Figure 1. Kaplan-Meier survival curve showing ALK gene expression level is correlated with worse event-free survival in various tumor types.

a. neuroblastoma, **b.** melanoma, **c.** lung cancer, **d.** hepatocellular carcinoma, **e.** glioblastoma, **f.** colon adenocarcinoma, **g.** breast cancer and **h.** rhabdomyosarcomas

revealed a significant correlation where higher ALK expression was associated with a decreased overall survival probability ($p < 0.01$) (Fig. 1).

Consequently, the development and clinical application of ALK inhibitors have been a focus for treating patients with tumors. In 2011, the FDA approved crizotinib, an ATP analogue that inhibits ALK, for treating patients with ALK-positive NSCLC, grounded in compelling clinical response data²². However, the efficacy of long-term crizotinib treatment is compromised by the emergence of drug resistance²³⁻²⁷. Resistance to ALK tyrosine kinase inhibitors (TKI), such as crizotinib, poses a significant clinical challenge not only in NSCLC patients with known ALK positivity, who may develop resistance mutations during treatment^{23,28}, but also in neuroblastoma cases. In neuroblastoma, emergent activating mutations in ALK have been observed in the advanced stages of the disease, independent of prior ALK TKI exposure²⁹⁻³¹. Recent studies have demonstrated that AUG- α and β regulate ALK expression and consequently inhibit tumor growth^{14,18-19,32-33}. Therefore, characterization of the ALK ligands AUG- α , β and interaction with ALK has opened a new path for exploration of potential therapeutic targets for tumors.

Cryo-electron microscopy (cryo-EM) of single particles is a biophysical technique that can be used to determine the structure of biological macromolecules and assemblies. Negative-stain transmission electron microscopy (TEM) is a technique that has provided nanometer resolution images of macromolecules, which is a quick method that can be used to screen for robust biochemical conditions, the integrity, binding, and composition of samples and to get an estimation of sample grid concentration³⁴. Negative stain TEM serves as a rapid and cost-effective method to evaluate protein homogeneity and behavior, laying the groundwork for more intricate studies using cryo-EM. Recent investigations into the ALK:AUG- α complex have proposed varying ligand-receptor stoichiometries and activation mechanisms^{1,3}. De Munck and colleagues identified a 2:1 ligand-to-receptor ratio, suggesting a unique binding stoichiometry that could play a crucial role in the activation of the ALK receptor¹. On the other hand, Reshetnyak et al. and Li et al. observed a 2:2 ratio, proposing an alternative interaction mechanism²⁻³.

To further understand the molecular and structural basis of the ALK interaction with its ligand AUG- α , the ALK receptor and AUG- α ligand have been successfully purified respectively to form the ALK:AUG- α complex. The negative-stain TEM image has visualized the overall morphology

of the ALK:AUG- α complex. The current negative-stain TEM work provides the basis for detailed cryo-EM analysis to better understand the binding interface between ALK and its ligand AUG- α .

Results

Characterization of ALK Binding Domain with AUG- α

The term “full-length” ALK receptor as used in this study refers to an ALK receptor variant comprising residues 673-1407, which includes part of the extracellular domain (ECR), the transmembrane helix, the intracellular kinase domain with a kinase-dead mutation, and a C-terminal Strep-tag (Fig 2a-b). The binding domain of ALK, specifically within the ECR region spanning residues 673-1025, was investigated for its interaction with the AUG- α ligand³ (Fig 2a). The expression of ALK was confirmed by Western blot (Fig. 2c).

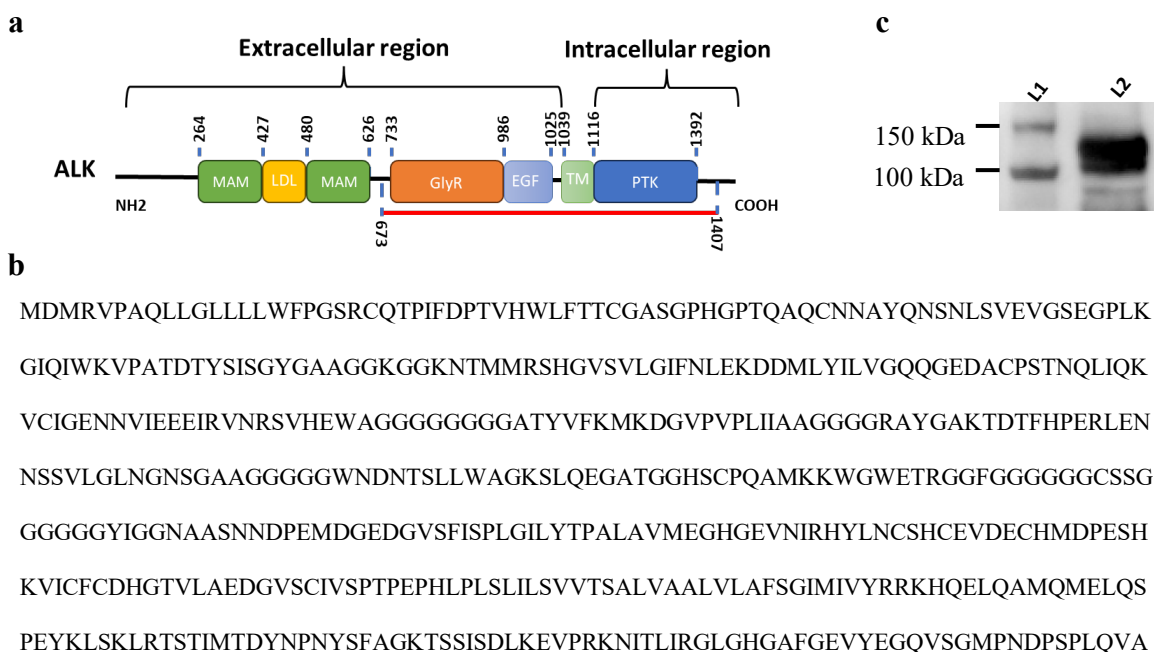


Fig. 2. Characterization of ALK.

a. Schematic representation of domain organization for human ALK receptor, is composed of two meprin, A-5, receptor protein-tyrosine phosphatase mu (MAM) domains separated by a low-density lipoprotein receptor class A (LDL) domain, a glycine-rich region (GlyR), a TNF-like15 domain and an EGF-like (EGF) domain, Transmembrane (TM) domain, and Tyrosine kinase (PTK) domain. **b.** Amino acid sequence of “full length” ALK, which included kinase dead mutant and Strep-tag. **c.** Western blot validated “full length” ALK expression. L1: Protein Ladder; L2: ALK

Affinity Purification of AUG- α

Nickel Affinity Chromatography was applied to extract His-tagged AUG- α from clarified lysate. Subsequent elution yielded five fractions, with Sodium dodecyl-sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) indicating fractions 2 and 3 contained the highest concentrations of AUG- α (Fig. 3a, L4-L5), which were pooled for further purification. Prior to Nickel Affinity Chromatography, a sample from the clarified lysate was analyzed by SDS-PAGE alongside a molecular weight marker confirming AUG- α at the anticipated 50 kDa size (Fig. 3a, L1). SEC was employed to further isolate the AUG- α protein. SEC further purified AUG- α , with the elution profile revealing two distinct UV absorbance peaks at 280 nm (Fig. 3b). SDS-PAGE resolved an elution profile with two peaks: the first peak corresponding to high molecular weight species, potentially misfolded or aggregated AUG- α with non-native disulfide bonds under non-reducing conditions; the latter peak was consistent with the expected monomeric form. SEC purification yielded 22 fractions; fractions 12-22 were analyzed via SDS-PAGE (Fig. 3c). Fractions 17-21 exhibited the highest concentration of monomeric AUG- α and were pooled for further application (Fig. 3c, L6-L10).

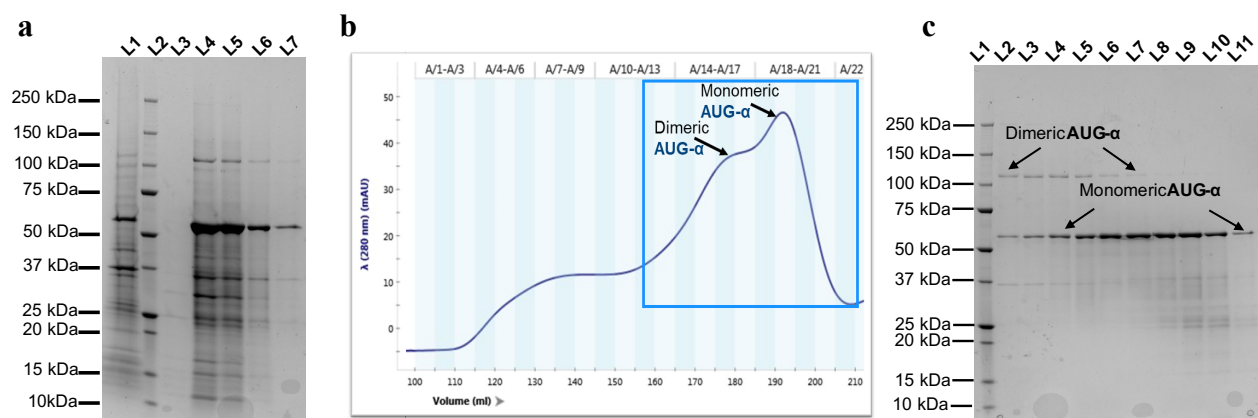


Fig. 3. AUG- α Purification.

a. Gel image of AUG- α sample before and after Nickel Affinity Chromatography. L1: Protein sample before Nickel Affinity Chromatography; L2: Protein Ladder; L3-L7: Nickel Column Elution fractions; L4 and L5 combined for SEC. **b.** Chromatogram from SEC of AUG- α . X-axis: Volume of protein sample eluted (first 100 ml of calibration buffer); Y-axis: UV detection at 280 nm. A/1-A/22 indicate all fractions collected. **c.** SDS PAGE analysis of AUG- α fractions after SEC. L1: Protein Ladder; L2-L11: SEC fractions A/12 – A/21; L6 – L10 combined for spin concentrator.

Formation of ALK:AUG- α Complex

Affinity purification on a Strep-Tactin column isolated ALK with a C-terminal Strep-tag, confirmed by UV absorbance at 280 nm returning to baseline post-washing (Fig. 4a). Purified AUG- α was subsequently introduced to bind with immobilized ALK, resulting in the formation of the ALK:AUG- α complex. Biotin buffer elution eluted the ALK:AUG- α complex from the Strep-Tactin column, as evidenced by a marked UV absorbance peak (Fig. 4a). SDS-PAGE of the eluate

identified fractions 3 and 4 as containing the highest complex density (Fig. 4b, L5-L6). Combined fractions underwent SEC, with UV absorbance at 280 nm revealing two distinct peaks: the initial peak corresponding to protein aggregates and the latter indicative of the ALK:AUG- α complex (Fig. 4c). Ten fractions were collected during these two peaks, and SDS-PAGE analysis was performed to assess protein distribution (Fig. 4d). Fractions A/6 to A/9, corresponding to the highest concentration of the ALK:AUG- α complex, were combined to yield a purified, concentrated sample for GraFix (Fig. 4d, L7-L10).

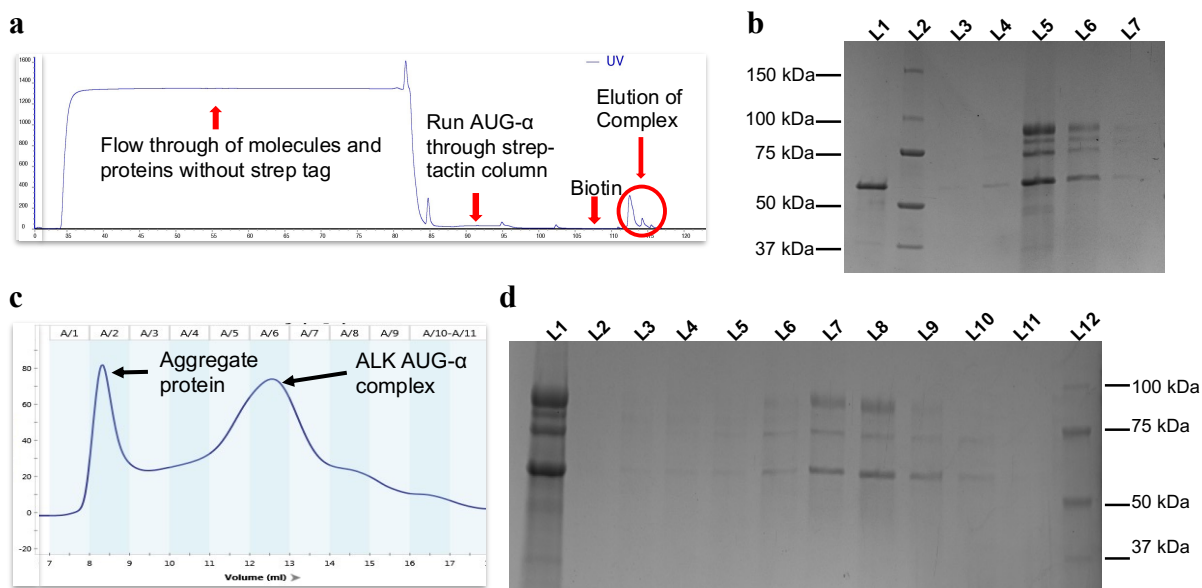


Fig. 4. ALK:AUG- α Complex Formation.

a. Chromatogram from Fast Protein Liquid Chromatography (FPLC) of ALK and ALK:AUG- α complex. X-axis: Volume of protein sample eluted; Y-axis: UV detection at 280 nm as sample elutes. **b.** SDS PAGE analysis of ALK:AUG- α complex fractions after FPLC. L1: AUG- α ; L2: Protein Ladder; L3-L7: FPLC fractions #1 - #5; L5 and L6 combined for SEC. **c.** Chromatogram from SEC of ALK:AUG- α complex. X-axis: Volume of protein sample eluted (first 7 ml of calibration buffer); Y-axis: UV detection at 280 nm as sample elutes. A/1-A/11 indicate all fractions collected. **d.** SDS PAGE analysis of ALK:AUG- α complex fractions after SEC. L1: Output from Affinity Purification of ALK:AUG- α complex; L2-L11: SEC Fractions A/1 – A/10; L12: Protein Ladder; L7 – L10 combined for GraFix.

GraFix and Negative Staining

Following its assembly and preliminary purification, the ALK:AUG- α complex was subjected to GraFix to enhance stability and facilitate size-based separation. Glycerol gradient centrifugation separated protein complexes, yielding two distinct absorbance peaks at 280 nm (Fig. 5a). The initial elution peak suggested the presence of lower-order ALK:AUG- α complexes, indicative of

1:1 or 2:1 stoichiometries. The subsequent peak corresponded to higher molecular weight entities, consistent with a 2:2 stoichiometry of the ALK:AUG- α complex indicating a complete receptor-ligand assembly. Fractions within this peak were combined for further application. Following GraFix stabilization, 2:2 ALK:AUG- α complex samples were subjected to negative staining with uranyl formate for TEM, providing a quality control step to assess complex morphology. Particle analysis confirmed uniformity in size and morphology, indicating a homogeneous sample suitable for further high-resolution structural analysis by Cryo-EM (Fig. 5b-c).

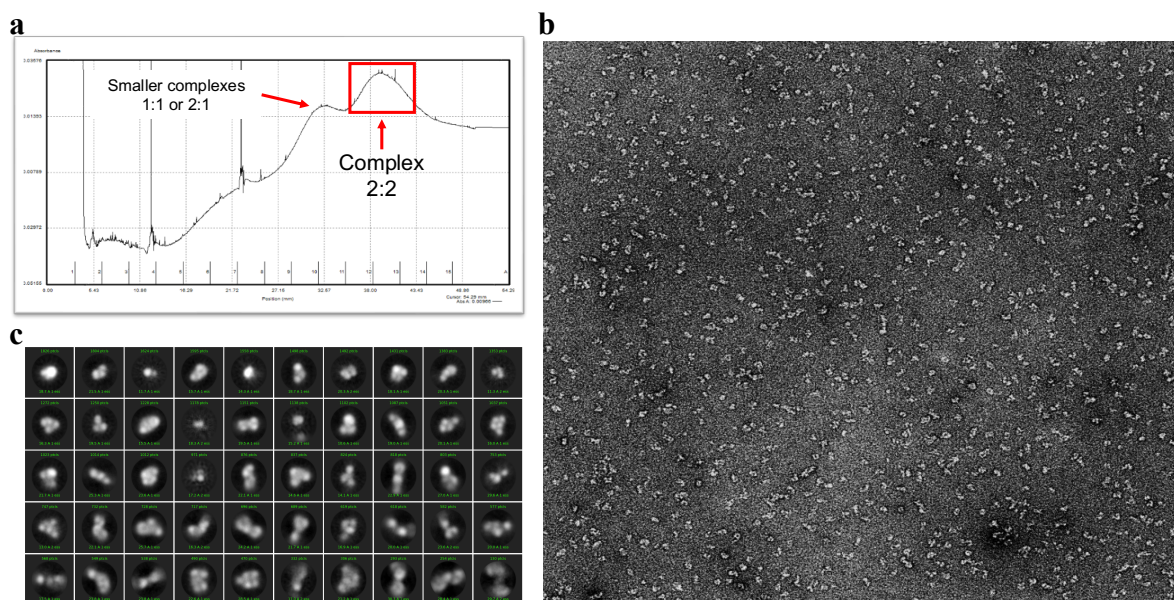


Fig. 5. GraFix and Negative Staining of ALK:AUG- α complex. **a.** Glycerol gradient ultracentrifugation profile of the ALK:AUG- α complex. Absorbance at 280 nm (y-axis) plotted against the position in the gradient (mm) on the x-axis. 2:2 receptor-ligand complex collected for negative staining analysis. **b.** Negative stain electron micrograph of ALK:AUG- α complex. **c.** Two-dimensional class averages obtained from negative stain electron micrograph of ALK:AUG- α complex.

Discussion

This study established a purification protocol for the ALK:AUG- α complex, pivotal for the structural analysis of this interaction. The protocol's efficacy was validated by negative staining TEM, which confirmed the high quality of the purified complex. The demonstration that the complex is suitably homogeneous and well-formed positions us for subsequent high-resolution cryo-EM studies. This preparatory work lays the groundwork not only for an in-depth structural understanding of the ALK:AUG- α interaction but also for the development of targeted therapeutic

interventions aimed at modulating this protein-protein interaction in various ALK-mediated diseases.

Methods

Data Mining

To investigate the prognostic value of ALK expression level, Kaplan-Meier analysis are used from R2: Genomics Analysis and Visualization Platform. First, Kaplan Meier option was selected from left side main menu. In the “Data set:” to choose data set according to tumor type need to be mined. Next, choose “Separate by ‘a single gene’” and click “Next”. In the next screen fill in ‘ALK’ in the Search by Gene field and use the first reporter in the list of the dropdown that popup. In the “Other settings” selection window, “Type of Survival” pulldown option choose “overall survival” option and leave the rest as is and click “Next”. The Kaplan scan generates a Kaplan Meier Plot based on the most optimal mRNA cut-off expression level to discriminate between a good and bad prognosis cohort.

Protein Purification of AUG- α Ligand

To enhance the solubility and facilitate proper folding of the AUG- α ligand, a fusion construct was engineered to include a maltose-binding protein (MBP) tag and a Cys66 to Phe mutation. This construct, also containing a polyhistidine (His)-tag for purification purposes, was transformed into BL21(DE3) C+ E. coli cells (Agilent, 230280) using heat-shock. After transformation, colonies that grew on LB agar plates containing 100 μ g/ml ampicillin were picked and cultured in LB broth supplemented with 100 μ g/ml ampicillin at 37°C with shaking at 225 rpm. Upon reaching an OD600 of 0.4, protein expression was induced by isopropyl β -D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, 16758) to a final concentration of 0.5 mM. After a subsequent 4-hour incubation at 37°C, cells were harvested by centrifugation at 5000g for 10 minutes at 4°C.

The resultant cell pellet was resuspended in lysis buffer: 15 mM HEPES (pH 7.4), 250 mM NaCl and 1% NP40. Sonication was performed in 5-second pulses with 10-second intervals for 5 minutes on ice (QSonica, Q500-110). Post-sonication, the lysate was centrifuged at 15,000g for 30 minutes at 4°C. The pellet, containing inclusion bodies, was isolated.

Inclusion bodies were resuspended in washing buffer: 15 mM HEPES (pH 7.4), 250 mM NaCl and 0.1% NP-40. After sonication under the same parameters, the solution was centrifuged at 15,000g for 30 minutes at 4°C. The pellet was retained.

Inclusion bodies were resuspended in a buffer containing 6 M GuHCl, 10 mM Tris-HCl (pH 7.5), and 10 mM DTT. After stirring at room temperature for 2-3 hours, the mixture was centrifuged at 20,000g for 30 minutes.

The solubilized protein was diluted to a final concentration of 50 mg/L in refolding buffer containing 2.6 M guanidium chloride, 500 mM arginine chloride, 50 mM Tris-HCl (pH 8.8), 10 mM reduced glutathione, 1 mM oxidized glutathione and 0.001% NP-40. Subsequently, the solution underwent dialysis against a dialysis buffer 10 mM HEPES (pH 7.4), 150 mM NaCl, and 0.001% NP-40.

The column, filled with Ni-NTA agarose beads, was equilibrated with wash buffer: 15 mM HEPES (pH 7.4), 500 mM NaCl, 0.5 M urea, 0.1% CHAPS, and 10 mM imidazole. After loading the refolded protein, the column was washed with at least 10x column volumes of the wash buffer. Elution was performed with buffer containing 15 mM HEPES (pH 7.4), 500 mM NaCl, 0.5M urea, 0.1% CHAPS, and 250 mM imidazole. Fractions were collected based on UV absorbance at 280 nm, analyzed using SDS-PAGE, and combined accordingly.

The refolded AUG- α ligand was subjected to SEC using a Superdex 200 column (Cytiva, 28990945), equilibrated with SEC1 buffer: 15 mM HEPES (pH 7.4), 500 mM NaCl, 0.5M urea, and 0.1% CHAPS. Fractions were collected, analyzed, and combined based on SDS-PAGE results.

The purified AUG- α ligand solution was concentrated with spin concentrator, by centrifugation at 10,000g for 10 minutes. The concentration was checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific, ND-ONE-W), before and after the process. The final product was stored at -80°C for subsequent usage.

Protein Purification of ALK

The ALK construct used for expression comprised the extracellular domain, transmembrane helix, a kinase-dead mutant of the kinase domain, and a C-terminal Strep-tag. It was cloned into a baculovirus expression vector for transduction into Sf9 insect cells (ATCC, CRL-1711). Notably, this construct did not include the NH2 terminal, MAM1, LDL, MAM2 domains, and the C-terminal tail, which are present in the native full-length receptor. The ALK gene was cloned into pFastBac baculovirus expression vector (Thermo Fisher Scientific, 10359016). The bacmid was generated according to Bac-toBac instruction manual. Baculoviruses were produced by transfecting Sf9 cells with the bacmids using TransIT (Fisher Scientific, MIR6104). After one rounds of virus amplification, it was used to infect Hi5 cells (Thermo Fisher Scientific, B85502).

Cell density was monitored, and 48 hours post-infection, cells from a 400 ml culture were centrifuged at 500 g for 15 minutes. The obtained pellet was resuspended in a lysis buffer (30 mM HEPES pH 7.5, 200 mM NaCl, 10% glycerol, flash-frozen, and stored at -80°C.

DNase 1 mg/ml and one tablet of cOmplete protease inhibitor (Roche, 11836153001) were added to cell suspension. Cells were lysed using an Avestin Emulsiflex C3 High-Pressure Homogenizer, ensuring three recirculation passes for uniform lysis. Following lysis and centrifugation at 10,000 g for 20 minutes to pellet cellular debris, the supernatant was analyzed via SDS-PAGE and subsequently transferred to a PVDF membrane (BioRad) for Western blot analysis probed with an anti-ALK antibody to confirm the presence of the ALK receptor (Cell Signaling Technology, 3633). Detection was carried out via chemiluminescence. The supernatant, containing the membrane fractions, was subject to ultracentrifugation using an Ti45 (Beckman) rotor at 40,000 RPM for 1 hour. The resulting membrane-enriched pellet was resuspended in a buffer comprising 30 mM HEPES (pH 7.5), 200 mM NaCl, 5% glycerol, and a cOmplete protease inhibitor mixture, adjusted to a protein concentration of 4 mg/ml, flash-frozen, and stored at -80°C.

The membrane fraction was solubilized using mixture of DDM/CHS (1%/0.1%) detergents, followed by a 2-hour incubation at 4°C to ensure complete solubilization of membrane proteins.

Formation of ALK:AUG- α Complex

The solubilized membrane fraction was loaded onto a 1 ml StrepTrap XT column (Cytiva, 29401322), equilibrated with wash buffer (30 mM HEPES pH 7.5, 200 mM NaCl, 10% glycerol, 0.1% DDM/CHS). 2 mg of AUG- α was loaded onto ALK-bound Strep-Tactin column. ALK:AUG- α complex was eluted using a 1x BXT buffer (100 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 50 mM biotin, pH 8) + 0.1% DDM/CHS). Five fractions were collected during the elution process, analyzed via SDS-PAGE to assess protein concentration, and the fractions with the highest concentration of the ALK:AUG- α complex were combined in preparation for SEC.

The affinity-purified ALK:AUG- α complex was subjected to SEC using a Superdex 200 Increase 10/300 GL column (Cytiva, 28990945). The column was equilibrated with SEC buffer (30 mM HEPES pH 7.5, 200 mM NaCl, 10% glycerol, 0.1% DDM/CHS). The protein complex was introduced to the column and eluted at a steady flow rate of 0.5 ml/min. The elution profile was continually monitored using UV detection at 280 nm.

ALK:AUG- α complex was stabilized by ultracentrifugation at 40,000 rpm for 16 hours at 4°C in a 5-20% glycerol gradient, with a 0-0.1% glutaraldehyde gradient, using a Beckman SW60Ti rotor.

Negative Staining of ALK:AUG- α Complex

The ALK:AUG- α complex fractions from GraFix were subjected to negative staining using a 2% uranyl formate solution (VWR, MFCD00041939). A 3 μ L aliquot of the protein sample was applied to a carbon-coated copper glow discharged grid (Millipore Sigma, 930334), and left for 1 minute. Subsequently, 3 μ L of 2% uranyl formate was applied for 1 minute. The grid was blotted and air-dried.

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