Extending The Frontiers Of Chemistry: Semi-synthetic Reconstitution Of Multi-domain Trans-membrane Receptors

Nikhil Singla, Dimitar Nikolov, Juha P. Himanen
Department of Structural Biology, Memorial Sloan Kettering Cancer Center, New York, NY
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FOCUS OF OUR RESEARCH
- A major impediment in conducting biochemical and structural studies on integral membrane proteins is the lack of expression systems to produce high amounts of purified proteins.
- Expressed protein ligation (EPL) has been previously used to modify intracellular proteins as well as small integral membrane proteins for structural and functional studies.
- Our specific aim is to further develop the intein based semi-synthetic technique of EPL to reconstitute the entire transmembrane Eph receptor tyrosine kinase.
- We propose to ligate the complete, multi-domain extracellular region of the Eph receptor to the intracellular tyrosine kinase domain by using a recombinant two-piece approach, and to introduce the transmembrane region by using a three-piece ligation.
- The semi-synthetic Eph receptors will serve as an attractive transmembrane protein, providing new insights into the mechanisms of receptor tyrosine kinase activation and for the structural characterization of single-pass membrane proteins.

BACKGROUND
- Domain Structure of Eph Receptor
- Mechanism of Expressed Protein Ligation (EPL)

RESULTS
- Expression of EphECD-Intein-Fc

EXPERIMENTAL DESIGN
- Generate protein fragments with reactive α-thioester and α-cysteine groups

CONCLUSIONS
- We have successfully fused an intein to a secreted protein and generated a reactive α-thioester after thiol treatment.
- Here we describe the semi-synthetic site-specific modification of the complete, multi-domain extracellular regions of both A- and B-classes of Eph receptor tyrosine kinases.
- We show that the complete extracellular region of these receptors can be ligated to different modified peptides under carefully established experimental conditions.
- Using this technique, we have also been successful in ligating the complete extracellular region of the Eph receptor with the intracellular kinase domain.
- This work extends the boundaries of the EPL technique for semisynthesis of multi-domain extracellular proteins and highlights its potential application for reconstituting the entire single-pass transmembrane proteins.

GENERATION OF EPH ECOD α-thioester
- Analysis of the EphB4ECD α-thioester generated after the thiolysis reaction is better in the H5 expression. (B: Protein A-Sepharose beads, S: supernatant)

OPTIMIZATION OF CLEAVAGE EFFICIENCY
- Effect of mutations at the C-terminus of Eph ECD on thiol cleavage efficiency. Efficiency decreases with Gly at the C-terminus. The efficiency is same with Ala and Phe at the C-terminus. EphA3 ECD has higher expression levels and hence, higher EphA3 ECD α-thioester generated after thiolysis.

LIGATION WITH KINASE DOMAIN
- Third cleavage provides an α-thioester at the C-terminus of the EphECD. Protease mediated cleavage provides an α-cysteine at the N-terminus of the Eph kinase domain. The EphA3ECD-EphA4Kinase ligation product confirmed by Mass Spectrometry.

EXPRESSION OF EPH KINASE DOMAIN FOR EPL
- The EphA4 kinase domain with an N-terminal GST purification tag and a TEV protease recognition site between them was expressed in E.coli. Cleavage with TEV protease generated an α-Cys at the N-terminus of the kinase domain.

PULL-DOWN WITH EPHRIN LIGAND
- The EphA3 ECD α-thioester obtained after thiolysis product were mixed with the Fc-tagged ephrinA5 ligand. The receptor-ligand complex binds to Protein A-Sepharose beads. The recombinant ectodomain of EphA3 is biologically active after thiol treatment and ligation as it forms specific complex with its ephrinA5 ligand.

GENERATION OF EPH ECD α-thioester
- The EphB4ECD α-thioester conjugated Streptavidin confirms the ligation product.

LIGATION OF EPH ECD WITH SYNTHETIC PEPTIDE
- Ligase of EphA3 ECD α-thioester with FLAG tag peptide that is biotinylated at its C-terminus (C-G-G-G-D-Y-K-D-D-D-D-K(ε-Biotin)-G). Western blot analysis with anti-FLAG antibody and AP-conjugated Streptavadin confirms the ligation product.