Role of SCAMP3 Regulating CXCR4 Trafficking

Angelika Chiang, Samantha Valaitis, Dr. Quyen Aoh
Department of Biology, Gannon University, Erie, PA 16501

GANNON UNIVERSITY

Abstract

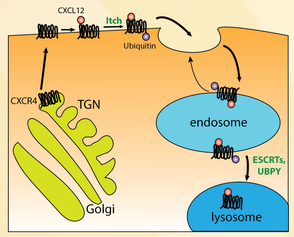
The CXC-Chemokine Receptor Type 4, CXCR4 is a G-protein coupled receptor that regulates cell growth and division, differentiation, and migration. Overexpression of CXCR4 has been linked to metastasis in cancer and promotes HIV infection. Decreased trafficking of CXCR4 to the lysosome can promote its overexpression. Upon binding to its ligand, CXCL12, CXCR4 is endocytosed. At the early endosomes, CXCR4 is ubiquitinated by the ubiquitin ligase Itch and then sorted into multivesicular bodies by the ubiquitin adaptor protein Hrs. It is then targeted for degradation in the lysosomes. We are interested in whether Secretory Carrier Membrane Protein (SCAMP) 3, which is known to interact with Hrs, also regulates CXCR4 trafficking. To test whether SCAMP3 regulates CXCR4 trafficking to the lysosome, we will use RNA interference to knockdown SCAMP3 and we will then monitor CXCR4's localization relative to markers of the early endosome and lysosomes. We have optimized the immunofluorescence assay to examine CXCR4 localization. We have found that staining for the lysosomes and early endosomes independently of CXCR4 reduces non-specific cross-reactivity between the antibodies and increases labeling specificity. Also, permeabilizing the cells before fixing them increases specific labeling of CXCR4. Our next step will now be to perform the immunofluorescence assay in the presence or absence of SCAMP3 to determine the role of SCAMP3 in CXCR4 trafficking.

Introduction

CXCR4 is downregulated through ubiquitylation and the ESCRT system

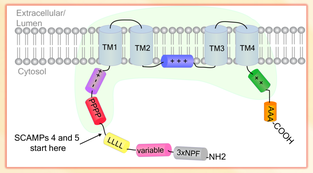
CXCR4 is a G-protein coupled receptor (GPCR) that has been linked to metastatic growth in cancer cells. In the presence of its agonist, the chemokine CXCL12, CXCR4 activates signaling pathways involved in chemotaxis, cell adhesion, and cell growth [4]. Subsequently, CXCR4 is downregulated after interacting with its agonist [1,2].

Downregulation of CXCR4 is mediated by ubiquitylation, followed by endocytosis and endosomal sorting of the receptor into multivesicular bodies [2]. CXCR4 is ubiquitinated by the ubiquitin ligase ITCH, allowing it to be endocytosed into the cell. The receptors are then transported to early endosomes where they are sorted into multivesicular bodies (MVBs) by the endosomal sorting complexes required for transports (ESCRTs). Just prior to sorting, the receptors are deubiquitinated by the deubiquitinase, UBPY [5]. The MVBs then bud from the early endosome and fuse with the lysosome



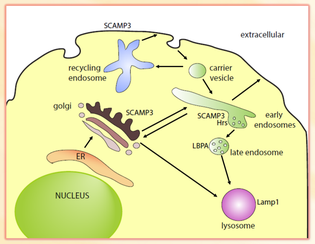
SCAMPS are transmembrane proteins that regulate membrane traffic

The SCAMP family consists of five isoforms, SCAMPs 1-5. SCAMPs have four transmembrane domains with cytoplasmic N- and C-termini that facilitate interactions with other proteins. SCAMPs 1-3 are ubiquitously expressed and SCAMPs4 and 5 are neuronally expressed [3].



SCAMPs are found in many places including the *trans*-Golgi network (TGN), endosomes, and plasma membrane. They function in the recycling and degradative process of cell surface receptors [3].

SCAMP3 specifically has been shown to regulate degradation of the epidermal growth factor receptor by interacting with and opposing the function of the ESCRT protein Hrs [3].

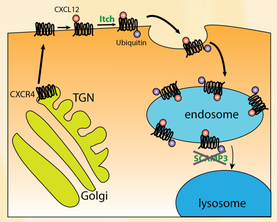


References
[1] Marchese, A. (2006). Assessment of Degradation and Ubiquitination of CXCR4, a GPCR Regulated by EGFR Family Members. Epidermal Growth Factor, 327, 139-146. [2] Mines, M. A., Goodwin, J. S., Limbird, L. E., Cui, F. F., & Fan, G. H. (2009). Deubiquitination of CXCR4 by USP14 is critical for both CXCL12-induced CXCR4 degradation and chemotaxis but not ERK activation. Journal of Biological Chemistry, 284(9), 5742-5752. [3] Aoh, Q. L., Castle, A. M., Hubbard, C. H., Katsumata, O., & Castle, J. D. (2009). SCAMP3 negatively regulates epidermal growth factor receptor degradation and promotes receptor recycling. Molecular biology of the cell,20(6), 1816-1832. [4] Busillo, J. M., & Benovic, J. L. (2007). Regulation of CXCR4 signaling.Biochimica et Biophysica Acta (BBA)-Biomembranes, 1768(4), 952-963.[5] Berlin, I., Higginbotham, K. M., Dise, R. S., Sierra, M. I., & Nash, P. D. (2010). The Deubiquitinating Enzyme USP8 Promotes Trafficking and Degradation of the Chemokine Receptor 4 at the Sorting Endosome. The Journal of Biological Chemistry, 285(48), 37895–37908.

Hypothesis

SCAMP3 may promote or inhibit CXCR4 trafficking to the lysosome

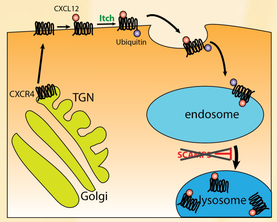
(A) SCAMP3 promotes CXCR4 trafficking



then, knockdown of SCAMP3 will:

↓ traffic of CXCR4 to lysosome

(B) SCAMP3 inhibits CXCR4 trafficking



then, knockdown of SCAMP3 will:

↑ traffic of CXCR4 to lysosome

Experimental Design

Knockdown SCAMP3 — HeLa cells are transfected with SCAMP3 –specific or control siRNA for two days to knockdown SCAMP3 expression

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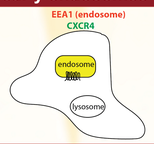
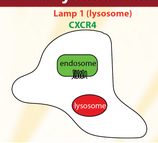
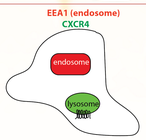
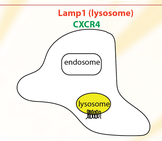
Stimulate endocytosis — The cells will be stimulated with CXCL12 for up to 3 hours to induce endocytosis and lysosomal degradation of CXCR4

↓

Determine localization of CXCR4 relative to endosomes and lysosomes — CXCR4 and markers for proteins found in early endosomes (EEA1) and lysosomes (LAMP1/2) will be labeled with different fluorophores for immunofluorescence microscopy. Colocalization between CXCR4 and the organelle will be indicated by overlap of the fluorescent signals.

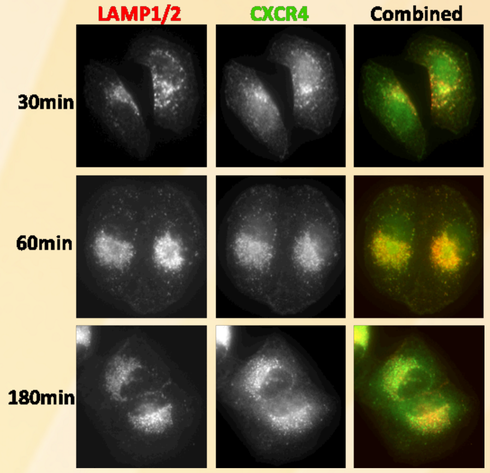
Possible outcomes of fluorescence studies

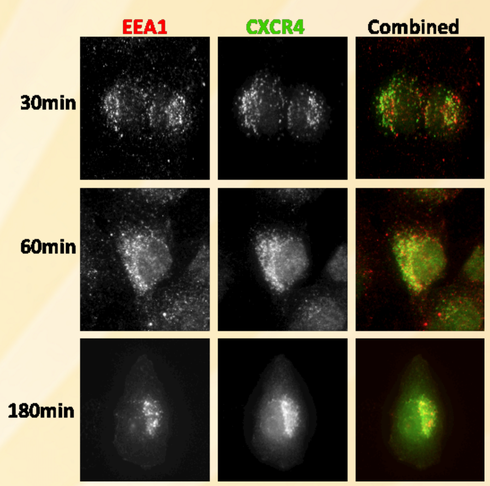
In SCAMP3 knocked-down cells, HA-tagged CXCR4 will be labeled with a green fluorophore while the early endosomal marker EEA1 or the lysosomal marker Lamp1/2 will be labeled with a red fluorophore. Colocalization between the CXCR4 and organelle markers will result in overlap of the red and green signals to produce yellow.

Result	Effect of SCAMP3 Knockdown	Localization relative to early endosomes	Localization relative to lysosomes
SCAMP3 promotes the degradation of CXCR4	CXCR4 will be retained in the early endosome		
SCAMP3 inhibits the degradation of CXCR4	More CXCR4 will be trafficked to the lysosome		

Results

CXCR4 is transported to lysosomes after 30 minutes, 60 minutes, and 180 minutes time course





HeLa cells incubated with CXCL12 for 30 minutes, 60 minutes, and 180 minutes to induce endocytosis and lysosomal sorting of CXCR4. CXCR4 and the early endosomal marker EEA1 or the lysosomal marker Lamp1/2 were labeled through immunofluorescence. To avoid cross-reactivity between the rat CXCR4 and mouse EEA1 and Lamp1/2 antibodies, the fluorescence labeling was done sequentially. Colocalization is indicated by the presence of yellow. The cells were then mounted and observed using fluorescence microscopy.

Conclusions and Next Steps

Based on our results we determined that SCAMP3 is ubiquitinated by ITCH, deubiquitinated by UBPY. Staining CXCR4 and organelle markers separately improved specificity of signal in the immunofluorescence. Also, using LAMP1 and LAMP2 together increased the colocalization of CXCR4 with the lysomoses. Our preliminary results show that CXCR4 is correctly trafficked to the lysosomes after three hours of stimulation. In future studies we will perform a knockdown of SCAMP3 using siRNA interference and used immunofluorescence assay to examine CXCR4 localization. We will perform the experiment in the same way by using EEA1 and LAMP1/2 organelle markers to observed CXCR4 localization when SCAMP3 is knockdown. We will also observe what effect the knockdown of SCAMP3 has in comparison to the control cells.