Examining differences of ubiquitination on KDEL receptor isoform expression

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Introduction

The disruption of vital cellular processes, commonly supported by the transport of proteins emanant from the endoplasmic reticulum (ER), is a primary factor in disease pathogenesis. Key proteins in this transport are KDEL receptors (KDELrs). Post-translation, nascent proteins are folded by ER chaperones, a subgroup of ER-resident proteins (ERPs), and moved them to the ER membrane (Trychta et al., 2018). They may also undergo post-translational modifications (PTMs). Post-secretion from the ER, they are trafficked through the Golgi, and to their final destination. However, ERPs are unique in this regard; ERPs return from the Golgi to the ER upon interaction with a KDELr (Raykhel et al., 2007). This property is due to the peptide sequence Lys-Asp-Glu-Leu (KDEL) or KDEL-like sequences, which interact with three KDELr isoforms. These interactions cause ERPs to return to the ER when secreted to Golgi, although, perturbations to the ER can hinder this retrieval pathway. Trychta et al. observed a mass release of ERPs following ER calcium depletion, a phenomenon coined exodosis (Figure 1). In human neuroblastoma (SY5Y) cells, relative steady-state levels of KDELr isoforms have been observed: KDELr1 exhibited greater levels than KDELr2 and KDELr3. This project seeks to observe ubiquitination's effects on the relative abundance of KDELr1 and KDELr2. It was hypothesized that when probed for ubiquitin, KDELr2 will be more abundant than KDELr1.

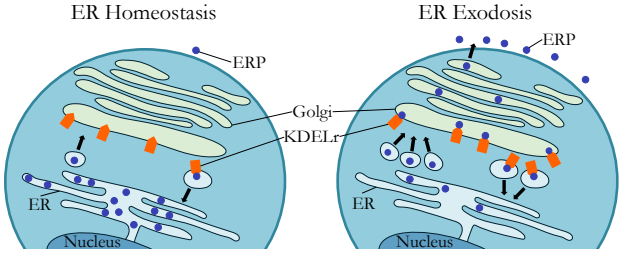


Figure 1 (above): Under homeostatic conditions, ERPs are retained within the ER, as KDELrs bind to them in the Golgi. Amid ER exodosis, ERPs are displaced and can be found in the extracellular space. KDELrs were interestingly found to be upregulated during exodosis. Trychta et al. postulated that this was an adaptive cellular response to maintain the ER proteome under conditions of stress.

Materials and Methods

Figure 2 (above): KDELr's FASTA sequence was identified in UniProt and used in Prediction of Ubiquitination Sites with the Bayesian Discriminant Method (BDM-PUB) to produced a table with potential-scores (p-scores) for predicted ubiquitinated residues. P-scores represent how highly a residue is predicted to be ubiquitinated.

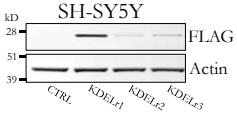
Materials and Methods (cont.)

Following the identification of putative PTMs (Figure 2), KDELrs were probed for ubiquitination. SY5Y cells were plated in a 24–well plate, and KDELr plasmids were transfected into cells with no additional treatment, vehicle, or a proteasomal inhibitor (MG132). Cells were then lysed and collected for immunoprecipitation (IP). IP experiments were performed with magnetic beads incubated with FLAG-antibodies, followed by PBS-T washes. Samples were centrifuged, incubated with cell culture media, and eluted. Samples were then run on a 4–12% Bis-Tris gel and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. For analysis of KDELr isoform relative abundance, PVDF membranes were probed with IR secondary antibodies and scanned with an infrared scanner. For analysis of ubiquitination, the membrane was probed with anti-ubiquitin (Figure 3).

Figure 3 (above): Summary of the experimental timeline.

Results

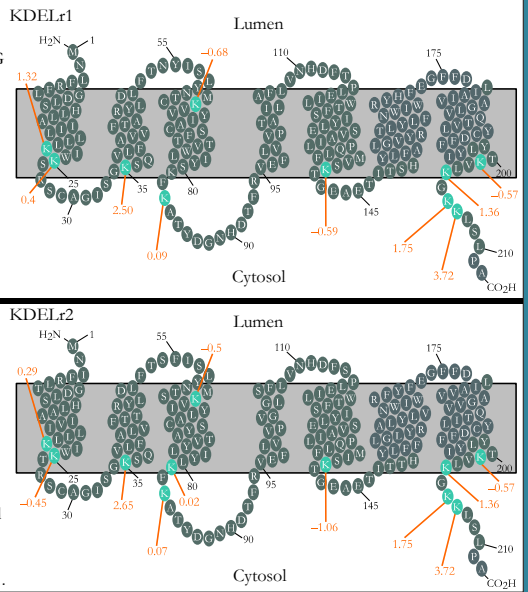
Figure 4 (below): Steady-state levels of KDELrFLAG were assessed by Wires et al. KDELr1 was more abundant than KDELr2; Actin acted as a control to ensure equal sample volumes were loaded.



Residue Number	P-score KDELr1	P-score KDELr2
64	−0.68	−0.50
140	−0.59	−1.06
201	−0.57	−0.57
83	0.09	0.07
25	0.40	−0.45
22	1.32	0.29
204	1.36	1.36
206	1.75	1.75
35	2.50	2.65
207	3.72	3.72
81	*	0.02

Table 1 (above): Ubiquitination data were sorted such that KDELr1 p-scores were ordered from least to greatest
*Residue 81 was not predicted to be ubiquitinated on KDELr1.

Figure 5 (below): The potential of ubiquitinated residues on KDELr1 and KDELr2 were shown on maps constructed using Inkscape. Each lettered circle represents a known residue on KDELr1 and KDELr2. Turquoise represents ubiquitinated residues, while gray represents non-ubiquitinated residues. Connected orange values indicate the p-score for each residue and the light-gray region represents the membrane.



Results (cont.)

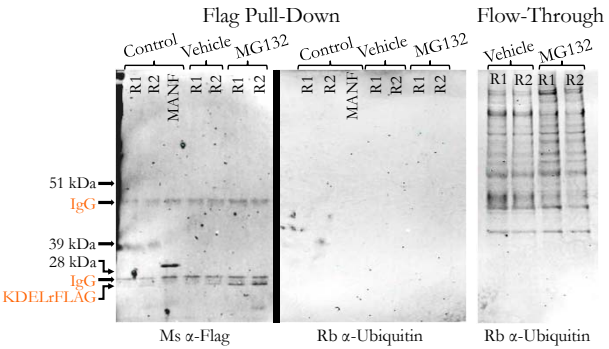


Figure 6 (above): After IP, samples transfected with KDELr1- and KDELr2-FLAG (R1 and R2) and MANFsp-FLAG eGFP-ASARTDL (MANF), were probed for FLAG (Ms α-Flag) and Ubiquitin (Rb α-Ubiquitin). Constructs with FLAG were successfully pulled down when probed for FLAG.

Previously observed differences in steady-state levels of KDELr1 and KDELr2 (Figure 4), prompted an investigation of influences of PTMs on KDELr isoform expression. BDM-PUB predicted ten ubiquitination sites on KDELr1 (M = 0.930, SD = 1.468) and eleven on KDELr2 (M = 0.662, SD = 1.515) (Figure 5 and Table 1). The pull-down of transfected cells were probed for ubiquitin, but no ubiquitinated protein was detected; when the flow-through was probed, several bands were detected (Figure 6).

Conclusions

To observe effects of PTMs on the steady-state levels of KDELr1 and KDELr2 in SY5Ys, both *in-silico* and *in-vitro* investigations were pursued. It was predicted that KDELr2 would be more ubiquitinated than KDELr1, though IP and western blot experiments failed to support this because no ubiquitin-KDELr protein was observed upon probing within any treatments. Ubiquitin detection in the flow-through signifies that the antibodies were functional, thus, there is no ubiquitination on KDELrs, or the transfection efficiency was too low to show bands on the western blot. Future experiments may require a more sensitive assay to detect KDELr isoform specific ubiquitination. Alternative PTMs such as phosphorylation may also warrant inquiry.

References

Raykhel, I., Alanen, H., Salo, K., Jurvansuu, J., Nguyen, V. D., Latva-Ranta, M., & Ruddock, L. (2007). A molecular specificity code for the three mammalian KDEL receptors. *The Journal of Cell Biology, 179*(6), 1193–1204. https://doi.org/10.1083/jcb.200705180

Trychta, K. A., Bäck, S., Henderson, M. J., & Harvey, B. K. (2018). KDEL receptors are differentially regulated to maintain the ER proteome under calcium deficiency. *Cell Reports, 25*(7), 1829–1840.e6. https://doi.org/10.1016/j.celrep.2018.10.055