Utilizing a plasma membrane enrichment protocol to optimize western blot detection of the CCK2 and $CCK2_{i4sv}$ receptors

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Abstract

Pancreatic cancer is currently the fourth most deadly form of cancer in the United States. One factor implicated in pancreatic cancer growth is the hormone signaling pathway between gastrin and its receptor, CCK2R. In the early 2000's, it was also discovered that pancreatic cancer cells can contain a variant ($CCK2_{i4sv}R$) of the normal receptor protein. Crucially, this longer variant has been shown to be hyper-stimulated and to drive increased cancer growth. Measuring the relative abundance of these two receptors at the protein level can help us understand their role in pancreatic cancer and may represent prognostic value as a biomarker. However, while RNA detection and measurement have been reproducible, protein detection has been problematic. Using western blot analysis, we have been able to detect the receptors in cells expressing them at high levels; however, detection in wild type and stably-transfected lines more representative of physiological expression has been unclear. Thus, we hypothesize that low, natural abundance of the receptors requires enrichment for reliable quantification. Herein, we report our initial attempt to enrich for green fluorescent protein-tagged variants, CCK2R-GFP and $CCK2_{i4sv}R$-GFP, utilizing a membrane extraction protocol based on the non-ionic detergent, Triton X-114. Following enrichment, western analysis demonstrated a significant decrease in cytosolic protein in control cells transfected with untagged GFP. Efforts to verify retention of membrane proteins in the hydrophobic fraction, and subsequent specific detection of the CCK2R variants, are ongoing.

Introduction

- The cholecystokinin 2 receptor (CCK2R) is a G protein-coupled receptor that has a regulatory role in the digestive pathway.
- The digestive hormone Gastrin and it's signaling cascade (via CCK2R) has been shown to drive increased cancer cell proliferation, especially in pancreatic adenocarcinoma.
- $CCK2_{i4sv}R$ is a splice variant of the CCK2R receptor that has only been detected in cancerous tissue. Additionally, this variant has increased affinity for Gastrin and elicits greater tumor growth.
- Our lab has previously detected both CCK2R and $CCK2_{i4sv}R$ in transiently transfected cell lines. However, detection of these receptors in wildtype and stably transfected cell lines has proven difficult.
- We believe the receptor is maintained at low abundance. Thus, we hypothesize that detection may be improved via a membrane enrichment protocol.
- Accurate detection of CCK2R and $CCK2_{i4sv}R$ at the protein level may have prognostic value for diagnosed patients.

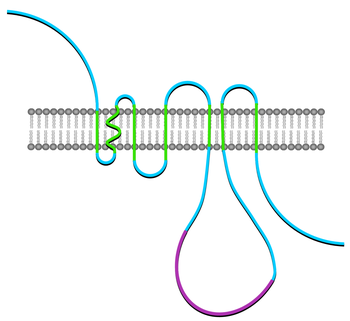


Figure 1. Depiction of $CCK2_{i4sv}$ Receptor. The gastrin receptor CCK2R is a G protein-coupled receptor. A splice variant of the receptor, $CCK2_{i4sv}R$, is the result of failure to splice intron 4 (shown in purple). As a 7-membrane spanning protein, the receptor is largely hydrophobic which can be exploited for enrichment.

Methods

Transient Transfection Panc02, murine pancreatic cancer cells, were transfected with the plasmids: pCAGEN.puro-EGFP, pCAGEN.neo-CCKCR*i3/UTR-EGFP, pCAGEN.neo-CCKBRi3/UTR-EGFP using Lipofectamine 2000 (ThermoFisher), Lipofectamine 3000 (ThermoFisher), FuGene 6 (Promega), Neon Electroporation (ThermoFisher). Transfection efficiency was assessed 24 hours post-transfection by fluorescence microscopy.

Cell Lysis Whole cell lysates were prepared via 8M urea and homogenized with a QIAshredder (Qiagen). Membrane enrichment was performed as outlined by Taguchi (2014) using Triton X-114 (Sigma) lysis buffer and wash buffer. Hydrophobic fractions were retrieved via chloroform methanol extraction (Taguchi 2013) and solubilized in 1x β-ME sample buffer.

SDS PAGE and Western Blotting Lysates were boiled 5 minutes and run on a polyacrylamide gel (29:1, 10%) for 200 minutes at 40 mV. Gels were transferred to a nitrocellulose membrane via Trans-Blot Semi-Dry Transfer Cell (BioRad) with running parameters of 12.3 V for 30 mins with a 500-mA current limit. Membranes were blocked overnight in 10% milk (TBST) at 4°C. Blots were incubated in primary (anti-GFP, abcam ab13970) and secondary antibody (anti-chicken, Sigma 6409) washes for 2 hours and 1 hour, respectively. Following the secondary wash, the membranes were incubated in SuperSignal™ West Dura Extended Duration Substrate for 5 mins. X-Ray film was developed following 5-minute exposure.

Results

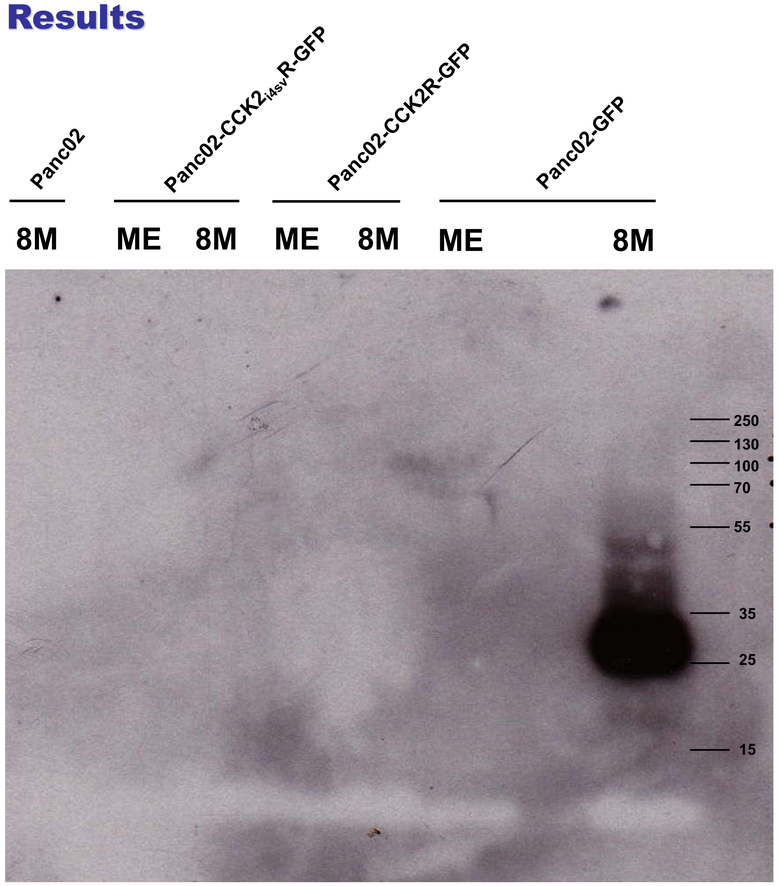
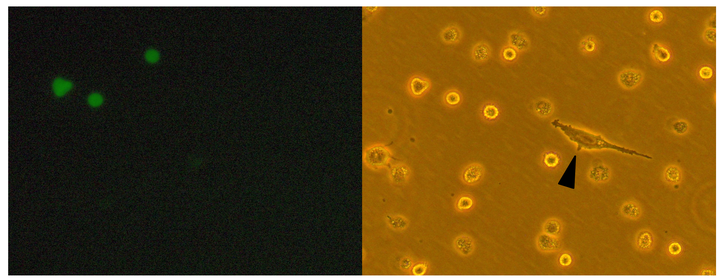


Figure 2. Hydrophilic/cytosolic protein fraction is successfully eliminated by membrane enrichment. Western analysis (anti-GFP) demonstrated a strong GFP band (27 kDa) in the 8M urea whole cell lysate (8M). Membrane-enriched (ME) lysate exhibits complete elimination of the cytosolic GFP protein. No signal was observed for any of the GFP-tagged receptor lysates (Panc02-CCK2R-GFP, Panc02-$CCK2_{i4sv}R$-GFP) regardless of preparation.



Method	Transfection Efficiency (%)
FuGene 6	2.86
Lipofectamine 2000	8.54
Lipofectamine 3000	12.5
Neon (Electroporation)	14.8

Figure 3. Panc02, murine pancreatic cancer cells, demonstrate poor transfection across multiple techniques. Neon (electroporation) exhibits a marginally improved transfection efficiency in comparison to cationic lipid-based reagents. Few cells were viable at 24 hours post transfection (arrowhead).

Conclusions and Future Directions

- **Western analysis suggests elimination of the cytosolic fraction of cell lysates after extraction utilizing the Triton X-114 membrane enrichment protocol.**
- **Poor transient transfection efficiency confounds reliable expression of positive control GFP-tagged receptors.**
- **To determine successful enrichment of hydrophobic membrane proteins, ongoing analysis will utilize endogenous membrane proteins such as VEGFR-2 and CCK1R.**
- **Human pancreatic cell lines (e.g., BxPC-3) demonstrate significantly higher transfection efficiencies of the pCAGEN.puro-EGFP plasmid (data not shown) but may display background endogenous receptor.**

Acknowledgments

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