Star Cell Bio Exercise

Focus on membranes, transmembrane proteins

Techniques: Western blot, FACS, microscopy (pics and movies if possible)

Background

Mamalian cell (mouse?)

WT and mutants 1 and 2

Look at an transmembrane protein (can be a transporter or maybe part of a signaling pathway?)

Mutant 1 and 2 appear to have a defect in glucose transport into cells from the blood stream. You know a major glucose transporter GLU1 is responsible for the majority of glucose transport in these cells, so your advisor suggests that Mutants 1 and 2 may be due to mutations in the GLU1 gene.

You decide to first look at the levels of the GLU1 protein by western blot. Luckily you have an antibody which is specific for the GLU1 protein, conjugated to \_\_\_\_\_ .

**Mutant 1 will be a truncated protein (deletion which includes a transmembrane region, flipping orientation of protein) that is still localized to the cell membrane – will show as a smaller molecular weight on western blot, but expression of level the protein is similar to WT. likely inactive protein. Or can have a pt. mutation instead of a deletion causing premature stop – doesn't have one of the transmembrane domains and maybe no activation loop for downstream signaling pathways to go.**

*-if mutant one is missing or has defect intracellular activation loop/where gprotein or signaling molecule binds,*

*- can study this with fret. WT would show fret with addition of glucose to media, while mut 1 would not because g protein/signaling molecule would not be able to bind*

*Note: can use lectins on a blot to detect sugars – maybe can use this to measure levels of glucose inside the cell? Or does lectin blotting only detect glycosylated proteins. May not be specific to this transporter. Nevermind.*

**Mutant 2 will be a protein in which the signal sequence is missing or mutated (deletion of signal sequence region of protein at N terminus) – about same size (resolution not high enough to detect on western blot), different subcellular localization – cytoplasmic?**

**Can this mutation possibly changed to nuclear localization sequence? Is a mut that will cause this possible?**

*-hmm but transmembrane proteins don't really have a signal sequence – if only one transmembrane domain is left, the protein will be secreted. So maybe this mutant will be secreted therefore not in correct place*

PROTEIN EXPRESSION

1. perform a western blot on mutant 1 and 2 whole cell lysate samples. Make sure to include all positive and negative controls.

a. what is/are your positive control(s), what is their purpose

WT, loading control

b. what is/are your negative control(s), what is their purpose

another mutant, MutantG, which is known to not express the Glu1 protein

c. Which antibodies do you use? What is the purpose of each.

primary, mouse anti glu1. Secondary conjugated goat anti mouse.

d. What do you observe in Mut 1 and 2? What can you conclude about protein expression and protein size in the mutants.

Mut 1 and 2 have expression levels (signal intensity) similar to WT – not a difference in total protein amount in cell.

Mut 2 is the same size as WT – not a truncated protein. Mutant 1 is smaller in size than WT, suggests a smaller protein.

*-if mut 2 is secreted, will show lower levels on WB (only internal protein being transported and what is in PM at time will be visible)*

e. Which of the following options could explain the observations in mutant 1? (multiple choice, circle ALL that apply)

i. a mutation in the GLU1 promoter region affecting the transcription of GLU1 gene

ii. a mutation in the GLU1 translation initiation sequence of the GLU1 gene

iii. Mutation in GLU1 gene resulting in a defective/inactive GLU1 protein

iv. a mutation in the GLU1 gene signal sequence (which targets glu1 to membrane).

v. none of the above

answer iii and iv. Transcription and translation not effective because same amt of protein in mutants and wt.

e. Which of the following options could explain the observations in mutant 2? (multiple choice, circle ALL that apply)

i. a mutation in the GLU1 promoter region affecting the transcription of GLU1 gene

ii. a mutation in the GLU1 translation initiation sequence of the GLU1 gene

iii. Mutation in GLU1 gene resulting in a defective/inactive GLU1 protein

iv. a mutation in the GLU1 gene signal sequence (which targets glu1 to membrane).

v. none of the above

answer iii and iv. Transcription and translation not effective because same amt of protein in mutants and wt.

*-if secreted mutant: lower levels on WB means all of above (just know there is less protein in the cell, don’t know why*

SUBCELLULAR LOCALIZATION

2. to see where the mutants localize their GLU1 protein, you perform a IF microscopy experiment.

- results: mut 1 localized to membrane (and vesicles??), mut 2 localized to cytoplasm

-where would secreted protein be found – in PM and vesicles??

- ??can you also use FC with anti-glu antibody – strength of signal relative to amt of glu1 in membrane, since AB cannot penetrate cell

b. what does this infer about mut 1 and 2?

Mut 2 is improperly localized and therefore unable to perform function (, mut 1 is in the proper location (membrane)

TRANSMEMBRANE PROTEIN ORIENTATION

3. look at orientation of protein in membrane with Western Blot or flow cytometry

create construct of glu1 (and mut glu 1 from mut 1 and 2) with myc tag at N terminal end and HA tag at C terminal end in vector, transduce cells with construct, wait for expression of the tagged glu1 protein. (there will be both endogenous and exogenous glu1, only the glu1 from the vector will be tagged). digest with endo to chew up protein outside cell

a) WB

- digest with protease k to chew up protein outside cell – cant get into cell

- lyse cells, blot with conjugated anti-myc andtibody, reprobe with conjugated anti-HA antibody.

* If protein shows both Myc and HA signal, both ends of protein inside cell
* If protein shows only Myc sicnal, N terminal end inside cell, C terminal end outside cell
* If protein shows only HA signal, C terminal end inside cell, N terminus outside cell
* If protein shows not singal, both ends outside cell

b) flow cytometry

incubate cells w/ anti-HA and anti Myc antibody, (is secondary fluorescent antibody fluorescent to increase signal??)

perform flow cytometry, 2 color

* Quad 1; no myc or ha - both ends inside cell
* Quad 2; low myc high HA - protein shows only HA signal, n terminal end inside cell, c terminus outside cell
* Quad 3; low HA high myc - protein shows only Myc sicnal, c terminal end inside cell, n terminal end outside cell
* Quad 4; high HA high myc protein shows both Myc and HA signal, both ends of protein outside cell

interpretations from both experiments should agree - but actual raw results are opposite (in one case measuring signal inside cell, in the other, measurieng signal outside cell).

mut 1 has a deletion which alters orientation. Mut 2 is not in membrane as determined above, which is why it gives no signal on flow cytometry, and signal for both myc and ha on western blot)

c. advantages/disadvantages of each method. When would you use one over the other?

Diff ways of getting the same result (ie measure inside vs outside cell)

d. Can classify protein (may need to give list) type I, type ii etc. or draw pic to represent orientation whether protein starts and/or ends inside or outside cell

e. is there a diff in protein orientation in mutants vs WT? what does this sugest about nature of mutants?

WT and mut 1 diff orientation (due to deletion of a transmembrane segment)

PROTEIN DYNAMICS/TURNOVER RATES – only applies to mut 1 and wt (mut 2 is not transmembrane)

4. Study protein turnover

Label cells with biotin – will tag all proteins in membrane but not proteins in cell. Remove aliquots at diff. time period, incubate with anti glu-1 antibodies conjugated to beads and pull down glu 1 from whole cell lysates. (immunoprecipitation)

WB the pellet proteins with conjugated streptavidin (will show proportion of Glu1 protein that was initially in membrane

See how this intensity changes over time, the rate of decrease is proportional to protein turnover

Alternative experiment to measure protein recycling

* label whole cells w/ biotin
* strip biotin
* measure biotin levels INSIDE cell (cytoplasmic levels) on wb w/ streptavidin?
* At different timepoints, measure biotin levels outside cell – rate of recycling will effect levels of biotynilasted proteins being reintegrated into membrane

a) summarize findings

b) sketch graphs for WT, Mut1 and Mut2 showing rate of decrease

c) conclusions: Is the rate of protein turnover of glu1 in WT, Mut1, and Mut 2 different? explain

\*\*\* is it possible to add densiometry function to WB to quantitively compare intensity of bands between lanes(we gice SCB this info anyway so it knows how intense to make the bands?)

VESSICLE TRANSPORT

Do something with microscopy – still images or preferentially videos (some available on ASCB cell image library website)

Gfp tagged glu1, in wt can se it in vesicles and membrane…

Have a mutant showing improper vesicle transport?

Maybe a mutant has improper folding, is sent to lysosomes instead of transported to membrane?

Watch retrograde transport?

Papers on egfr

<http://www.jbc.org/content/273/52/35000.full>

Royston E. Carter and Alexander Sorkin

Fluorescent Protein Chimera

Growth Factor Receptor-Green

1998 paper

Microscopy images:

-pictures of EGFR localization in cell

-co-localization of EGF-TR and EGFR-GFP during endocytosis

-Dynamics of EGFR-GFP-containing endosomes in living cells

<http://www.jbc.org/content/288/21/14824.full?sid=5ae44e60-cb01-4b62-9f5a-35ff9ff1d3b9>

*Transforming Growth Factor-β1 (TGF-β1)-stimulated Fibroblast to Myofibroblast Differentiation Is Mediated by Hyaluronan (HA)-facilitated Epidermal Growth Factor Receptor (EGFR) and CD44 Co-localization in Lipid RaftsMay 24, 2013* The Journal of Biological Chemistry, *288, 14824-14838.*

-frap on egfr shows its not dynamic (v. low recovery) stays photobleached

-can compare this to controls, one of which is recovered quickly, one that is not dynamic – conclude wt is not dynamic

<http://www.jbc.org/content/284/25/17243.full?sid=3a7f2b6c-586a-4df2-bc77-948285e03f87>

Systems Biological Analysis of Epidermal Growth Factor Receptor Internalization Dynamics for Altered Receptor Levels\*

Hannah Schmidt-Glenewinkel et al. 2009 jcb

-flow cytometry to measure dynamics of egfr internalization in presence of egf

<http://www.sigmaaldrich.com/life-science/cells-and-cell-based-assays/egfr-biosensor-cell-line.html#imaging>

sigma website: link to pub

<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3277729/#!po=5.76923>

nice pics of fluorescent egfp, shows movt from PM into cell w/ confocal microscopy in presence of egf ligand

<http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0058148>

shows pics of egfr internalization and maybe an inhibitor?