Bi/Ch 113 Midterm Instructions

This is an open-book, open-notebook exam. Your class notes, the two texts (Lodish et al and Alberts et al), and the assigned section and review papers may be consulted. The use of the internet and discussions with other persons are not allowed. Do not exceed 3 hours for the exam.

The best answers are clear and concise, but complete. For each question, the correct answer is the simplest answer consistent with all the data.

The midterm is due back at the beginning of class (11 am) next Tuesday, May 3.

There are no extensions.

There are 5 pages, including this page.

Question 1: Trafficking of a plasma membrane protein
You have identified a membrane protein produced by cultured fibroblasts, and are interested in characterizing its trafficking. This protein is present in the plasma membrane.

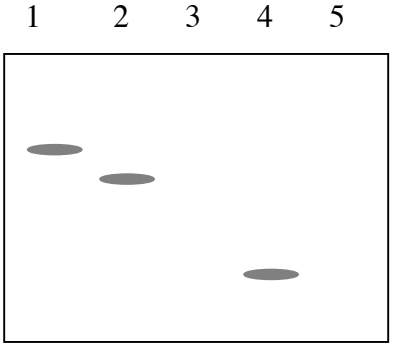
1) You identify the gene for this protein. The opening reading frame predicts the following polypeptide.

MDLWQLLLTLALAGSSDAFSGSEATAAILSRAPWSLQSVNPGLKTNSSKEPKFTKCRSPE
RETFSCHWTDEVHHGTKNLGPIQLFYTRRNTQEWTQEWKECPDYVSAGENSCYFNSSF
TSIWIPYCIKLTSNGGTVDEKCFSVDEIVQPDPPIALNWTLLNVSLTGIHADIQVRWEAPR
NADIQKGWMVLEYELQYKEVNETKWKMMDPILTTSVPVYSLKVDKEYEVRVRSKQRN
SGNYGEFSEVLYVTLPQMSQFTCEEDFYFPWLLIIIFGIFGLTVMLFVFLFSKQQRIKMLIL
PPVPVPKIKGIDPDLLKEGKLEEVNTILAIHDSYKPEFHSDDSWVEFIELDIDEPDEKTEES
DTDRLLSSDHEKSHSNLGVKDGDSGRTSCCEPDILETDFNANDIHEGTSEVAQPQRLKGE
ADLLCLDQKNQNNSPYHDACPATQQPSVIQAEKNKPQPLPTEGAESTHQAAHIQLSNPS
SLSNIDFYAQVSDITPAGSVVLSPGQKNKAGMSQCDMHPEMVSLCQENFLMDNAYFCE
ADAKKCIPVAPHIKVESHIQPSLNQEDIYITTESLTTAAGRPGTGEHVPGSEMPVPDYTSI
HIVQSPQGLILNATALPLPDKEFLSSCGYVSTDQLNKIMP

You purify some of the protein from the culture media and use Edman degradation to sequence the N-terminus. The first 5 residues are FSGSE. Why does this sequence not match the beginning of the open reading frame?

2) Analysis of the hydrophobicity profile of the protein sequence indicates a single transmembrane segment in the middle of the open reading frame.

When the protein is translated (with radiolabeled amino acids) *in vitro* in the presence or absence of microsomes and tested for protease sensitivity, you get the results below. Explain the protein band patterns in each lane. (6 points)



Lane 1: no protease, no microsomes
Lane 2: no protease, plus microsomes
Lane 3: protease, no microsomes
Lane 4: protease, plus microsomes
Lane 5; protease, plus microsomes, plus Triton X-100

3) You figure out that the N-terminus of this transmembrane protein is facing the outside of the cell. Explain in detail how you expect this protein co-translationally import into the ER membrane. In particular, how does it interact with the Sec61 translocon to be placed in the proper topology on the ER membrane?

4) Suppose you had a way to acutely inactivate NSF (NEM-sensitive factor) in these cells (for example, assume you have a drug that specifically blocks NSF function). What would you expect will happen to the secreted factor? How could you experimentally demonstrate this? (6 points)

5) Suppose you want to reproduce, in these fibroblasts, the famous Palade experiment showing the pathway of secreted proteins. You incubate the fibroblasts briefly with radiolabeled leucine, change to media with unlabeled leudine, and track the destination of radiolabeled protein by electron microscopy. What do you expect the results to be?

Question 2: Sar1 in COPII vesicle formation
In yeast, formation of COPII vesicles from the ER is controlled by the GTPase Sar1.

1) The transmembrane protein Sec12 residues on the ER membrane. You can recombinantly produce a cytosolic fragment of Sec12 in *E. coli*. The Sar1 is complexed with [^{32}P]GDP and incubated under a variety of experimental conditions. After the incubation, [^{32}P]GDP association with Sar1 is measured using a filter binding assay. [Sar1, being a protein, binds to the filter, whereas small molecules are washed through. You measure whether radioactivity is retained on the filter or washed away.]

When Sar1-[^{32}P]GDP is incubated with the cytosolic fragment of Sec12 and nonradioactive GTP, you find that the filter has little radioactivity. In contrast, when the same experiment is done with Ypt1, ARF, or Ras2 (these are 3 other small GTPases) results in retention of radioactivity on the filter. What is your interpretation?

2) Genetic studies show that overexpression of the cytosolic region of Sec12 leads to a defect in secretion. Overexpression of Sar1 is able to suppression this effect. Explain these two observations. (5 points)

3) You would like to analyze the trafficking of Sar1 in normal cells and cells lacking Sec12. Moreover, you want to do this analysis in living cells. How would you set up the experimental system, and what results do you expect?

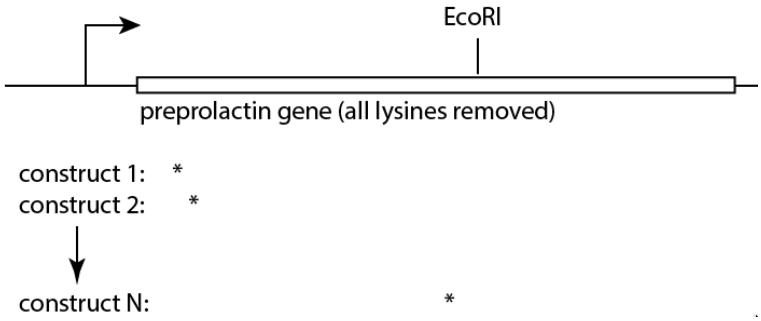
4) COPII-coated vesicles bud off from the ER, but the coat is shed shortly after budding. How is the timing of coat shedding coordinated with vesicle budding? What kind of mutations might delay coat shedding?

5) There are *in vitro* assays for COPII vesicle budding. Suppose GTPγS or the cytosolic fragment of Sec12 were added to such reactions. What would be the likely result in each case?

Question 3: Translocation of the polypeptide into the ER

Crosslinking experiments have been informative in terms of understanding what proteins are in contact with the nascent polypeptide chain during translocation through the ER. A typical secreted protein, preprolactin, was used as a model system. A special chemically modified lysy-tRNA is used in the translation reaction. The modified lysine residue could be photoactivated to crosslink to immediately adjacent proteins.

To get precise control of the crosslinking, it was useful to start with a gene that encodes no lysines and then make variants in which single lysine residues could be placed in all possible positions. The preprolactin gene was cloned into a vector suitable for *in vitro* transcription. The gene was mutated to remove all lysine residues. Using this parental construct, a systematic series of constructs could be made, in which single lysine codons were introduced at precise locations along the protein (see diagram below; * indicates the introduced lysine codon; arrow indicates start of transcription).



The plasmid is cut with EcoRI and an *in vitro* transcription reaction is performed. This produces a truncated RNA that encodes only the first 170 amino acids of preprolactin. The RNA is then added to a translation reaction containing wheat germ extract, ^{35}S-methionine, the chemically modified lysyl-tRNA, SRP, and rough microsomes.

1) During the translation process, what does the ribosome do when it gets to the end of the truncated RNA?

2) Note that because the translation reaction occurs in the presence of rough microsomes, translation and translocation are coupled. After translation, the reaction is treated with UV light to activate the crosslinking. The membrane fraction is recovered and run on an SDS-PAGE gel. If the lysine residue in a particular construct is in the correct position to crosslink a protein, that protein will become crosslinked to the nascent polypeptide chain. For constructs that lead to crosslinked products, what do you anticipate will be the major crosslinked protein?

3) In constructs where lysine is present in the last ~30 residues (amino acids 140-170), no crosslinking to membrane proteins is observed. Explain this result.

4) What positions along the polypeptide chain do you expect to be able to crosslink to the major membrane protein referred to in part (2)? Explain using a diagram.

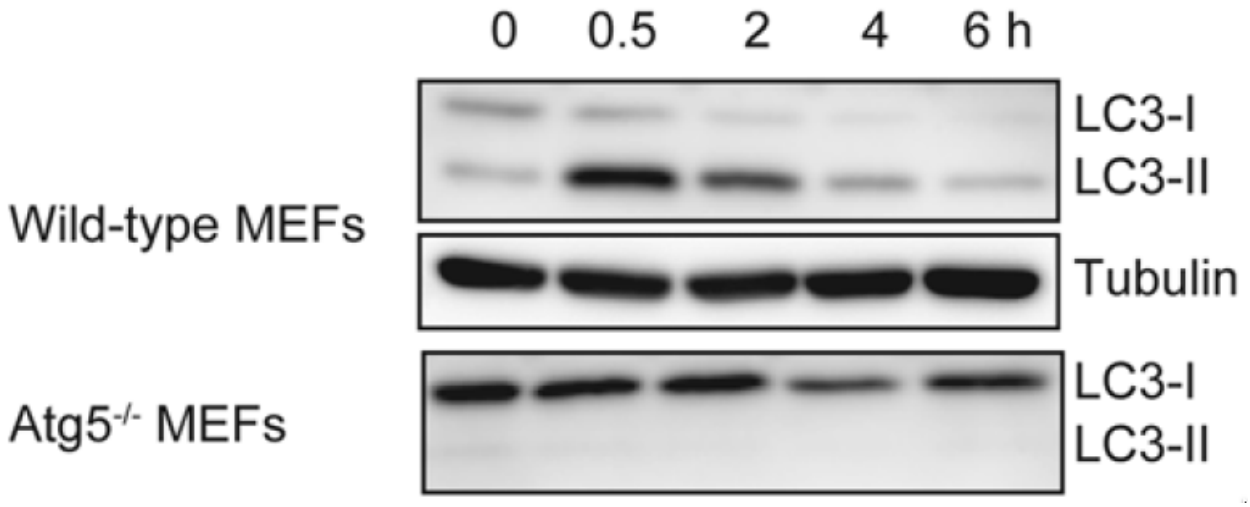
5) What happens to a polypeptide chain when puromycin is present during protein translation? Compare this process to normal translational termination.

Question #4: Autophagy
The yeast vacuole is functionally similar to the mammalian lysosome. It has been observed that autophagy occurs in yeast, with autophagosomes fusing with the vacuole, where the autophagosomes are degraded. When autophagy is activated in mutant yeast lacking vacuolar proteases, they accumulate autophagic bodies with the vacuole.

1) Described how the observations above can be used to screen for yeast mutants defective in autophagy.

2) Suppose mutants defective in autophagy are recovered using the screen in part (1). You would like to determine whether such mutants have growth defects under specific culture conditions. What culture conditions would be most promising?

3) One of the proteins isolated in such yeast screens is ATG8, which is called LC3 in mammalian cells. The Western blots below show LC3 protein in response to starvation conditions in mouse embryonic fibroblasts (MEFs). Data for both wildtype and Atg5-null MEFs are shown.



Explain how the two forms of LC3 are produced and interpret what the results mean.

4) Why does the total level of LC3 decline with prolonged starvation?

5) The blot above shows that LC3-II accumulates when autophagy is induced (see the 0.5 and 2 hour lanes). Is increased LC3-II a reliable criterion for increased autophagic flux? Explain and suggest any further experiment to confirm.