Biology 7.06 Exam 2 November 8, 1994

Question 1 (20 points)

A. (5 points) You are interested in acidification of the CURL in yeast cells. You isolate two proteins (CRL1 and CRL2) which you believe are required for CURL acidification. CRL1 is a ATP driven proton pump. CRL2 is a transmembrane protein with homology to a family of proteins known collectively as channel proteins. Suggest a likely function for CRL2 and explain why it is necessary for CURL acidification.

CRL2 is most likely a channel through which anions (probably Cl-) are pumped into the lysosomal lumen. This is necessary for CURL acidification because a proton pump alone (CRL1) would quickly cause an electrical gradient to build up that would not allow further proton pumping. However, if a Cl- channel can pump these anions in, the + charge can be neutralized and the energy of ATP hydrolysis can be used solely to overcome the proton gradient and not the electrical gradient.

B. (5 points) You isolate a strain of yeast that carries a temperature sensitive mutation in the gene that encodes CRL2. Within two hours of shifting the mutant cells to the restrictive temperature, proteins normally found in the lysosome are present bound to the extracellular face of the plasma membrane. Explain this observation.

Without proper CURL acidification, proteins targeted to the lysosome via the M-6-P modification could not dissociate from the M-6-P receptor (need acidic environment). In some cases these receptor-ligand complexes would cycle to the plasma membrane and the ligand (lysosomal enzyme) would remain bound.

We also gave credit for saying that proteins normally internalized from the plasma membrane could not dissociate from their receptors and would also recycle back to the plasma membrane.

C. (5 points) What would be the fate of these lysosomal proteins if the mutant cells were grown in medium containing tunicamycin. Explain your answer.

The answer to this question was stated during class and therefore we were not as lenient with partial credit.

The proteins would be secreted. This is due to the fact that the M-6-P signal (for lysosomal targeting) is dependent on prior N-linked glycosylation. Since tunicamycin blocks the first step in N-linked glycosylation, the M-6-P signal can not be put on and the proteins get secreted by default.

D. (5 points) You isolate the human version of the CRL2 gene and use it to create a human cell line which is unable to make functional CRL2 protein. You observe that this cell line is resistant to infection by influenza

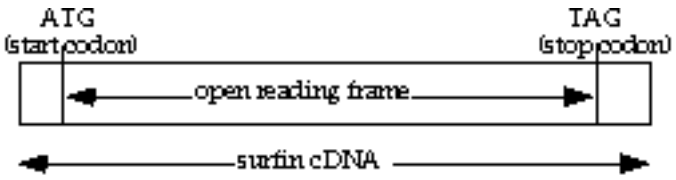
(flu) virus. Provide a reasonable explanation for this observation.

Influenza virus normally binds to sialic acid on the cell surface and gets endocytosed. When the endocytosed vesicle fuses with the CURL, a pH-dependent conformational change occurs which causes the virus to fuse with the CURL membrane and eject its genome and protein coat into the cytoplasm. Without proper acidification (dependent on CRL2), the virus can not undergo the conformation change and therefore can not infect the cell.

This was discussed in length during class ("Safety pin mechanism").

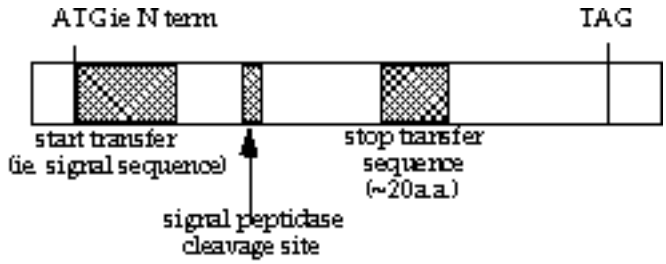
Question 2 (20 points)

Celia the cell biologist is interested in the plasma membrane protein surfin, which is found in abundance on the surface of B cells. Using a B cell line, Celia purifies surfin, raises antibodies against it, and uses the antibodies to clone a cDNA encoding surfin from a B cell library. A crude stretch of the cDNA is shown below:

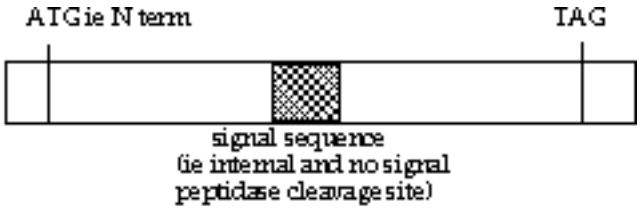


A. (4 points) Given your knowledge of the protein translocation process, annotate the diagram appropriately if surfin were a single membrane spanning region protein with the N-terminus on the extracellular side and C-terminus on the cytoplasmic side. Indicate the size (in amino acids) of the domain(s) that you identify.

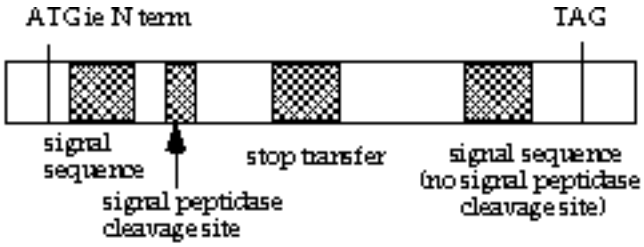
The important thing to remember for all these questions is that as proteins translocate into the ER and move out to the plasma membrane, eventually the cytoplasmic face of the protein remains facing the cytoplasm - reversing the orientation was the most common mistake. If everything was correct but reversed, half credit was given.



B. (4 points) Annotate the diagram appropriately if surfin were a single membrane spanning protein with its C-terminus on the extracellular side and N-terminus on the cytoplasmic side. Again, indicate the size of the domain(s) that you identify.



C. (4 points) Annotate the diagram appropriately if surfin spanned the membrane twice such that both the N and C terminus were on the extracellular side connected by a loop of protein located on the cytoplasmic side. Again, indicate the size of the domain(s) that you identify.



D. (4 points) Based on the actual sequence of the surfin cDNA, Celia concludes that the surfin has the topology described in Part A. Using her B cell line and any other necessary reagents, how could she confirm that the N-terminus was on the outside of the cell and the C-terminus was not.

There are many possible answers here but all involve having monoclonal Abs (antibodies)to each terminus of surfin. If one had fluorescent - tagged or gold - conjugated Abs to each terminus one could add these Abs to intact B-cells in culture and see if they stained the outer cell membrane (using imunoflourescence microscopy or EM, respectively). One can do this with each termini's Ab - would want to confirm Ab not getting inside cell - could also do positive control and break open cell and show C-terminal Ab now stains inner face of plasma membrane as well. Some used proteases to digest outer portion of protein and then lysed cells and immunoprecipitated with each Ab to see which terminus is left.

Other approaches valid but needed to indicate how identifying / visualizing termini with Abs.

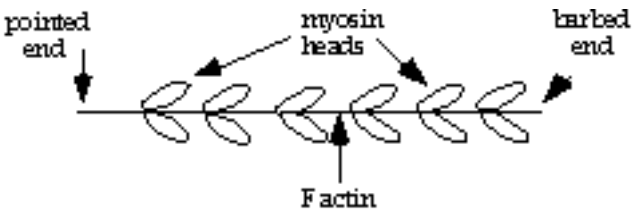
E. (4 points) In the course of analyzing the surfin cDNA by deletion mutagenesis, Celia removes the first fifty codons of the open reading frame (and replaces them with a single ATG start codon). To her surprise, when Celia expresses this deletion mutant in the B cell line, she finds the mutant protein almost exclusively in the nucleus. Provide an explanation as to why the mutant surfin protein localizes to the nucleus and why the wild-type protein is not normally found there. Again, assume the wild-type protein has the topology described in part A.

(2 points) Deletion of first 50 codons definitely removes signal sequence so SRP will not recognize and bind this protein as its being synthesized therefore it will never be brought into ER and will be translated fully in cytoplasm. This deletion may have also "uncovered" a nuclear localization signal which proteins in the cytoplasm bind to, and thereby bring the surfin mutant to the nuclear pores.

(2 points) The w.t. protein is not normally found in nucleus because normally after the signal sequence is translated it binds SRP which halts further translation until the protein is translocating simultaneously into the lumen of the ER. The internal nuclear loc. signal is internalized into the ER as it is translated and thus does not ever "see" the proteins in the cytoplasm which bind to it and target it to the nucleus. Also, the nuclear loc. signal may be hidden by N-terminal structure in the w.t. protein.

Question 3 (20 points)

When the isolated head region of myosin II (generated by proteolytic cleavage of intact myosin II) is bound to F actin in vitro, it "decorates" the filaments in a way that reveals the intrinsoc polarity of F actin. As shown below, all of the myosin heads appear in the electron microscope to point in one direction. Thus, the filament is said to have a "pointed" end and a "barbed" end.



A. (4 points) Using any necessary reagents, design an in vitro assay to determine whether the pointed or barbed end of F actin is the + end. You can assume that the isolated myosin heads become irreversibly associated with F actin once bound.

Isolate the actin filament decorated with myosin

Add G-actin to it (above critical concentration)

Observe which end the actin adds faster to

This is the + end.

One common (very minor) mistake was using fluorescent or radiolabelled actin. Since this is an electron microscopy based assay, you can't use fluorescence or radioactivity. You just observe under EM.

B. (6 points) Assuming that the critical concentration of actin is 1µM for the + end and 8µM fro the - end, describe the changes in a preformed actin filament in vitro if it were incubated with G actin at a concentration of 0.1µM, 4µM and 10µM.

0.1 uM: - end depolymerizes

+ end depolymerizes

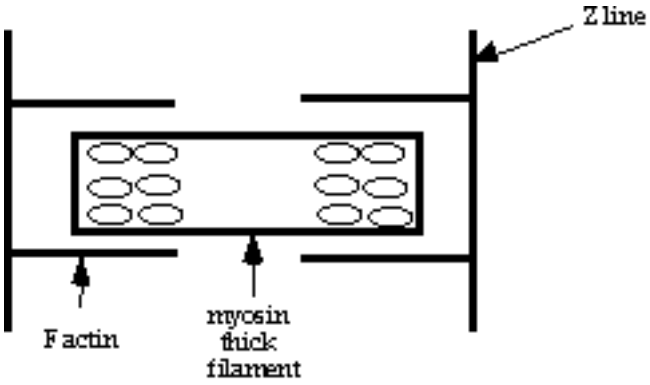
4 uM: - end depolymerizes

+ end polymerizes

10 uM: -end polymerizes

+ end polymerizes

C. (10 points) The diagram below represents a cross section of a sacromere. An important component of the Z line is a protein called cap Z, which binds to the + end of F actin and blocks further polymerization at that end. Using the assay that you developed in part A of this question, describe in detail a biochemical purification protocol that would allow you to purify cap Z. You should include your source of cap Z protein, at least three purification steps, and a clear description of how the assay will be used. Note: you should have a way of overcoming the problem that some cellular proteins (e.g. G actin binding proteins) might simply block polymerization altogether.



Source (1 point) - skeletal muscle

Purification(6 points) - to get the full 6 points you had to include 3 purification steps in a logical order and in a way that showed you understood how these steps were done. You could use the following purifications:

Ammonium Sulfate Precipitation; Affinity, Ion-Exchange, or Molecular Sizing Columns, or SDS gel electrophoresis. You also had to show us some how that you understood you needed to collect fractions in some way and assay them.

Assay(3 points) - You had to show that you needed to take the fractions and assay them individually and then take the positive fractions and do the next purification. The assay was based on adding fractions to the assay in A and looking for fractions that blocked polymerization only at the + end and didn't block the slower polymerization at the - end.

Question 4 (20 points)

A fictious neuronal cell line called 706 normally stores several peptide neurotransmitters, including excitin, in secretory vesicles. When 706 cells are injected with a buffer containing high [Ca^{++}], the secretory vesicles fuse with the plasma membrane and release their contents into the culture medium.

A. (5 points) Using 706 cells and any other necessary reagents, explain (IN DETAIL) how you would determine how long it taked for newly synthesized excitin to be packaged into mature secretory vesicles. You can assume that secretory vesicles can be purified away from other cellular constituents.

(2 points)Label multiple plates of 706 cells with a radioactive a.a. for a short time (pulse) then chase by adding a large excess of that amino acid unlabelled.

(1 point)Chase for multiple time points and at each, homogenize cells and use cellular fractionation to isolate secretory vesicles (SVs) - could also isolate Golgi, ER, etc.

(1 point)For each isolated fraction from each timepoint, use an Ab to excitin to immunoprecipitate it.

(1 point)Run out each immunoprecipitate on an SDS - PAGE gel and use autoradiography to detect the labelled band. When the labelled excitin first reaches the SVs it will appear in the immunoprecipitate of that fraction for the timepoint it takes for newly synthesized excitin to reach SVs.

One common mistake was to just run out all radiolabelled proteins in each fraction for a given timepoint - this will only show when some proteins can reach SVs - you want to know when excitin reaches SVs. Other ways to do this are possible and received full credit as long as it was possible to show how long it takes for excitin to reach the secretory vesicles.

B. (5 points) You clone the gene for excitin and determin that the open reading frame should encode a protein of 30 kiloDaltons (kD), while the excitin found in the mature secretory vesicle is just 12kD. In light of what you know about other proteins that go through the regulated secretory pathway, explain this apparent discrepancy. Your answer should include a brief description of both how and why this difference exists.

(2 points)The difference is most attributable to cleavage of excitin from a precursor, inactive form.

(1 point)As stated in lecture, often secretory proteins undergo pH-dependent protealytic cleavage to their final active form in maturing secretory vesicles.

(2 points)The cleavage occurs at this point so that active neurotransmitter does not go through the biosynthetic ER - Golgi pathway, so it does not interact with its receptor or any other proteins to which it might bind which are also going through that pathway, as this could have a biological effect or prevent its proper secretion.

Any signal sequences removed or other modifications cannot explain this molecular weight difference.

C. (5 points) When 706 cells are injected with a high [Ca^{++}] buffer containing GTPγS, no secretory vesicle fusion occurs. This observation indicates to you that normal membrane fusion requires the action of a small G protein along with its associated guanine nucleotide exchange factor and GTPase activating protein. Explain the likely role of these three proteins in the fusion event.

Following the increase in Ca++, the guanine nucleotide exchange factor is stimulated and causes GTP to replace GDP on the small G-protein (placing it in the active conformation and allowing it to bind to the vesicle). The GTP - bound G-protein bound to the vesicle then (along with other proteins) helps target the vesicle to the proper

membrane and regulates the vesicle - plasma membrane recognition. The GTPase activating protein (GAP) is associated with the inner face of the plasma membrane and binds to the GTP - bound G-protein or the vesicle, stimulating the G-proteins intrinsic rate of GTP hydrolysis. When the GTP is hydrolyzed to GDP, the vesicles can fuse.

For full credit you needed to explain what each protein does and the likely order of events in vesicle fusion. You did not need to mention SNARES in your answer as the question does not ask about them.

D. (5 points) Treatment of 706 cells with colchicine prior to the injection of high [Ca^{++}] blocks secretory vesicle fusion. Based on this observation, you suggest that the movement of these vesicles to the plasma membrane requires the action of a motor protein. Explain why you think so and describe one additional experiment that would strengthen your claim that a motor protein was involved in the movement of secretory vesicles.

(2.5 points)Colchicine depolymerizes microtubules (MTs). Motor proteins fxn by transporting vesicles along MTs using energy from ATP hydrolysis. Thus if one depolymerizes MTs, motor proteins will be unable to function.

(2.5 points)To further test the involvement of motor proteins one could add a nonhydrolyzable ATP analog (eg. AMPPNP) to normal (ie. non-colchicine treated) 706 cells and determine if vesicle movement can still occur. Some of you correctly worried that AMPPNP might interfere with other processes and as on your vesicle transport handout, vesicle fusion is ATP dependent - therefore you really need to assay vesicle movement only.

The best experiment would be to use purified vesicles, MTs, and do an experiment in vitro adding AMPPNP to 706 cell extract to see if an activity exists in the 706 lysate which is capable of still transporting vesicles. Using purified motor proteins from other systems in vitro would not address if these vesicles in 706 cells were being moved in vivo on MTs by motor proteins - would probably work in vitro even if irrelevant to these cells. You needed to address specifically if motor proteins were involved and not just test MTs involvement.

Question 5 (20 points)

Bob the Bio major is given a set of four L cell lines (Line A, Line B, Line C, and Line D) with which to do cell-cell adhesion assays. As shown in the table below, he observes that in buffer containing calcium, all cell lines will adhere to themselves, but when EDTA is added to the buffer, only Line A continues to adhere to itself. He also determines that Line A will

adhere to Line D in the calcium-containing buffer, but these cells fail to adhere when EDTA is added. Finally, no other pair of cell lines will adhere under either condition.

Cell-cell adhesion in Ca2+ containing buffer (+/- EDTA)

Line:	A	A	B	B	C	C	D	D
EDTA:	+	-	+	-	+	-	+	-
A	yes	yes	no	no	no	no	no	yes
B	no	no	no	yes	no	no	no	no
C	no	no	no	no	no	yes	no	no
D	no	yes	no	no	no	no	no	yes

A. (5 points) Bob concludes that Line A expresses two different species of cell surface adhesion molecules while Lines B, C, and D express just one. Suggest what cell adhesion molecules these lines might express. Your answer should include an explanation of the results shown above.

EDTA chelates Ca^{++}, thereby effectively removing it from the media. CAMs are cell adhesion proteins that are Ca^{++} independent and thus fxn in the presence or absence of EDTA. Cadherins are Ca^{++} dependent cell adhesion proteins and thus function only in the absence of EDTA. Both of these protein families exhibit homophilic (homotypic) binding meaning that they only interact among their specific types.

eg. N-cadherin only binds to N-cadherin and not to P or E cadherins.

Given this:

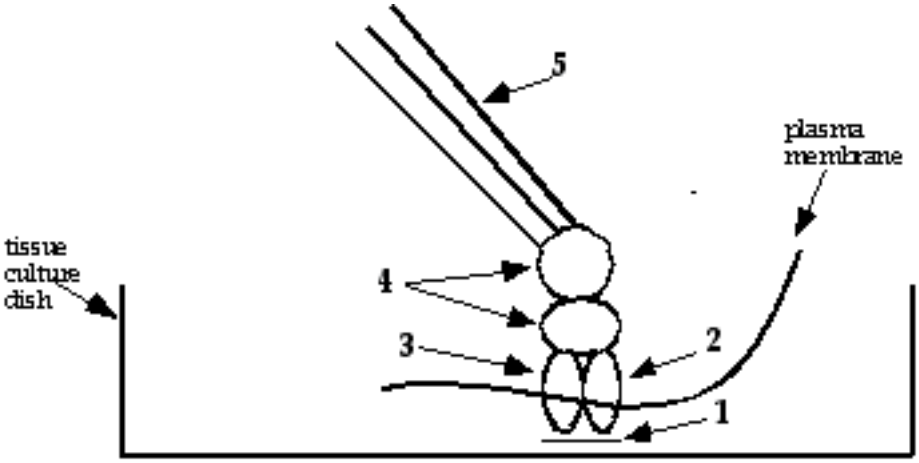
Line A: expresses a CAM (ex: N-CAM) and a cadherin (say A cadherin).

Line B: expresses a cadherin that no other cell line has (B-cadherin).

Line C: just as B, expresses a unique cadherin only (C-cadherin).

Line D: expresses a cadherin, but can also interact with line A in the presence of Ca^{++}

B. (5 points) Bob also observes that all of these cell lines will adhere to tissue culture dishes that are coated with the extracellular matrix protein fibronectin. Label the diagram shown below which depicts a site of focal adhesion between the cell and the dish.



1. fibronectin

2. α -integrin

3. β -integrin

4. linker proteins

5. actin filament (or intermediate filaments)

C. (5 points) Fibronectin is a very large molecule with domains that mediate binding to the cell as well as to other components of the extracellular matrix. Describe an experimental strategy that would allow Bob to localize the fibronectin domain required for binding to the cell.

One way would be to make deletion mutants in the fibronectin gene. Then, you would take protein expressed from these deletion mutants and see which mutant could still bind to cells. By doing this, you could localize the domain required for the interaction. A good extension of this was to take this binding domain and fuse it to another protein and now see if that new fusion protein could bind cells.

Other methods were accepted as long as you could provide a good rationale for how the method would work.

D. (5 points) Having localized the domain of fibronectin required for binding to the cell, Bob raises an antibody to it. Upon injection of the antibody into an amphibian embryo, he observes that the migration of a certain cell population is inhibited. What conclusions does he draw from this observation? Describe an experiment that

would allow Bob to determine which cell surface protein was normally responsible for this migration.

Conclusion(2 points): The antibody blocks fibronectin from binding to a receptor on the cell surface necessary for proper migration.

Experiment(3 points): A variety of answers were accepted.

You could cross-link or immunoprecipitate the fibronectin to whatever it was binding on the cell surface (and at least briefly explain how you would isolate the protein).

-or-

You could inject antibodies to different integrins (which bind fibronectin) to see which of these antibodies might also block the migration.

Other answers were also accepted provided they were logical and practical (and explained well enough).