Kinesin: Introduction: BACTERIAL EXPRESSION AND PURIFICATION OF KINESIN-GFP
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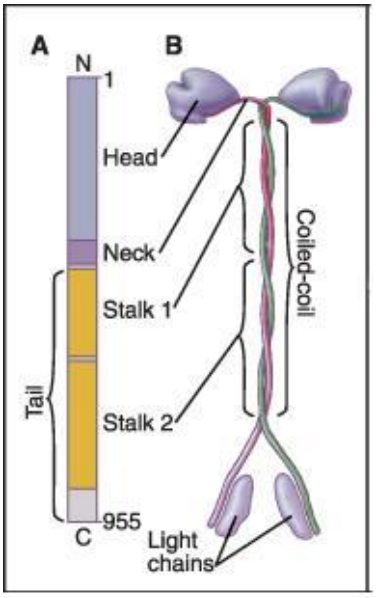
Pre-Lab Assignment:

Cells are dynamic. Scientists have been able to observe the movements of cells and the movements of components of cells through the microscope for hundreds of years. It has only been in the last 40-50 years that the molecular components responsible for those movements have been identified and well understood. In this series of labs, we will be following in the footsteps of cell biology pioneers to recreate some classic experiments in the study of cellular movements. Over the next three weeks, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analysis of protein concentration, we will track the progress of a protein purification procedure. We will also attempt to show that the purified motor protein is capable of moving in a reconstituted system by using computers to collect and analyze data from fluorescence microscopy images.

The performer

We will be studying the motor protein kinesin in this series of labs. Kinesins are proteins that use conformational changes induced by ATP hydrolysis to move in a particular direction along microtubules. Kinesins contain three important functional regions, or domains. The "motor" domain of kinesin is responsible for microtubule binding and ATP hydrolysis. The kinesin neck linker domain converts the conformational change into movement along the microtubule. The kinesin tail is responsible for attaching the motor to whatever it happens to be responsible for moving. You can think of kinesins like a tractor-part of a tractor-trailer or an 18-wheeler, or a lorry (if you are British). The engine (kinesin motor) converts the chemical energy stored in the fuel (ATP) into force that drives the wheels (neck linker), aiding in the transport of whatever is hitched onto the back (cargo attached to the tail).



Specific amino acid sequences (A) fold to create domains in the kinesin quaternary structure (B). The amino and carboxy termini are labeled "N" and "C", respectively. The expressed construct contains the amino acids 1-560, encoding for the head, neck, and a portion of stalk 1. ©Elsevier Ltd. Pollard and Earnshaw: Cell Biology www.studentconsult.com

The performance

Kinesins move on microtubules and we will be asking them to do just that, but in reverse. Rather than the kinesins moving on microtubules, the microtubules will be moving on kinesins. It all depends on your point of view. The motility assay, or microtubule gliding assay, is a microscopic assay that measures the velocity of molecular motors. Both kinesins and microtubules are too small to resolve via light microscopy, but fluorescently tagged microtubules are easily visible by fluorescence microscopy. For a typical gliding assay, kinesins are attached to the surface of the cover slip. Microtubules are added, and the kinesins stick to the microtubules. With the addition of ATP, kinesins begin their cycle of ATP hydrolysis and conformational change. Since the motors are attached to the cover slip, it is the microtubules that move in response. If we take a fluorescence image at regular intervals, we can see the microtubules move. If we measure the distance that they have moved over a particular amount of time, then we can calculate the velocity of the molecular motor. But first, in order to do any of this, we need relatively pure samples of kinesin protein.

Molecular Biology, the Universal Genetic Code, and Reductionism

Many cell biologists (especially cytoskeletal biologists) have taken a reductionist approach to studying cellular phenomena. That is, they break a complex system down into its most basic components and study each component individually before studying them in combination. In the molecular motor world, that meant isolating large quantities of a particular motor from a tissue source that had an abundance of that motor. For example, the microtubule motor kinesin was originally isolated from tissues that had high requirements for microtubule-based transport (i.e. nervous tissue). The identification of kinesin from squid optic lobe and bovine (cow) brain happened at the Marine Biological Labs on Cape Cod in the mid-1980s. The problem with this approach is that it requires two things: whatever you are studying must be present in great abundance, and you must have easy access to the tissue source. The solution for kinesin biologists was to go summer on the Cape, or to visit their local slaughterhouse. Not every scientist is lucky enough to have ready access to starting materials.

A more elegant solution to this problem came with the revolution in molecular biology. If you know the coding sequence for the gene that you want to study, then you have enough information to generate large quantities of it. This is because, for the most part, the genetic code is universal. The bacterial transcription/translation machinery will produce the correct sequence of amino acids if given a eukaryotic gene with the proper prokaryotic start and stop signals. In other words, scientists can coax bacteria into producing large quantities of a particular protein, if they can get the DNA into the bacterium.

Once the desired DNA is in the cells, you have to be able to get the resultant protein out of the cells and purify it. In our case, the protein will only be expressed when the cells are in the presence of lactose, or a molecule that looks like lactose (IPTG). When IPTG is present, it binds to a transcription factor that blocks transcription of the kinesin mRNA. The binding of IPTG turns off the transcription blocker, allowing for the message to be made, and thereby producing the protein.

Under these conditions, the kinesin will be in the bacterial cytosol. The simplest way to get the protein out of the cells is to lyse them, or break them open. In our case, bacterial lysis involves freezing the cells in liquid nitrogen. The extreme cold forms ice crystals quickly. The ice crystals weaken the plasma membrane and cell wall. The near instantaneous freezing minimizes damage to the proteins. Addition of an enzyme called lysozyme also aids in the breaking open of the cells by catalyzing reactions that further weaken the cell wall. The cells are then exposed to high frequency sound waves (sonication), which also helps to break them open.

Once the cells are broken open, it is important to remove all of the insoluble material (cell wall, membranes, DNA) from the proteins that are to be purified. This can be accomplished by centrifugation. Exerting a large gravitational force on materials while they are in suspension can separate them by their differing densities. Like the amusement park rides that force you to stick to the wall of a spinning cylinder, centrifuges spin quickly to produce g-forces ranging from a few hundred times the force of gravity, to around 150,000 times g. By spinning the lysed bacteria at around 40,000 x g, the insoluble material (pellet) settles at the bottom of the tube while the protein of interest remains in suspension.

New-fangled biochemistry and protein purification

If you can make a culture of bacteria produce a protein that you want to study, how do you harvest that protein? The keys to this process are being able to "tag" the protein that is being expressed with something that allows you to isolate it from the thousands of other proteins in the bacterium, and keeping that purified protein intact. This tag is usually the addition of unusual amino acid sequences that can be used as "tags." In our case, there are two tags, and we have taken some preventative measures to keep the purified protein from breaking down.

The demonstration samples that you will be using in these labs mimic those that would come from bacterial cells that were made to express the motor and coiled-coil domains of kinesin, along with these two tags: a green fluorescent protein (GFP) domain, and a series of six histidines (6xHis). The GFP allows for the visualization of purified protein in single-molecule biophysics experiments. The 6x-His tag allows for purification. Histidines in series will bind noncovalently to Ni^{2+} ions. Ni^{2+} ions can be immobilized in a resin within an affinity chromatography column, and if you expose this resin to a homogenate from cells expressing 6x-His-tagged protein, then these proteins will stick to it, and most of the other untagged proteins will not. So if your cell lysate contains kinesin-GFP-6x His, it will stick to the column. The resin in the column can then be washed to remove any protein that isn't tightly bound.

The tightly bound protein (in this case, the kinesin-GFP-6xHis) can then be eluted from the resin by adding imidazole, a compound that looks like the ring structure of histidine, and competes with the histidine for Ni^{2+} binding sites. By adding a huge molar excess of imidazole (compared to Ni^{2+} binding sites) then the chances for the 6x-His-tagged protein being bound to the resin greatly decrease. The tagged protein comes off the column and can be collected as a relatively pure sample. The last step is a buffer rinse through a buffer exchange column that separates molecules by size. It effectively traps any imidazole and separates it from the purified protein, which is collected in the column flow-through. Protein expression and purification systems such as this allow

for the generation of useful quantities of rare proteins, mutant versions of proteins, and even single domains.

To keep the proteins intact, you have to protect it from degradation along the way. Inside a cell, the concentration of proteins and solutes is very different from the concentrations of those components in a lysate. Additionally, lysis results in the mixing of compartments and substructures that had been kept separate within the cell. As a consequence, reactions that might have been energetically unfavorable in the cell (for a variety of reasons) may be energetically favorable in a lysate. Specifically, cellular enzymes that degrade proteins are particularly troublesome in a cell lysate. These enzymes, called proteases, will catalyze hydrolysis reactions that cleave peptide bonds within the protein. There are multiple ways to minimize this activity. The simple (and less effective) way is to keep your samples cold. By keeping samples on ice, the low temperature will slow down the progress of proteolysis reactions because the average energy of the collisions between molecules will be lower. Additionally, it is common practice to add protease inhibitors to cell lysates. Some of these inhibitors have been developed to inactivate proteases by binding covalently to their active sites. Others function by binding to cofactors required for the function of some proteases. For example, EDTA binds to Ca^{2+} and other divalent cations with high affinity, stripping the divalent cations from certain proteases that require divalent cations for catalysis. Any biochemist worth her salt will pay careful attention to minimize proteolysis of her samples, as these processes can never be completely eliminated. Even without enzymatic catalysis, proteolysis (cleavage of peptide bonds) is an energetically favorable process in aqueous solution. Luckily for us, proteolysis is not instantaneous and requires some time. However, keep these potential problems in mind as you analyze the fractions that mimic the purification steps outlined above.

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