Synthetic Protease Switch

SIGHTINGS

Every graduate student has been quizzed at least once on the life cycle and genetics of the bacterial phage lambda. This virus is perhaps the most well-characterized piece of genetic information in all of biology. Over the years, lambda has served mainly as a dependable laboratory workhorse for everyday molecular biology experiments. This bacterial virus is well suited for cloning purposes and can propagate complex mixtures of exogenous DNA of a convenient size. For genetic experimentation, sophisticated assays of gene and protein function have been designed based on the molecular components of the virion. The protein components of lambda gene regulation and their interactions are known at the most detailed level. In fact, many of these proteins have been examined at the atomic level through x-ray crystallography. Investigators can use their molecular knowledge of lambda to design experiments that manipulate the regulatory circuits in ways that produce defined results.

The lambda life cycle consists of two distinct phases, lytic and lysogenic. The lytic phase is characterized by infection of a bacterial host followed by intense phage replication and the production of a large number of progeny. Released virions infect adjacent cells and amplify quickly. The lysogenic phase is characterized by infection and integration into the host genome. No progeny are produced, and the phage is effectively dormant. A molecular switch composed of transcription factors and proteases controls which direction a phage will go. Sices and Kristie at the National Institutes of Health (NIH) took advantage of their knowledge of the molecular switch to alter it so that it could now be used to detect the presence of a specific protease within a bacterium (*1*).

The lambda repressor protein is a DNA-binding protein that binds to a sequence of DNA adjacent to the lytic genes. When the repressor is functional, lytic gene products are silenced and the phage enters a lysogenic phase. The repressor also binds upstream of a negative regulator of its own transcription, a gene called *cro*. Together, the concentrations of lambda repressor and *cro* protein determine whether a phage will lyse a bacterium or just remain dormant. An endogenous bacterial protease, RecA, cleaves the repressor at a specific molecular hinge region. When cleaved, the repressor cannot complex efficiently with DNA. Lytic genes are then turned on, and the phage produces infectious progeny and kills the bacterium.

How does one modify this system to study other proteases? First, the NIH group altered the repressor gene sequence to insert a restriction site at the natural RecA protease–sensitive site in the protein. Testing in vivo, they showed that the mutant form was now resistant to RecA cleavage. Next, they used the inserted restriction site to create a new protease target site in the repressor that was patterned after one of the target sites of the human immunodeficiency virus–1 (HIV-1) protease (repressor-HIV). The basic idea was to make a synthetic molecular switch in lambda that could be toggled by introduction of active HIV-1 protease.

They next introduced the HIV-1 protease (in a phage with an intact *cro* circuit) along with a plasmid that contained repressor-HIV. Gel electrophoresis showed the cleavage of the repressor-HIV polypeptide in vivo. In addition, examination of the growth rates of the HIV protease–containing phage showed that they replicated 7000 times more efficiently than controls. To see if they could use this growth advantage to select for a protease-containing phage in a complex mixture of phage in vivo, the researchers designed a mock experiment. They infected bacteria with a 10^5 mixture of control to HIV-1 protease phage and examined plaques for the presence of the protease. Amazingly, after only three rounds of selection, 40% of all phage contained the HIV-1 repressor.

They also went on to show that drugs that act as inhibitors of the HIV-1 protease would also alter the synthetic molecular switch in vivo. In fact, the inhibition by drugs such as crixivan seen in the phage assay paralleled the effective concentrations of the drug determined in humans.

The system described should be useful for selecting a protease in a mixed population of clones, as would be done in a cloning experiment. It can be used to test inhibitors of the isolated proteases or of known proteases. Because proteases are involved in a wide range of biological processes, from apoptosis to Alzheimer's disease, the new assay could have broad utility. This work also shows that the old lambda system still has more uses up its molecular sleeve.

–Robert Sikorski and Richard Peters

References

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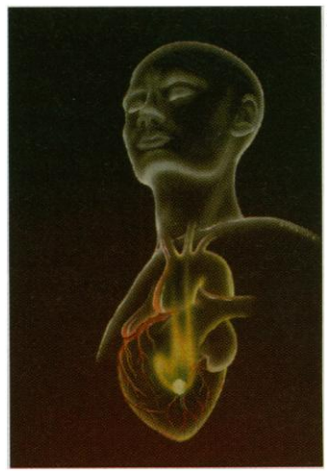
Beauty in Simplicity

SIGHTINGS

Each year, cardiovascular diseases cause approximately 12 million deaths worldwide. In developed countries, heart attack is the number one killer. The problem is simple on paper: Clots or plaques block the arteries that supply the heart itself. To open these blockages, hundreds of thousands of patients each year undergo some sort of revascularization procedure such as percutaneous coronary angioplasty or coronary artery bypass surgery. Researchers have been busy trying to come up with new methods to improve revascularization of damaged heart tissue.



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One experimental approach involves angiogenic factors that stimulate the growth of new blood vessels. Several methods are being investigated to achieve this. They include delivery of growth factors systemically, via gene therapy, or locally with microspheres. Intravenous administration of growth factors [such as fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF)] is the simplest method, but, because of intravascular dilution, large amounts of proteins have to be delivered, which may result in potentially dangerous side effects (such as hypotension). The promise of gene therapy has been that growth factors could be expressed locally in high concentration by targeting genes to damaged areas only. However, the technology behind gene therapy is still quite complicated, and it continues to face significant challenges in progressing to a clinical setting.

A more straightforward approach has been to use drug-containing microspheres to deliver growth factors. Several groups have attempted this by delivering the beads systemically or directly implanting them in the heart during open heart surgery. Intracoronary delivery of microspheres coated with basic FGF (bFGF) has been done in infarcted swine myocardium, and has resulted in increased angiogenesis (*1*).

Unfortunately, the beads also caused extensive myocardial necrosis, probably because of their large size (75 to 150 mm). Could smaller beads that would lodge more distally in the terminal capillaries provide a safer delivery system? A report by Arras *et al.* seems to show that this is indeed the case (*2*). The approach they used is elegant in

its simplicity and could pave the way to a new adjuvant form of therapy during revascularization procedures.

They used resin-coated chromatography beads 7 mm in size in their studies. These beads are coated with SO_3^- so they bind basic, positively charged proteins. The beads were incubated with bFGF, and binding experiments indicated that approximately 2 pg of bFGF was bound to each bead. The bead preparation (10^5 beads per gram of targeted myocardium) was then injected percutaneously in the left circumflex coronary artery of healthy pigs. The control group was injected with beads that had not been incubated with bFGF. Hearts were removed at various time intervals up to 7 days after the single injection together with tissue samples from various other organs such as the spleen, liver, lung, kidney, bowel, and so on.

Microscopic sections stained with toluidine blue indicated that about 60% of the beads had obstructed small capillaries within the distribution of the left circumflex artery. The rest of the beads could not be accounted for and the authors assumed that, because of their very small size, they had been recirculated and evenly distributed within the vascular tree. Immunohistochemical staining of heart sections indicated that bFGF was present in the tissue sections, but had disappeared within 7 days. Tissue sections of control hearts, injected with beads without bFGF, failed to stain for bFGF, indicating that the bFGF identified in the tissue sections was not endogenous. On the basis of those data, the authors estimate that the concentration of beads in the targeted coronary artery area was 1500-fold that in the rest of the body and that 0.12 mg of bFGF was delivered to each gram of targeted myocardium.

Histologic studies of the myocardium showed a lack of inflammatory response to the beads as well as an absence of tissue necrosis. There was an increase of proliferating fibroblasts and endothelial cells around the microspheres. There was, however, no increase in vessel growth. The authors postulate that this indicates that not only an increase in growth factor but also an increase in growth factor receptor is required, such as happens following ischemia. Others have indeed shown that bFGF can induce angiogenesis in ischemic myocardium (*1*).

One can easily imagine having this simple method available in the cardiac catherization lab in a hospital setting. Physicians could inject coated beads in coronary arteries that they have just opened by angioplasty. Before this day comes, though, key questions still need to be answered. The most important is whether this approach will actually lead to vessel growth when applied to ischemic myocardium and whether the neovascularization will be of physiological significance for myocardial perfusion. Indeed true collateral arteries that are potentially capable of compensating the loss of the main arterial supply tend to grow from preexisting arteriolar connections between nonoccluded and occluded vessels mainly in the non-ischemic territories. So, it is concievable that squirting the microspheres in the periphery of the perfusion territory of the diseased vessel may not lead to the induction of tru collateral arteries (*3*). In addition, whereas healthy myocardium did not seem to be negatively affected by the beads, it is conceivable that ischemic myocardium will be more sensitive to the occlusive effect of the beads, hence, leading to additional myocardial necrosis.

This study is elegant in its simplicity: mixing small chromatography beads with commercial preparations of a growth factor and injecting them percutaneously into the coronary artery. Sometimes, just reassembling off-the-shelf components may be all that is needed to do the job.

–Richard Peters and Robert Sikorski

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Showing Off Your Lab

NET TIPS

These days, just about every lab has their own home page on the Internet. Laboratory Web sites are great because they provide a medium to exchange detailed versions of protocols and data that augment those sent to peer-reviewed journals. This month, we look into the technology needed to rapidly create digital images that can be added to your Web site. We'll move from digital camera to Web site to give you a sense of the hardware and software needed.

A digital camera can be viewed as a hybrid between a conventional film-based camera and a scanner. Instead of storing the picture on film, the image is recorded in a digital form, compressed, and stored in either a built-in memory chip or a removable PC card. There are several issues that users need to be aware of before making a significant investment (prices range from $300 to $1200; a high-quality single lens reflex model may cost $4000 or more) in a digital camera.

First, depending on the quality of the camera, there can be a significant delay of up to 1.5 s between the time you press the shutter and the time the picture is actually taken. If you are recording the phenotypes of a twitching mouse, this could be a problem. For fast moving objects, it is probably best to stick to conventional cameras for now. These pictures can then be scanned and digitized later.

After you take the picture with a digital camera, the camera will remain "busy" for 4 to 9 s, during which time it is converting the pictures to digital form, compressing, and archiving them. You cannot take rapid-fire pictures with conventional digital cameras. Another issue is the amount of memory you will need in the camera. Picture quality (and image size) is determined by the density of pixels used. The quality chosen can be altered so that you can take high- or low-resolution images. For the highest resolution images, you may be able to store only three pictures at a time on a 2-megabyte (MB) standard memory card. For the lowest quality, you may be able to store about 25, depending on the camera. Additional, removable memory can be purchased for about $50 for 4 MB.

Although it is improving, the picture quality of digital cameras is still less than the quality of traditional cameras. Quality varies significantly from camera to camera.

Perhaps the most surprising feature of digital cameras is just how fast they use up batteries. It is not unusual to go through four AA batteries after taking only 30 pictures. Power cables are available, but they obviously limit your flexibility.

When you are ready to format the images for the Web, things get a bit easier. Most digital cameras come with software packages for picture editing. In fact, the images are usually delivered in a form known as JPEG or jpg, one that can go straight to the Web without any editing at all.

In the end, the field of digitizing your data with a camera is getting easier, but it is still a work in progress. For more info, we have collected online reviews of the most popular cameras at www.medsitenavigator.com/tips.

–Richard Peters and Robert Sikorski

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(continued on page 458)

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