MOLECULAR BIOLOGY

New Way Found to Study Closely Related Proteins

Rather than designing specific inhibitors for closely related proteins, researchers are remodeling the proteins to make them uniquely susceptible to inhibition

In large families, siblings sometimes get lost, especially when they look a lot alike. But people can modify their appearance—combing their hair into spikes, for example—so that they stand out in a crowd. Now, scientists have applied this same principle to mark individual proteins so that their activities can be distinguished from those of other family members—an accomplishment that should help pin down the functions of the many closely related proteins found in cells.

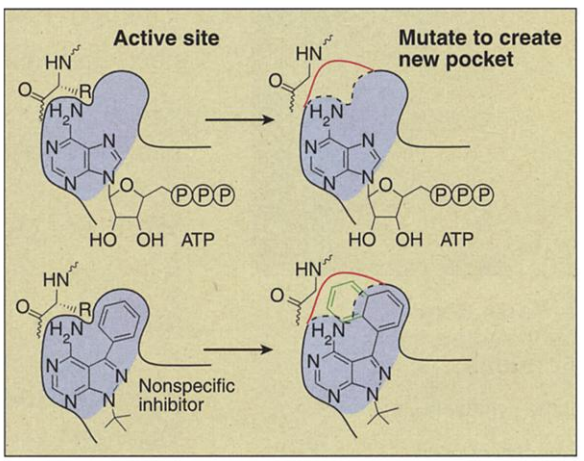
The technique, devised by Kevan Shokat, a chemist at the University of California, San Francisco (UCSF), and his colleagues, involves enlarging the active site of an enzyme so that it can bind an inhibitor that won't fit into the active sites of related—but unaltered—enzymes. Researchers can then insert the gene that encodes the modified enzyme into cells or living animals and turn off that enzyme by feeding them the inhibitor—without affecting other, very similar, enzymes. "This is a beautiful way to get around the limitation that we can't yet design inhibitors for every natural protein in the cell," says Tim Clackson, a protein engineer at ARIAD Pharmaceuticals Inc. in Cambridge, Massachusetts.

What's more, the technique may have some advantages over other approaches to studying the functions of individual proteins, such as mutating or knocking out the genes that encode them. Knockouts, for example, may disrupt embryonic development, producing abnormal animals, or even no animals at all. But as Tony Hunter, a molecular biologist at the Salk Institute in La Jolla, California, points out, "Here you take a fully developed animal in which everything is normal until you turn off the [enzyme]."

The Shokat team began its work about 3 years ago, focusing on a key family of regulatory enzymes, the protein kinases, which transfer a phosphate group from the high-energy molecule ATP to any of a large number of target proteins. The kinases have been tough to study individually, because they are numerous—yeast has more than 100 and humans about 900—and have very similar active sites, making it tricky to design specific inhibitors.

Instead of trying to tease out distinguishing features of individual kinases that might serve as inhibitor targets, the Shokat team used genetic engineering to create such sites. They started with *v-src*, one of the cancer-causing oncogenes, which encodes a kinase. They modified the gene so that the bulky amino acid threonine in the part of the kinase that binds ATP was replaced with the smallest amino acid, glycine. "We decided to cheat," says Shokat. "We carved a new hole in the active site."

As reported in the February 1998 issue of *Current Biology*, this modification had little effect on the catalytic activity of the Src kinase. Although the altered version showed slightly decreased activity in the test tube, it transferred phosphate efficiently when introduced into cells. Furthermore, it still conferred unrestrained growth on cells in culture. But adding a specially synthesized kinase inhibitor blocked that aberrant growth. Because the technique was tried on just one protein, the team did not know how widely applicable it might be. Quite applicable, suggests new results reported in the 21 September issue of *Nature*.



Opening up. Replacing a bulky amino acid in the ATP-binding site of a kinase with glycine enlarges the site. That doesn't affect ATP binding or catalytic activity (top), but as shown below, it's now possible to convert a nonselective kinase inhibitor to one that will fit—and block—only the altered enzyme.

In this work, Shokat and his colleagues created new inhibitor-binding pockets in seven protein kinases from five distinct families, again by replacing a bulky amino acid with a glycine. The researchers then dove into their collection of chemical inhibitors, some synthesized by their group and others by chemist John Wood at Yale University. They found compounds that inhibited the altered enzymes but not the normal ones. "We can now say it's going to be a generally useful procedure, because they've used it successfully on very disparate kinases," says Clackson.

Shokat's team also showed that they could inhibit the modified kinases in yeast cells, which lend themselves particularly well to genetic manipulation. The researchers targeted a gene that encodes the Cdc28 kinase, which is needed for progression of the cell division cycle. Because Cdc28 knockouts are lethal, researchers have previously studied the gene's function by generating mutations in which the gene product functions normally at low temperatures but is inhibited at higher ones. But results with such temperature-sensitive mutants can be hard to interpret, because the temperature change may affect cellular processes other than those that directly involve the catalytic activity of the target protein. The new method may help circumvent that problem.

Working with David Morgan's group, also at UCSF, Shokat's team substituted a modified *Cdc28* gene for the normal one in yeast cells. The researchers found that the altered cells showed few changes in gene expression and they divided normally—until the researchers added the kinase inhibitor, which blocked the growth of the cells. In contrast, the inhibitor did not affect proliferation of cells containing the normal *Cdc28* gene, except at 1000-fold higher concentrations.

Further analysis revealed that the inhibitor arrests the modified cells after DNA duplication has occurred, but before the two daughter cells have separated. This contrasts with the results of previous experiments with the temperature-sensitive mutants, most of which indicated that loss of Cdc28 function blocks the cycle at the point when cells start copying their DNA. The fact that the results were different when using the temperature-sensitive mutants than when inhibiting the kinase implies that the protein may have more than one role in the cell. Shokat notes that temperature-sensitive mutations sometimes disrupt the structure of the altered protein, which can lead to loss of all of its activities, including interactions with other proteins. Consequently, he suggests, such interactions of Cdc28 may be important at the DNA-copying stage of the cell cycle, whereas its kinase activity may be what's more impor-

ILLUSTRATION: MODIFIED FROM K. SHOKAT

tant at the cell separation stage.

Because the inhibitor acts quickly, the new technique can also be used to assess the effects of blocking a protein's activity at specific points in the cell cycle. David Drubin's group at the University of California, Berkeley, has been doing just that. In work reported in the October issue of *Nature Cell Biology*, the researchers used the technique to study a protein called Cla4, a kinase involved in forming the bud that expands to form the daughter cell when a yeast cell divides. Analysis of this protein using temperature-sensitive mutants has been difficult because a shift to the higher temperature temporarily disrupts the cell's internal skeleton, interfering with bud formation even in normal cells.

In their experiments, Drubin and his colleagues replaced the normal *Cla4* gene with an inhibitor-sensitive version. They found, as others had using different techniques, that the protein is needed to build the collar that eventually squeezes off to separate the daughter cell from its parent. They were also able to pin down exactly when Cla4 acts. The researchers gathered cells that they had arrested at various points in the cell cycle, added the inhibitor, and then allowed the cells to resume growth. Cells that had started to bud before addition of the compound divided normally. But buds formed after inhibitor addition continued to elongate without pinching off. The observations suggest that Cla4 kinase activity is needed at or before an early stage of bud emergence, even though the defect isn't evident until much later when mother and daughter cells try to separate.

Now that the technique has worked in yeast and in isolated mammalian cells, scientists are trying it in whole animals, such as mice. The results have not yet been published, but they look promising. And although Shokat has so far applied the system only to enzymes that use ATP, he says it should work with proteins in other large families. The similarity of the members, he notes, has been a hindrance to researchers. But the new technique may turn that around, because a mutation and inhibitor that work with one family member may work with others as well. Says Shokat, "We've turned the disadvantage into an advantage." –EVELYN STRAUSS

MEETING ECOLOGICAL SOCIETY OF AMERICA

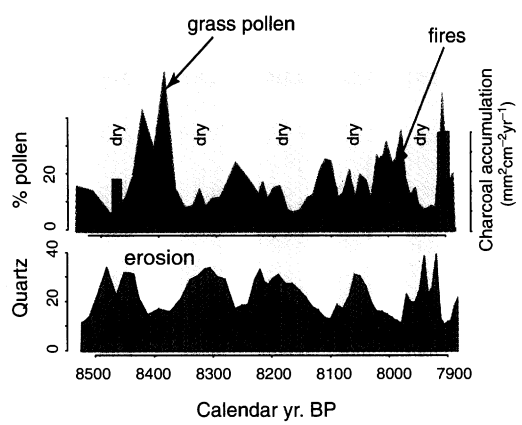
Global Warming, Insects Take the Stage at Snowbird

SNOWBIRD, UTAH—Some 2600 ecologists made their way to this sun-soaked canyon last month for the Ecological Society of America's (ESA's) 85th annual meeting. Topics ranged from ancient droughts to photosynthesis beneath snow and how trees resist insects.

Could Past Portend 50-Year Droughts?

The Dust Bowl that struck the southern plains of the United States in the 1930s devastated millions of hectares of rich farmland, leading 750,000 people to flee, burying houses with dirt, and darkening the skies for days. But that 7-year drought was a mere taste of what global warming may bring, warned ecologist Jim Clark of Duke University. Sediments from a North Dakota lake reveal that 8000 years ago, the plains were seesawing through droughts and wet periods that lasted a whopping 40 to 50 years. Similarly long drought cycles could happen again, asserts Clark, as accumulating greenhouse gases turn continental interiors warmer and drier.

By probing tree-ring records and other evidence, researchers have recently found hints that Dust Bowl–scale droughts were frequent over the past 2000 years. But to find out what might happen to ecosystems under much more arid conditions than today's, Clark's team looked even farther back in time, to the mid-Holocene, when the U.S. plains were arid. To do so, they went to Kettle Lake in North Dakota, which contains 20 meters of mud deposited over millennia. Clark, Eric Grimm of the Illinois State Museum in Springfield, Joe Donovan of West Virginia University in Morgantown, and others studied a 50-cm sediment core dating from the mid-Holocene. By examining minerals, charcoal, and pollen in the core, they have illuminated in unprecedented detail the wildly shifting ecology of the region during a 600-year period.



CREDIT: J. CLARK *ET AL.*

Shape of things to come? U.S. plains flipped between decades-long droughts and wet periods in the warm, arid mid-Holocene.

Clark and his colleagues found long wet periods, characterized by pollen, diatoms, and charcoal from naturally burned grasslands. They also charted a shift in plant type from cool-season grasses to warm-season grasses. Then the pattern flipped to drought: Quartz dust from erosion becomes abundant, while grass pollen and charcoal levels plummet. The repeating cycles lasted about 80 to 100 years. By contrast, a core from 2000 years ago showed no distinct patterns. "I was just blown away" by the work, says Brown University paleoclimatologist Tom Webb. "To get that high a frequency [of climate swings] and to get it so neatly told among the chemical data and the pollen is rather astounding."

Because the driver behind the warm, arid climate of the Holocene was a different tilt and orientation of Earth, not the rising carbon dioxide levels that seem to be contributing to warmer temperatures today, these results may not predict what's to come in a greenhouse world, concedes Clark. But even so, his group's data are worrisome, says ecologist Peter Leavitt of the University of Regina in Saskatchewan. "We've been adapting to some of the mildest possible droughts. These are beyond anything we've known in human society."

Snow Falling on Tundra

Few ecologists visit the Alaskan tundra before the winter snowmelts. They've long assumed that there's little biological activity to warrant the trip. But the notion of a snow-cloaked ecosystem too cold and dark for photosynthesis may no longer hold. At the meeting, ecologist Gregory Starr of the University of Florida, Gainesville, reported that he's peered under the snow on Alaska's North Slope and found plenty of photosynthesis by evergreen tundra plants—enough that estimates for how much carbon the tundra soaks up may need to be adjusted.

Along with other scientists doing separate arctic experiments, Starr may have stumbled upon a small but significant hidden "sink" for global carbon dioxide: early spring tundra growth. "It seems to be a pret-