Clues to Cell Growth and Differentiation

Phorbol esters—compounds derived from plants—offer insights into the regulation of processes involved in tumor promotion

On 16 March, James Neidel of Duke University gave a lunchtime seminar at the National Institutes of Health on phorbol esters. The seminar room was overflowing, the crowd being evidence of a recent explosion of interest in phorbol esters as scientists finally develop insights into how these compounds control cell growth and differentiation. "The system is now experimentally accessible," says Neidel.

Phorbol esters, which are derived from plants and used as herbal medicine in China, are now known to be tumor promotors. Although they do not themselves cause cancer, they amplify the effects of a low dose of a carcinogen. The classic demonstration is to paint a patch of a mouse's skin with a very small amount of a carcinogen—so low a dose that the carcinogen by itself would have no effect. If the mouse skin is then painted twice a week for 12 weeks with phorbol esters, skin tumors appear.

According to Peter Blumberg of the National Cancer Institute (NCI), "it is increasingly thought that tumor promotion plays a role in causing human cancers." As potent tumor promotors, phorbol esters may yield clues to how promotors and carcinogens work in general.

Phorbol esters are known to have quite specific effects on certain cells. Very low concentrations—in the nanomolar range—can induce some cells to differentiate and others to proliferate. For example, phorbol esters cause leukemia cells, which are immature white blood cells, to differentiate into mature white cells or macrophages. On the other hand, they inhibit the differentiation of certain melanoma cells.

Phorbol esters give cells a signal but how that signal is interpreted depends very much on the particular kind of cell. The question is, What is that signal?

It now appears that the signal may be the phosphorylation of cellular proteins. This is a well known way for cells to control growth and differentiation, and phosphorylation seems to be the way certain tumor viruses exert their effects. But the phorbol ester system is different from any other yet studied and the hope is that it may lead researchers to a naturally occurring cellular control system that happens to be activated by these compounds. The phorbol ester story is now beginning to come together.

The story began a few years ago when Blumberg, then at Harvard Medical School, and his associates decided to focus their efforts on the initial steps of tumor promotion in the hopes of eventually learning enough to block those steps. Since phorbol esters are active in nanomolar concentrations, it had seemed likely that the compounds bind to specific cell surface receptors. Based on their analysis of the relationship between the structure and activity of various phorbol ester derivatives, Blumberg and his colleagues predicted that a phorbol dibutyryl derivative should be best for detecting the putative receptor. They synthesized a radioactively labeled dibutyryl phorbol ester and showed that it bound specifically to both whole cells and isolated cell membranes. The receptor binding required calcium and phosphatidylserine. Receptors, they found, are present in all cells except red blood cells but their highest concentration is in the brain. And the receptors seem to be evolutionarily conserved—they are in all animals from worms to humans.

These properties of the phorbol ester receptor were remarkably similar to properties of an enzyme, called a kinase, that phosphorylates cellular proteins. Yasutomi Nishizuka and his colleagues at Kobe University School of Medicine in Japan discovered and characterized this kinase in the cytoplasm of rat cells. Because it is dependent on calcium as well as phospholipids, the kinase was called calcium phosphatidyl-dependent protein kinase, or c-kinase.

Last year, Monique Castagna of the Institut de Recherches Scientifiques sur le Cancer in Villejuif, France, who had previously spent a summer in Blumberg's lab, collaborated with Nishizuka to test whether the c-kinase and the phorbol ester receptor are related. The researchers found that phorbol esters could replace a requirement for the membrane phospholipid diacylglycerol to directly stimulate the kinase.

The receptors, however, seemed to be membrane-bound, whereas the c-kinase was in the cytoplasm. So, says Blumberg, "we had initially thought the kinase activation was a step or two down the pathway." But when Blumberg's group and, independently, Niedel and G. R. Vandenbark at Duke looked at broken cells and at partially purified kinase, they discovered that the phorbol esters seem to be binding to the kinase.

This led them and others, including Nishizuka, to reason that perhaps the kinase was the receptor. But what was needed were experiments with whole cells. These came when Wayne Anderson, Julie Sando (who is now at the University of Virginia), and their asso-

Croton tiglium

The phorbol esters are obtained from crotin oil, which comes from the seeds of Croton tiglium. *[Picture from an 1886 text on medicinal plants]*

ciates at the NCI, looked at the effects of phorbol esters on mouse thymoma cells. Phorbol esters specifically bind to these cells, whereupon the cells produce a growth factor and proliferate.

When Anderson and Sando treated the thymoma cells with phorbol esters, they noticed a dramatic decrease in c-kinase activity in the cell cytoplasm. "As soon as we added phorbol esters to the cells, as soon as we could measure it—within seconds—there was a dramatic decrease in kinase activity," Anderson recalls. And the abilities of various phorbol ester derivatives to decrease the kinase activity correlated with their abilities to bind to the phorbol ester receptor.

The reason Anderson and Sando saw a decrease in kinase activity following phorbol ester treatment is that they were looking for the enzyme in the cytoplasm. Upon the addition of phorbol esters, however, the enzyme becomes tightly associated with the cell membrane. "It is *really* tight," Anderson remarks. "The only way to get it off is with detergent." He believes that the kinase may be loosely associated with the membrane and that the kinase may be the receptor that the phorbol esters recognize. The esters are lipophilic so they glide easily through the cell membrane. Once through, they clamp onto the c-kinase.

In most cases of receptor binding to hormones or viruses or other compounds, the receptor is on the outside of the cell membrane. The phorbol ester system, where the receptor is on the inner surface of the membrane, then is highly unusual.

Researchers in the field believe that the c-kinase normally is activated by some sort of membrane compound that phorbol esters mimic. Whatever this compound is, it most likely is a key to controlling cell growth and differentiation. Nishizuka notes that the membrane compound diacylglycerol, along with calcium and acidic phospholipids, activate the c-kinase. Since phorbol esters are able to replace the requirement for diacylglycerol, he reasons that phorbol esters may stimulate the effects of this membrane phospholipid.

Another open question is, What is the c-kinase phosphorylating? It is known that the kinase phosphorylates a variety of proteins in vitro and it phosphorylates them on serine residues. And once the c-kinase is associated with the cell membrane, cells are much more susceptible to differentiate or to proliferate—depending on the type of cell. Clearly, there are some important clues here to how cells control their growth.

—GINA KOLATA

Interferon Activity Without the Interferon

In the mid-1970's, Ian Kerr, now at the Imperial Cancer Research Fund in London, observed that double-stranded DNA introduced into cells provokes a sharp inhibition of protein synthesis. Several investigators subsequently found that the DNA activates a nuclease that inhibits protein synthesis by destroying the messenger DNA that codes for the proteins. In 1978, Kerr isolated a low molecular weight substance that initiates this process in cells treated with the naturally occurring antiviral agent interferon. The compound was 5′-*O*-triphosphoryladenylyl(2′→5′)adenylyl(2′→5′)adenosine or 2-5A, an RNA analog in which the nucleosides are linked by a 2′→5′ bond rather than the conventional 3′→5′ bond. It was the first naturally occurring compound found to have this type of linkage.

2-5A is an extremely potent inhibitor of protein synthesis, and investigators hoped it would be a useful antiviral and anticancer agent. Unfortunately, the compound is rapidly degraded by a specific phosphodiesterase, so that its half-life in the cell is only about 20 minutes. The molecule is also very polar and does not pass through the cell membrane, and therefore studies must be conducted by direct injection into the cell or in disrupted cells. At the recent meeting of the American Chemical Society (ACS)* two groups of investigators reported that they have to overcome some of the problems.

Paul F. Torrence and his colleagues at the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases have solved the longevity problem. They reacted the terminal sugar of 2-5A with periodate to give a dialdehyde, which was reacted with hexylamine and reduced to give a 2-5A analog in which the sugar is replaced by a substituted morpholine ring. This analog persists in the cell about 15 times as long as 2-5A and is as active as 2-5A at one-tenth the concentration. It still does not pass readily through the cellular membrane, however, and Torrence is working with longer amines to make the analog more lipophilic.

*185th National Meeting of the American Chemical Society, held 20 to 25 March in Seattle.

Opendra K. Sharma and Biswendu B. Goswami of the AMC Cancer Research Center in Lakewood, Colorado, have prepared analogs of 2-5A in which the 3′-hydroxyls of the sugar moieties are methylated. Sharma reported that these analogs strongly inhibit the growth of vaccinia virus in intact cultured cells without affecting cell replication. Corrado Baglioni of the State University of New York at Albany has shown that these analogs are as effective as 2-5A when injected into cells and that they persist longer. It is still not clear, however, whether the analog added to the culture medium is transported into the interior of the cells or if it is degraded to 3′-*O*-methyladenosine, which also has antiviral activity.

New Agents Active Against Herpesviruses

Herpes has become one of the most feared viral diseases because there is no vaccine to protect against it and no good therapy once it has been contracted. The two best antiviral agents, 9-β-D-arabinofuranosyladenine (known as ara-A or Vira-A) and 9-[(2-hydroxyethoxy)methyl] guanine (acyclovir or Zovirax) have limited effectiveness. Ara-A is too polar to pass through the skin and is not used against herpes. Acyclovir penetrates the skin more readily but it, like ara-A, is easily degraded by an enzyme called adenine deaminase that is found throughout the body. In the cell, both drugs are activated by a viral enzyme, thymidine kinase, which converts them to a triphosphate ester that inhibits a viral DNA polymerase. Viruses that lack this enzyme are resistant to chemotherapy with these drugs.

Several investigators reported at the ACS meeting on new ways to overcome these problems. William M. Shannon of the Southern Research Institute, David Baker of the University of Alabama, and William I. Higuchi of the University of Utah School of Medicine have been working with ara-A that has been esterified at one or more of the sugar hydroxyls. Esterification makes the molecule more lipophilic, so that it passes through the cell membrane more easily, and also makes it more resistant to deamination. Shannon reported that the