EDITORIAL
The Search for Oncogene Targets

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"We are searching for the essence that lies behind the fortuitous."—PAUL KLEE

Many clinicians and naturalists among oncologists look skeptically at the efforts of the school of abstract oncology. In the year Picasso painted the Man With Guitar, without a clearly visible man or guitar, Rous (1) published a paper on the transmission of a chicken sarcoma by a filtrable virus.

Between 1943 and 1952, I was on the faculty of New York University School of Medicine in the Department of Bacteriology. Our chairman, Colin MacLeod, had come from the Rockefeller Institute where he had participated with Avery and McCarty in the revolutionary experiments on gene transfer with pneumococcal DNA. In 1947, MacLeod asked me to organize a 3-month laboratory and lecture course on infectious and tumor-forming viruses. No precedent existed for such a course, but I received advice on the design of laboratory experiments from MacLeod's friends at the Rockefeller Institute—Horsfall, Kidd, Rous, and Shope—and from many other investigators who participated in the course by giving lectures.

Being a naturalist, I was at first skeptical of the significance of transmitted animal tumors as models for human cancer. I was more impressed by the overwhelming evidence for environmental factors documented by the old histories of scrotal cancer in chimney sweeps and the linking of chemicals to bladder cancer, factors since overshadowed by the demonstration that carcinogens are present in tobacco smoke. But it has also become apparent that cancer in humans is a multifactorial disease involving several mutational events that are only partly orchestrated by carcinogens in the environment. The panorama of infectious animal tumors has become a favorite target for molecular biologists, and the advances made in the past 15 years on the role of specific DNA sequences in transformation are truly impressive.

In 1978, a breakthrough occurred in cancer biochemistry (2-4). The oncogene of the Rous sarcoma virus (v-src) responsible for tumorigenicity was found to code for an enzyme, a protein kinase that phosphorylates tyrosine residues of several proteins including itself. From a conceptional and experimental point of view, this transforming v-src oncogene is a most important model. It tells us that one oncogene, one protein kinase, is responsible for the formation of a sarcoma in chickens—a breathtaking picture, even if we cannot as yet recognize the man with either a tumor or a guitar. The naturalists, however, are naturally still skeptical.

Thus, in view of the questionable relevance for man, it is clear that all animals should work on this problem and that we animal-loving creatures should help them. The evidence for natural transmission of tumors in animals is persuasive. Even the evidence for natural transmission in humans is slowly mounting, although we recognize that it is mainly circumstantial.

I cannot avoid staring at the v-src model—a single enzyme causing cancer! How does it do this? Unfortunately, despite a decade of intensive research, we still have no clue to the targets of this protein kinase that change the course of the life of the infected cell. There are several intracellular proteins that become phosphorylated at tyrosine residues following infection and were claimed to be pertinent to transformation, but these suspects had to be released without bail after a short period of "incarceration." They did not withstand the tests of molecular genetics experiments showing that these proteins can be phosphorylated without a resultant transformation or that they are not obligatory for transformation.

The reason for this sad state of affairs lies in the promiscuity of protein kinases. Not every protein phosphorylated at a tyrosine residue deserves a grant. We have shown that protein tyrosine kinases of oncogene products and growth factor receptors phosphorylate a synthetic random polypeptide that contains only two amino acids, glutamate and tyrosine (5). We believe that a tyrosine residue at an appropriate distance from negatively charged glutamic or aspartic acid residues is the signal for attack by these protein tyrosine kinases. Such constellations may be present in the primary sequence of a protein or may be induced by the three-dimensional conformation of the protein. Thus, a small fraction of some innocent bystanders (e.g., lactate dehydrogenase or enolase) become victims of the undiscriminating v-src enzyme. Although studies with low-molecular-weight synthetic peptides have produced evidence that the so-called amino acid consensus sequences determine the suitability of these substrates, the significance of such studies for high-molecular-weight proteins is problematic. For example, the protein encoded by the RAS2 gene in yeast is an excellent substrate for the cAMP-dependent protein kinase, even though it does not have a consensus sequence for this enzyme (6).

How can we recognize the target in the lineup of a multitude of phosphorylated proteins that keep increasing with the improvements in the sensitivity of detection, e.g., by the use of phosphotyrosine-specific antibodies? We have one important clue. The biologically significant target is not likely to

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be an enzyme like lactate dehydrogenase with a narrowly defined function such as the conversion of pyruvate to lactate. The biologic target or targets should be capable of participating in a chain reaction responsible for the pleiotropic responses of the infected cells. Good candidates are other protein kinases, other enzymes causing post-translational epidemics, or structural proteins (e.g., Ca^{2+}-binding proteins) that influence several aspects of cell metabolism.

Research in many laboratories is focused on these metabolic chain reactions, not only those involving oncogenes but those affecting normal physiologic events induced by hormones, neurotransmitters, and growth factors. In many aspects, signal transductions by these agents resemble the activity of oncogene products. The signal-transducing ligands come from outside the cell, whereas the oncogene products act mainly, but not entirely, from inside the cell. Oncogene products are the fifth column in a war for the control of metabolism and growth. Their weapons resemble those of some normal receptors. The first act of many hormones and growth factors is to stimulate protein tyrosine kinase activities. A large fraction of oncogene products are also protein tyrosine kinases; a few catalyze phosphorylation of serine residues. Tumor promoters such as the phorbol esters activate protein kinase C (PK-C), an enzyme specific for serine (threonine). The products of some oncogenes (e.g., members of the ras family) have no kinase activity but induce the excretion of growth factors such as transforming growth factor α or platelet-derived growth factor, which in turn activate protein tyrosine kinases.

There is yet another group of normal genes that code for products participating in phosphorylation events. They have been named protooncogenes because they can be mutated to become oncogenes. Protooncogene is not a very good name. Obviously, these genes are not in the cell to punish tobacco smokers. Evidence points to their function in normal cell growth and differentiation (7). The protooncogene c-src codes for a protein kinase that is also abundant in certain neurons and in platelets, neither of which are capable of growth. Nevertheless, some protooncogenes, such as c-ras, when expressed in excess, render suitable cell lines of questionable "normality" tumorigenic (8). Most oncogenes, however, arise from protooncogenes by alteration in the DNA sequence induced by carcinogens, be it tobacco smoke or sunshine.

Once again, the gene product of the v-src oncogene has yielded important clues. How does it differ from the protein product of the c-src protooncogene? It appears that the difference lies in control of enzyme action. The protooncogene product is restricted in its kinase action by the autophosphorylation of a tyrosine residue, which is absent in the v-src oncogene product (9,10). This marvelously simple and impressive explanation is not likely to be the whole story. Albert Einstein once said, "Scientific explanations should be as simple as possible but not simpler." All protein kinases are controlled either directly or indirectly by numerous factors: directly by the presence of regulatory subunits, inhibitors, activators, and post-translational modifications or indirectly by counterbalancing protein phosphatases that in turn are also under control, sometimes by protein kinases. These various control mechanisms that may influence the v-src protooncogene tyrosine kinase activity are still mainly unknown, nor do we have sufficient information on differences in substrate specificity between c-src and v-src oncogene products.

The major subject of this article is cross talk among protein kinases and among protein phosphatases. Several years ago, I proposed a "bargain" hypothesis (11). It was cheap, probably obvious, and at the time, not very useful. But new methods have made it a valuable working hypothesis. It postulates that the key lesion in cancer is an alteration in the fine-tuning balance among the various phosphorylation and dephosphorylation reactions. As mentioned earlier, oncogene products such as ras p21 do not catalyze phosphorylation reactions, but stimulate phosphorylation-dephosphorylation by secretion of transforming growth factors. The immediate target of the ras oncogene in animal cells is still unknown, but RAS proteins in yeast control the production of cAMP, a kinase activator (12). Protooncogenes such as myc, fos, and jun code for proteins that become phosphorylated and are involved in gene activation.

Examples of cross talk go far back in the history of protein kinase studies. Glycogen phosphorylase activated by phosphorylase kinase was the first example (13); it was followed by phosphorylase kinase kinase (14), and glycogen synthase kinase (15). Glycogen synthase is thus far the most battered enzyme subject to phosphorylation. In vitro, at least 10 different protein kinases phosphorylate this protein (16,17). Some influence the catalytic activity of glycogen synthase directly and some indirectly, by making it a better substrate for a second kinase. Some may just be doing it for fun, expressing their promiscuity. One of my current lectures is entitled: To be or not to be phosphorylated, that is not the question. The promiscuity of protein kinases remains a serious problem, particularly since failure to detect a functional change is always met with the objection: How do we know what happens in vivo? Most of the time we don't. The same question of biologic significance can be raised when we do see a functional change in in vitro experiments. As an aging enzymologist, I have heard all of this before. Enzymes are artifacts, postdoctoral modifications of native catalysts. I have learned to be patient until we have answers, and answers will come as they have come before—from experiments with intact cells with and without the tools of molecular biology.

A few specific examples of cross talk have been studied in my laboratory. These studies have revealed surprising features, e.g., cross talk at the substrate level that causes alterations in the surface charge of a substrate. For example, an acidic protein (such as casein or acetyllipocortin) is activated as a kinase substrate in the presence of spermidine or histone. Such interactions could provide us with new clues to cancer metabolism, e.g., interactions by increased production of polyamines, which are positively charged molecules that influence the activity of some protein kinases.

We have described a protein serine kinase (PK-P) present in plasma membranes of placenta, tumor cells, and yeast (18). This enzyme, which was purified to homogeneity from yeast plasma membranes, is a casein kinase that is greatly stimulated by basic polypeptides such as histones, polylysine, basic myelin protein, and peptides present in plasma mem-
branes. It bears some similarity to a heterogeneous group of enzymes called casein kinase 2. Like casein kinase 2, spermine or spermidine stimulates PK-P, but only with some substrates (casein). Other substrates, such as band 3 of erythrocytes, are phosphorylated by PK-P only in the presence of histone. The enzyme is a hydrophobic protein extracted from plasma membranes with detergents and requires the presence of a detergent for stability. It alters the activity of the protein tyrosine kinase of the epithelial growth factor (EGF) receptor in a complex manner. The EGF-stimulated protein tyrosine kinase activity of the receptor (19) is inhibited by low PK-P activity, stimulated by higher activity, and inhibited by excessive activity. The stimulation of tyrosine kinase by PK-P in the presence of histone or polylysine is seen at two levels—at the enzyme level (increase in autophosphorylation of the receptor) and at the substrate level (phosphorylation of endogenous membranous substrates that do not appear to be phosphorylated in the absence of the basic polypeptides).

These observations led us to propose an activation of substrates similar to earlier formulations on the mode of action of spermine and spermidine in stimulating casein kinase 2 activity (20,21). The specificity of the enzyme is not altered by the activators, but the substrates are altered and rendered susceptible to phosphorylation by interactions with the basic activators. These experiments emphasize the importance of charge distributions in the substrates, which were also observed in our work with synthetic random polypeptides. EGF receptor protein kinase phosphorylates a 35-kilodalton protein that has several aliases. First described by its size (35 kilodaltons) as a primary substrate for EGF receptor protein kinase (22), it belongs to a group of lipid- and Ca$^{2+}$-binding proteins known by names such as lipocortins, calpactins, and calcimedin. Among these proteins are also victims of v-src tyrosine kinase. Lipocortin 1 (22,23), a substrate for EGF receptor kinase, has an isoelectric point of 6.8. We acetylated it with acetic anhydride to yield a protein with an isoelectric point of 4.9, which ceased to be a substrate for EGF receptor protein kinase. Addition of suitable amounts of polylysine restored its capability to serve as the substrate for the EGF receptor tyrosine kinase. Curiously, when lipocortin 1 is exposed to highly purified preparations of the EGF receptor, it becomes phosphorylated at tyrosine residues as well as at threonine (serine) residues. Both reactions were enhanced by addition of EGF. A similar observation with insulin receptor has been recorded (24). These observations help to explain previous observations of intact cells that respond with enhanced serine or threonine phosphorylation after exposure to insulin (25) or EGF (26). There are several protein serine kinases (27) that may contribute to such cascade effects. Stimulation of two different S6 kinases by nerve growth factor receptor and EGF receptor has been described recently (28). Observation of threonine kinase in highly purified EGF receptors purified by affinity columns was surprising. This finding suggests a close physical association between the two protein kinases. Indeed, it raises the possibility that some of the pleiotropic consequences of EGF binding or insulin binding may be caused by a cascade induced by protein threonine and/or serine kinases.

The complexity of cross talk among phosphorylating enzymes is staggering. Protein serine kinases stimulate other serine kinases, protein serine kinases stimulate or inhibit protein tyrosine kinases, protein tyrosine kinases stimulate protein serine kinases, and protein kinases stimulate or inhibit protein phosphatases—an orchestration as inspiring as a symphony by Beethoven and as difficult to comprehend as a symphony by Husa. If the bargain hypothesis is correct, however, we have no choice; we must unravel the individual reactions by resolution and reconstitution of the phosphorylating network section by section.

How are we going to pick up oncogene targets in this cross talk puzzle? Previous approaches have perhaps been too naive. Investigators have compared phosphorylation in normal and transformed cells exposed to pulse labeling with $^{32}$P. This approach ignored our lack of knowledge about the turnover of various phosphoproteins. If a protein phosphorylated by the v-src kinase has a low turnover, we might expect a lower incorporation of $^{32}$P by pulse labeling rather than an enhanced one. In that case, a new search should be started for proteins that are less phosphorylated in transformed cells after pulse labeling, perhaps with the aid of phosphorytrosine antibodies that detect phosphoproteins independently of labeling with $^{32}$P. It is a good approach, but it is limited by our insufficient knowledge about the specificity of these antibodies and their ability to precipitate minute amounts of phosphoproteins. Another novel approach is the use of specific inhibitors for different protein tyrosine kinases (29).

We have recently discovered that many protein kinases have specific protein phosphatase activity (30). In other words, in the reverse direction, they can use water instead of ADP as the phosphoryl acceptor. This vanadate-resistant protein phosphatase activity is low, and the reaction is clearly not of physiologic significance. But with the availability of highly purified protein kinases, it serves as a powerful tool in the specific dephosphorylation of native phosphoproteins. Casein phosphorylated with [γ$^{32}$P]ATP by protein kinase A (PK-A) can be specifically dephosphorylated by PK-A but not by PK-P. Casein phosphorylated by PK-P is not susceptible to dephosphorylation by PK-A. More surprisingly, the synthetic random polymers of glutamate–tyrosine are specifically phosphorylated and dephosphorylated by EGF receptor protein kinase and insulin receptor protein kinase. Thus, the specific charge distribution is recognized by the protein kinase as well as by the phosphatase of the individual receptors. Should this specificity operate among the oncogene tyrosine kinases, the usefulness of this approach to proteins phosphorylated in vivo is obvious. Moreover, a cycle of dephosphorylation by the specific phosphatase activity, followed by rephosphorylation by the same enzyme, allows us to estimate the steady-state concentration of phosphoproteins in vivo, provided that we can avoid nonspecific dephosphorylation of the phosphoproteins during their isolation.

This approach will be of immense value in the analysis.

of the stimulation of DNA synthesis controlled by transcription factors. Some protooncogene products such as jun and fos protein are now recognized to function as transcription factors (31). In some instances, phosphorylation of transcription factors influences DNA binding (32), but thus far, little is known about the protein kinases that phosphorylate transcription factors. We have observed phosphorylation of nuclear proteins by PK-C (33), PK-P, PK-A, and casein kinase 2 (Leister K: unpublished data). A casein kinase 2 phosphorylates topoisomerase 1 (34), and casein kinase 1 phosphorylates RNA polymerase 2 (Dahmus M: personal communication). Once again, we are facing the dilemma of protein kinase promiscuity. Functional assays of transcription factors in well-defined systems of RNA synthesis are still in their infancy, but progress is being made.

Equipped with these new tools of immunology, enzymology, and molecular biology, we should be able to approach the problem of battles of phosphorylation–dephosphorylation and cross talk in the control of growth as influenced by the appearance of a new oncogene protein kinase activity when cells become transformed.

Finally, let me return to the reality of cancer in humans. Abstract oncology with all of its limitations is having a great impact on clinical oncology. For example, the high incidence of the mutated ras oncogene (p21) in human colon cancer (35,36) and pancreatic tumors (37) as well as correlations between several other oncogenes and human cancer are not likely to be coincidental. Our increasing knowledge about animal oncogenes and transforming growth factors will have a great impact on both diagnosis and therapy of human cancer, but we must not forget one aspect of oncology that is likely to be coincidental. Our increasing knowledge about molecular biology, we should be able to approach.

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