CELL SIGNALING
PHOSPHORYLATION, KINASES,
AND KINASE INHIBITORS

Cell signaling is a broad and frequently confusing field. This overview describes some of the exciting new technologies that promise to drive research via more specific, user-friendly assays, reagents, and services, and the development of new inhibitors. By Lynne Lederman

Understanding the signaling pathways, networks, and interactions that cells require to function is a major scientific challenge. Kinases are involved in the many signaling pathways cells use for growth, proliferation, differentiation, and death. Assays to elucidate kinase-related components of signaling pathways may rely on “classic” methods using radioactive phosphates to detect kinase activity (phosphorylation), inhibition, or dephosphorylation, or they may rely on more recently developed fluorescent or colorimetric technologies. Antibodies specific to phosphotyrosine, phosphoserine, and phosphothreonine and to specific target sequences are also useful for assay development. Companies with an interest in cell signaling provide a variety of products that are driving basic research, including enzymes, substrates, inhibitors, antibodies, and off-the-shelf or custom assay kits.

Keeping Track
PhosphoSite is a unique online resource developed at Cell Signaling Technology (CST), to provide information about all in vivo mammalian phosphorylation sites. Funded by both the company and three grants from the US National Institutes of Health (NIH), it identifies over 15,000 unique sites from the literature and over 8,000 previously unpublished sites discovered by scientists at CST and elsewhere. PhosphoSite identifies sites of phosphorylation, and contains extensive information curated from the literature about the regulation and biological consequences of protein phosphorylation. By mid 2007, an enhanced version of PhosphoSite is expected to be launched. Roberto D. Polakiewicz, chief scientific officer, expects that the site will be freely accessible by the academic world, and that there will probably be a subscriber’s fee for other users. “We need the funds to keep adding information,” Polakiewicz says, “so the database won’t become obsolete.”

Huda Shubeita, technical service scientist, EMD Biosciences, an affiliate of Merck KGaA, which includes the Calbiochem brand, notes that a big focus of the company is signal transduction. Calbiochem’s Interactive Pathway Resource provides over 20 interactive signaling pathways, including Akt and PI3 kinases, and mTOR signaling. There are also disease-specific pathways, including ones for diabetes and inflammation. Users select a target within a pathway to obtain lists of available assay kits, antibodies, enzymes, substrates, inhibitors, and other products. “Kinases are involved in every cell signaling pathway you can think of, and, directly or indirectly, in most disease processes,” Shubeita says. “The differential regulation of molecules dictates the characteristics of cells, whether they remain normal or become pathological.”

Ambion, an Applied Biosystems business, has a website where customers will be able to search about 300 pathways for their protein of interest to see what is up- and downstream and whether its activity is associated with the cell

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Antibody-Based Tools in Combination Technologies
Polakiewicz believes Cell Signaling Technology “leads the pack in terms of tools to study signal transduction, having developed [as part of New England Biolabs] the first commercially available antibodies specifically to phosphoproteins in their activation state. More recently,” he says, “we have been developing antibodies capable of detecting phosphorylation in the context of fixed amino acid residues corresponding to kinase recognition motifs, which help to identify and detect multiple novel kinase substrate proteins in a biological sample.” These and other tools like general phosphotyrosine antibodies, Polakiewicz observes, are becoming useful to study the phosphorylation of proteins using CST’s PhosphoScan technology. PhosphoScan can identify hundreds of phosphotyrosine sites in a single analysis. It uses an immunoaffinity step to isolate phosphopeptides from protease-digested cellular or tissue extracts. The peptides are then identified using tandem mass spectrometry. “If you want to globally interrogate the phosphotyrosine proteome in a cell type or tissue in one shot, it’s a robust and powerful method,” Polakiewicz concludes.

According to Roberto Campos-Gonzalez, director, cell signaling research, BD Biosciences, “One of the most exciting recent developments in the phosphorylation field for BD Biosciences is the development of BD Phosflow, a unique way to monitor protein phosphorylation states in kinase signaling pathways.” The technology is based on phospho-specific, fluorochrome-labeled antibodies that are detected using flow cytometry. “It has a unique appeal to the pharmaceutical segment where it could be used to monitor the effectiveness of a compound in clinical trials,” he observes. “In an ideal situation, patients’ blood could be monitored for the drug’s effect on signaling pathways, for example, the Akt or MAP kinase pathways, in specific cell types because flow cytometry allows discrimination between different cell immunophenotypes, such as different subtypes of B or T cells.” Experiments have been done with up to 11 different colors of labels, and Campos believes that this is not the upper limit. “It’s a powerful way to monitor in vivo studies where primary cells are not as abundant as are cell lines used for in vitro studies, and are present in mixed cell-type populations,” he says.

Circumventing the Use of Antibodies
According to Annegret Boge, director for reagent and assay R&D, Molecular Devices, her company’s IMAP TR-FRET (time-resolved fluorescent resonant energy transfer) detection system is helping drive drug discovery in cancer research, among other areas. IMAP uses a high-affinity reagent, trivalent metal complex immobilized on nanoparticles bound via a linker to a terbium (Tb) donor, to bind phosphate. When a fluorescent peptide substrate containing tyrosine, serine, or threonine is phosphorylated by a kinase, the fluorescent phosphopeptide product binds to the IMAP reagent, bringing it closer to the Tb-donor complex, resulting in resonance energy transfer from the donor to the acceptor (peptide substrate) and emission of a stable signal as measurable light. In addition to TR-FRET, IMAP can be performed using fluorescence polarization (FP) readout. This assay circumvents the need for highly specific antibodies as well as the effort and cost required to generate them.

Another advantage of antibody independence is the ability to test lipid kinases and phosphodiesterases. “IMAP fits very well in the cell signaling field. We have a portfolio of over 150 fluorescently labeled substrates for tyrosine, serine, and threonine kinases. Our products are geared toward research where there is pressure to quickly validate a high quality assay for high throughput screening.” Boge says the assay also allows testing the activity of potential kinase inhibitors.

Molecular Devices sells substrates, buffers, and the IMAP binding system primarily to users who “don’t need us to tell them how to design an assay. We provide a modular approach with dedicated support,” Boge notes.

Inhibit and Protect
“A product we’re really excited about for 2007 is PhosSTOP,” says Jeffrey Emch, product manager, Roche Applied Science. “These phosphatase inhibitor cocktail tablets greatly increase convenience, stability, and reliability, allowing users to make a preoptimized inhibitor solution as needed.” PhosSTOP is designed to inhibit the broad spectrum of phosphatases that are released when cells are lysed, and to protect the phosphorylation state of proteins from being altered, which can affect the detection and isolation of functional proteins of interest. This should allow researchers to capture more accurately the phosphorylation state of their protein at a particular moment in time.

At Your Service
Those who don’t have the time, resources, or inclination to perform their own kinase assays can turn to companies who will perform them as a service. Jeff Till, group product manager, drug discovery, Millipore, observes that, as Upstate, the company was the first to sell recombinant kinases, and today kinases are still big targets in cancer therapeutics. Millipore has a portfolio of over 250 recombinant, disease-relevant kinases which form the basis of KinaseProfiler, a direct radiometric kinase inhibitor profiling service for the evaluation of candidate pharmaceutical compounds. Till refers to the assay, which was introduced about five years ago, as the “tried and true gold standard.” The service, performed in Dundee, Scotland, allows clients to avoid the use of radioactive isotopes. Till says, “Most of our clients are looking for kinase inhibitors, primarily in the oncology setting.” The company also has a specificity testing service for PI3-kinases, called PIProfiler. Because PI3 kinase is a lipid kinase, it is not as amenable to direct radiometric-formatted testing as are the protein kinases. PIProfiler uses HTRF (homogeneous time-resolved fluorescence), the company’s copyrighted name for its continued ›
TR-FRET-based technology, for lead identification and evaluation against the human PI3 kinases beta, gamma, and delta.

Biomol International, in partnership with Innova Biosciences, launched SpeedLead-P in July 2005, according to Mark Engleka, technical marketing specialist. SpeedLead-P is a service for selectivity screening against a panel of 19 highly active phosphatases. The turnaround is two weeks, and compounds can be screened against the entire panel or a subset of the enzymes for percent inhibition or IC50. “Our service allows researchers to determine the inhibition profile of a given molecule against a panel of phosphatases without having to clone and express many phosphatases and develop assays in house,” says Engleka. Biomol also has a large collection of assay kits, recombinant kinases and phosphatases, kinase inhibitors, including a kinase inhibitor library, phosphospecific antibodies, and peptide substrates.

Disease-Specific Applications
At Pfizer’s Research Technology Center, the kinase platform has a mandate to identify members of the kinase gene family as drug discovery targets, according to Jessie M. English, director of the Kinase Center of Emphasis. The platform partners with Pfizer scientists, determining where roadblocks in the discovery process may occur, and looking for approaches to address needs within the company. “Pfizer is a drug discovery company,” English says. “Our primary goal is to identify novel, effective, and safe therapeutics for human disease. There is a substantial need to better understand the molecular events underlying efficacy and safety as well as more clearly delineate the implications of inhibiting specific kinase targets. There sometimes is a gap between what is seen in in vitro kinase assays and what happens in cells.”

James Christensen, associate research fellow, PGRD (Pfizer Global Research and Development), is working in the oncology therapeutic area in drug discovery and clinical development. His projects include researching kinase inhibitors in early clinical development. Of interest is determining the subtypes of patients whose disease will be most likely to respond to kinase inhibitors. “We are looking at kinase pharmacogenomics—targeting polymorphisms, gene amplifications, mutations and other molecular profile analyses, and determining their relationship to patient clinical benefit from kinase targeted therapies,” he says. This information could be used to analyze clinical trial results retrospectively to define the molecular profiles of patients whose disease would respond favorably to a treatment; it could eventually be applied as a prospective diagnostic. “Another area of interest is to better define the molecular events downstream of kinase signaling, utilizing genomic and proteomic approaches in nonclinical models to identify biomarkers that are predictive of clinical response, and ultimately to apply the most robust biomarker approaches for clinical use,” Christensen says.

Express and Silence
Another approach to dissecting cell signaling pathways is to use small or short interfering RNA (siRNA) to silence the expression of genes of interest in viable cells. A number of well-known companies offer kits and reagents for kinase-related gene silencing studies relying on RNA interference (RNAi). Ambion Silencer siRNA libraries have three individual siRNAs per target, and include human kinase, phosphatase, and druggable genome libraries, which comprise transcription factors and other sequences that may be of therapeutic relevance. Kathy Latham, Ambion’s senior product manager, RNAi, says the company also makes custom siRNA libraries. “RNAi as a technology has revolutionized how people think of target validation. Instead of finding inhibitors to figure out the role of kinases in a particular biologic event, RNAi allows you to knock down expression if you know the sequence of the gene,” she observes. David Dorris, senior director and general manager, cell biology and RNAi, agrees. “It’s a great tool. Pharmaceutical companies have adopted the technology most quickly, particularly in the kinase field. RNAi can be used to see how specific the mechanism of action of a drug is. RNAi can knock down a target in a signal transduction pathway, and the effects on the pathway up- and downstream of the target can be seen.” As part of Applied Biosystems, the company has field-based scientists worldwide, who provide technical support.

Sigma-Aldrich has design services for customized siRNA oligonucleotide sequences that are analyzed to reduce nontarget binding. It also provides a wide range of kinase and phosphatase reagents and kits as well as the individual enzymes, substrates, activators, and inhibitors. Similarly, Qiagen offers a portfolio of high throughput–tested siRNA sets in addition to providing custom siRNAs and controls. In addition to human and mouse sets against the whole genomes, druggable genes, and phosphatase and phosphatase-associated genes and kinases, Qiagen’s newest products include siRNA against rat kinases, phosphatases, and phosphatase-associated genes. Its new FlexiPlate siRNA system allows custom selection of siRNAs targeting human and mouse genes along with controls, scales, and plate layout for efficient, economical screening with minimum off-target effects and maximal knockdown efficiency.

Driving Forces
As more information accumulates about cell signaling pathways, it will be crucial for researchers to be able to access databases and interactive pathways to see where their kinase of interest fits in the big picture. Combination technologies, too, will continue to drive research and development.

Lynne Lederman, Ph.D., is a freelance medical and science writer based in New York.
New Products

Next-Generation Coulter Counter

The Multisizer 4 Coulter Counter, the latest advancement in a long line of particle counting and sizing instruments, is designed to deliver an increased dynamic range. Its unique digital pulse processing provides size analysis results in real time. Originally developed to count blood cells, the Coulter Counter has a broad range of applications, from cells and bacteria to food and hydraulic fluids. The Multisizer 4 features new sample management technology to ensure reproducibility. The EZAccess fluid management system, software wizards, and automated functions improve ease of use and increase productivity. Software functions include an automated time stamp for real-time sample tracking and electronic blockage detection. The digital pulse processing scans and stores data for additional analyses and reporting.

Beckman Coulter
For information 714-993-8955
www.coultercounter.com

Cell-Based ERK1/2 ELISA

The dual-fluorescence PhosPho-ERK1/2 Cell-Based ELISA (enzyme-linked immunosorbent assay) is the first to measure total and phosphorylated proteins simultaneously in the same well, without the need for specialized equipment. Sample preparation is simple: Cells are grown, permeabilized, and blocked in the same 96-well plate. Primary antibodies against phosphorylated ERK1/2 or total ERK1/2, combined with species-specific horseradish peroxidase-conjugated or alkaline phosphatase-conjugated secondary antibodies and specific fluorogenic substrates allow for measurement using a standard fluorescence plate reader. Normalizing the fluorescence of phosphorylated ERK1/2 to total ERK1/2 makes it easy to account for well-to-well variations such as cell number.

R&D Systems
For information 800-343-7475
www.RnDSystems.com/go/CellBasedELISA

Protection from Phosphatases and Proteases

The combination of PhosSTOP Phosphatase Inhibitor Cocktail Tablets and cOmplete Protease Inhibitor Tablets can protect proteins from both phosphatases and proteases. PhosSTOP is a proprietary blend of phosphatase inhibitors that acts on a broad spectrum of acid and alkaline phosphatases, serine/threonine phosphatases, and tyrosine protein phosphatases. The cOmplete Protease Inhibitor Tablets inhibit serine, cysteine, and metalloprotease activity, and are available in tablets for either 10 ml or 50 ml of lysate, with or without ethylenediaminetetraacetic acid (EDTA). Both products are nontoxic and effective across a wide range of sample materials, including animals, plants, yeast, and bacteria.

Roche Applied Science
For information 317-521-2000
www.roche-diagnostics.us

Multiplexed Phosphorylation Analysis in Single Cells

BD Phosflow is an innovative flow cytometry–based technology that enables activation-state analysis of multiple proteins at single-cell levels. BD Phosflow reagents used in combination with cell surface markers make it possible to study phosphorylation events directly in small subpopulations of complex primary samples. The reagent line includes close to 100 directly conjugated phosphorylation-site-specific antibodies. A proprietary buffer system enables Phosflow analysis directly in whole blood samples. This technology not only boosts cell-signaling network analysis and high content secondary screening for drug discovery and development, but also enables the identification of signaling pathway–dependent biosignatures for disease.

BD Biosciences
For information 877-232-8995
www.bdphosflow.com

Protein Interaction Analysis

Biacore X100 is a system for label-free protein interaction for multiproject life science research laboratories. This general purpose system, designed to be readily accessible to multiple users, includes state-of-the-art software that leads the user from preparation to result within a day. The Biacore X100 enables scientists to draw conclusions based on comprehensive characterization of how proteins interact with other molecules in real time. This single instrument can determine affinity and rate constants, binding specificity, concentration, and thermodynamic parameters of the interaction. It provides deeper understanding of molecular mechanisms and interaction pathways to determine protein functionality and elucidate disease mechanisms.

Biacore/GE Healthcare
For information +44 (0) 79 710 8311
www.biacore.com

Tyrosine Phosphorylation Discovery Tool

The PhosphoScan (P-Tyr-100) Kit features patented technology that enables investigators to identify hundreds of thousands of phosphorylated sequences and observe the state of protein tyrosine phosphorylation in cells and tissues. The PhosphoScan technology was recently used in the discovery of a novel activating mutation in the JAK3 kinase in acute myeloid leukemia cells. The method underlying the kit involves the specific enrichment of phosphotyrosine-containing peptides from protease-digested cell extracts using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 coupled to protein G agarose beads. Phosphopeptides eluted from the beads are subsequently identified by liquid chromatography/tandem mass spectrometry. Typically, several hundred phosphotyrosine sites from one sample can be identified in a single analysis, depending on the sample's phosphorylation level and the sensitivity of the mass spectrometer. Researchers at Cell Signaling Technology have used PhosphoScan to determine cellular phosphorylation profiles in hundreds of cell lines, xenografts, and primary human tumors.

Cell Signaling Technology
For information 978-867-2300
www.cellsignal.com