3.1 Discovery of Signal Transduction Pathways: Current Tools of Chemical Biology and Other New Technologies

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Our laboratory focuses on the development of novel chemically based tools to decipher signal transduction pathways on a genome-wide scale. We have developed a method for producing small molecules that are specific for any protein kinase of interest in a signaling cascade by combining protein design with chemical synthesis. These highly specific inhibitors of individual kinases have been used to identify a number of new principles of signal transduction that have complemented genetic and biochemical studies of cell signaling. Examples where new pathways and new functions can be revealed by small molecule inhibitors of protein kinases will be highlighted. A second area of interest in our laboratory is the tracing of direct kinase substrates. We have designed and synthesized unnatural ATP analogs which are substrates of our engineered kinases but are poorly accepted as substrates of wild-type kinases. This specific nucleotide substrate of any kinase of interest allows for the radiolabelling of the direct substrates of a wide variety of protein kinases including both serine/threonine and tyrosine kinases. New methods for the isolation and identification of low abundance substrates of kinases from cells will be discussed. Once a phosphoprotein substrate of a kinase is identified, the specific phosphorylation site is often difficult to identify using traditional tryptic peptide phosphorylation site mapping. Using a novel strategy based on the design of tailor made proteases which specifically cleave proteins after sites of phosphorylation, we have developed a rapid means to map protein phosphorylation patterns. Finally, a potential link between the unnatural ligands of engineered kinases and a set of plant hormones, the cytokinins, will be discussed in the context of a custom designed database created for the genome wide analysis of protein kinase catalytic domains.

3.2 A Quantitative View of Protein Phosphorylation

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While phosphosite mapping by several techniques including mass spectrometry is now fairly reliable, understanding which phosphorylation sites modulate protein function or are active in a given biological pathway is still a difficult problem. Adding to the complexity of this problem is the fact that phosphorylation-dependent function may not depend on activity at a single site, but rather be dependent upon serial activation of several sites, and that multiple phosphorylation sites on a given protein may control multiple functions. Multisite phosphorylation of individual proteins appears to be quite common, and may be more the rule than the exception. In order to unravel phosphorylation dependent structure-function relationships, a thorough quantitative analysis of the phosphorylation profile of a protein is useful. This talk describes how we are attempting to understand phosphorylation-dependent regulation of protein function by quantitating protein phosphorylation profiles. Three approaches to measuring changes in phosphorylation stoichiometry are used in our laboratory. A phosphosite-specific mass spec Western is used to target specific phosphorylation sites in one or a few proteins. A second strategy uses an LC-MS based phosphopeptide profiling technique to give a quick readout of phosphorylation changes in response to a cellular signal. Data from these experiments are used to initiate results-driven phosphopeptide mapping experiments. Finally, an isotope encoded strategy is used to provide accurate relative quantitation of phosphorylation sites on one or more proteins. Examples of each of these three approaches used to help understand phosphorylation dependent protein function will be presented. The strengths and weaknesses of each of these three approaches will be discussed.

3.3 Gentle Methods for Probing Protein Post-translational Modifications with Mass Spectrometry

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Mass spectrometry is a sensitive and precise method for investigating protein structures, but even the so-called “soft” ionization processes often impart excess energy to the analytes and the methods employed for dissociation frequently cause rupture of the covalent and non-covalent bonds that link together the protein backbone and all or part of its post-translational modifications. Yet these labile groups may be the key elements that regulate the targeting, interactions and/or turnover of the proteins, so it is important that they be preserved in a manner that allows their identities (and heterogeneity) to be fully elucidated and their locations to be precisely determined. We are engaged in the development of methods that “preserve, protect and defend” the molecules of interest until we wish to dissect them step-by-step by means which generate the maximum information while minimizing the sample requirement. Approaches that we are developing include high pressure matrix-assisted laser desorption/ionization Fourier transform MS, with collisional cooling of desorbed ions, a qQq system for introduction of electrosprayed ions to the FTICR cell, and exploitation of various dissociation modes, with our current emphasis being placed on electron dissociation processes. With this instrument, as with our quadrupole orthogonal time-of-flight, ion trap and reflection time-of-flight instruments, we are optimizing ionization and dissociation conditions to study glycosylated, phosphorylated, sulfonated and nitrosylated species, among others, with and without chemical modification prior to mass spectral analysis. The present instruments and a novel instrument now under development, the cryoFTMS, are designed to achieve high sensitivity and will also have the potential for high throughput (although this feature is not the primary requirement in many detailed structural studies). In this lecture, the implementation of these emerging sample-handling and analysis techniques and their utilization to address questions that arise in proteomics, glycomics and glycoproteomics will be illustrated with examples from research projects ongoing in our laboratories.