Correlating Enzyme Activity and Cellular Behavior

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A photoactivatable phosphorylation sensor used in conjunction with isoform specific inhibitors of protein kinase C enables the enzyme's activity to be monitored inside cells at specific time points during mitosis. PKCβ was found to be active before, but not after, nuclear envelope breakdown [1].

Light is a powerful tool for monitoring biological processes in real time and in living cells. Many fluorescent dyes and proteins have been developed to report on a variety of activities within a cell, tissue, or whole organism [2–4]. Despite this fact, conducting experiments to correlate enzyme activity and cellular behavior remains difficult. One of the challenges has been to control the start of the observation period in the cell. Loading a sensor into a cell can take time, and without a well-defined start of the observation period, one cannot accurately measure the kinetics of an intracellular process. Sometimes enzymes cycle between active and inactive states, which obscures the interpretation of the signals from the fluorescent sensor. The enzyme might have been active at an earlier stage, so during the observation period, the sensor would indicate enzymatic activity. The observed signal might not correlate with the current activity of the enzyme. Delivering a fluorescent sensor of enzymatic activity through microinjection or permeabilizing reagents is stressful for cells and can create false responses. The cells require an incubation time to recover from the delivery of the probe.

One solution to these problems is to create a light-activatable sensor that would enable the experimentalist to turn on the sensor at precisely the desired time. Several light-activatable sensors of signaling mechanisms that involve phosphorylation have been developed. Vázquez et al. developed sensor **1** (Figure 1) to study phosphorylation-dependent protein-protein interactions in cell cycle regulation [5]. A peptide recognition element has a phosphorylated serine that is protected by a 2-nitrophenethyl (NPE) protecting group. After irradiation, the phosphoserine in the peptide mediates binding to the enzyme target, and the fluorescent reporter's emission blue shifts and increases in intensity. Sensor **1** could conceivably be used in vivo, but no experiments have been reported. In another example, Yudushkin et al. conducted in vivo Michaelis-Menten kinetics experiments on protein-tyrosine phosphatase 1B (PTP1B), which removes the phosphate from phosphotyrosines on receptor tyrosine kinases (RTK) [6]. The probe, compound **2**, contains a peptide recognition element and a 4,5-dimethoxy-2-nitrobenzyl-protected (DMNB) phosphotyrosine, which prevents dephosphorylation prior to a brief flash of 365 nm light. A FRET acceptor indicates when the sensor is bound to PTB1B, which is engineered with a GFP FRET donor tag. The FRET signal disappears once the tyrosine is dephosphorylated and the probe diffuses out of the PTP1B catalytic site. Good kinetic data on the activity of PTB1 can be obtained because the start and end points of the experiment are well defined. While this is a powerful method, one drawback is that the system requires engineering a protein fluorophore to the enzyme in order to observe the activity. Interference with the enzyme's activity due to the attachment of the large GFP and overexpression of the enzyme might present problems when applied to other systems.

In this issue of *Chemistry & Biology*, Dai et al. [1] describe the use of a sensor for PKC phosphorylation (**3**, Figure 1; [7]) to explore the role of phosphorylation by PKC in mitosis, the complex pathway by which a cell divides itself into two identical daughter

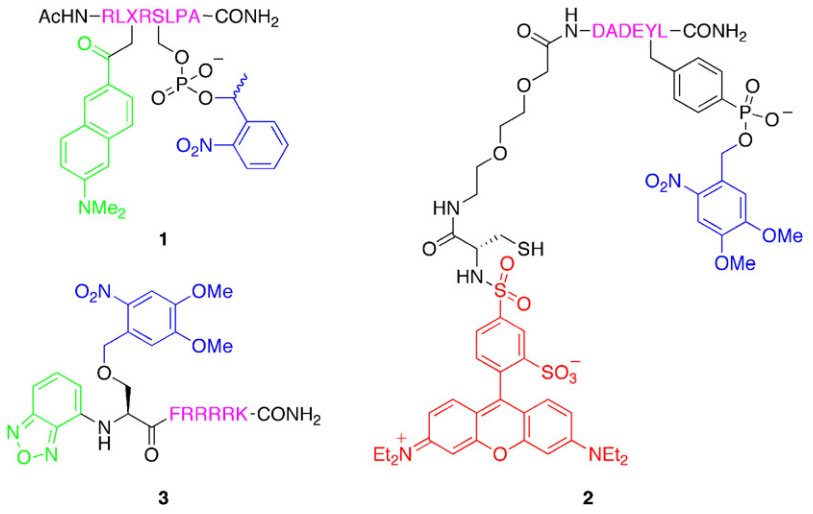

Figure 1. Light Activatable Sensors of Enzyme Activity
Pink = peptide recognition element, blue = photoremovable protecting group, green = fluorescent reporter, and red = FRET acceptor.

Figure 2. Timeline of PKC Activity around Nuclear Envelope Breakdown

cells. The transition between prophase and metaphase involves breakdown of the nuclear envelope (NEB), a group of membranes that provide the boundary for the nucleus. This step is necessary to enable the microtubules of the mitotic spindles access to the chromosomes in order to mediate chromosome segregation to the poles of the cell. Protein kinases are critical to the process [8, 9] especially PKC [10–14], but it is not known when (before, during, or after NEB) PKC is active or what isoform of PKC is responsible for triggering NEB.

To determine the timing of PKC-mediated phosphorylation at the prophase/metaphase junction, the researchers employed sensor **3**, which is comprised of a 6 amino acid peptide that is recognized by PKC, a DMNB-protected serine residue, and a nitrobenzofurazan fluorophore that is sensitive to phosphorylation of the adjacent serine. Prior to exposure to light, the serine on the sensor cannot be phosphorylated by PKC, but after a 1 s irradiation at 355 nm, the sensor can be phosphorylated, which is detected by a change in fluorescence intensity at 560 nm. When the probe is activated in PtK2 cells 5–12 min prior to NEB, large increases in fluorescence intensity result, indicating PKC activity; however, activating the probe after NEB shows no change in fluorescence activity. PKC is active before, but not after, NEB.

In a second set of experiments using isoform-specific inhibitors of PKC [15, 16] in conjunction with **3**, the researchers found that after injection of a PKCα inhibitor at concentrations that also inhibit PKCβ, the indicator did not show any PKC activity and the cells failed to undergo NEB. At low concentrations of the PKCα inhibitor [15, 16], which do not inhibit PKCβ, the indicator shows PKC activity and NEB. The indicator can be phosphorylated by other isoforms of PKC and Nek-2 protein kinase present in PtK2 cells, but using a highly specific PKCβ inhibitor [15, 16] that has no activity against the other isoforms or Nek-2, the cells did not proceed through NEB. Taken together, these results demonstrate that PKCβ is responsible for phosphorylation just prior to NEB (Figure 2). PKCβ is presumably phosphorylating lamins on the nuclear envelope [12–14], but because the probe is not the endogenous target of PKCβ, it does not indicate the target of the kinase.

The use of photoactivated sensors for kinase activity is a powerful tool for correlating cellular behavior and enzymatic activity. One drawback to the technique is that it is introduced into the cells through microinjection. This is stressful to the cells and some do not survive the ordeal, which can negatively impact an experiment. Finding less invasive methods for delivering the sensor- and peptide-based inhibitors would be a welcome improvement. The nitrobenzofurazan is probably not the optimal fluorophore; it is relatively dim and the fluorescence intensity change upon phosphorylation of the adjacent serine is small. Brighter fluorophores with larger changes in fluorescence intensity upon sensor activation would improve the sensitivity. Nevertheless, the probe will prove useful for conducting other in vivo experiments. The combination of the isoform-specific inhibitors and a light-activated sensor of PKC activity should enable researchers to look at other time-dependent PKC activities. One could envision engineering a fluorescent protein to PKC and observing steady state phosphorylation kinetics intracellularly, similar to the method of Yudushkin et al. [6]. Because the specificity of the PKC recognition element on the caged sensor is a peptide, one could imagine replacing it with a peptide sequence that is specific to other kinases of interest and observing their time-dependent activity.

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