signaling in response to the Hh signal. In the absence of Hh ligand, Ptc restrains Smo activity; binding of Hh to Ptc releases Smo, which then transmits the intracellular signal. The regulation of Smo activity therefore occupies a pivotal position in the cellular response to Hh. Previous work had identified small molecules that inhibit or activate Hh signaling by interacting with Smo<sup>6–8</sup>. These include the antagonist cyclopamine as well as further sets of structurally unrelated antagonists and an agonist.

To identify the molecular target of purmorphamine, Sinha and Chen took an elegant chemical genetics approach. Using a reporter assay for Hh-mediated transcriptional activation, they first demonstrated that Ptc is not required for purmorphamine activity. Cell lines derived from knockout mice lacking Ptc have constitutive Hh signaling. This is suppressed by the addition of cyclopamine; however, exposure to both cyclopamine and purmorphamine restored Hh signaling. Moreover, in normal cells, exposure to cyclopamine increased ten-fold the concentration of purmorphamine necessary to elicit an Hh response. Conversely, compared to Sonic Hedgehog (Shh) treatment, purmorphamine significantly increased the dose of cyclopamine required to inhibit Hh signaling. The opposing activities of the two compounds strongly hinted that purmorphamine acted on Smo. Consistent with this, purmorphamine was unable to induce pathway activation in cells lacking Smo, but reintroduction of Smo restored purmorphamine sensitivity. Finally, the authors provided evidence that purmorphamine interacts directly with Smo by demonstrating that even in fixed cells, purmorphamine was able to compete with the binding of a fluorescent derivative of cyclopamine that had previously been shown to interact with Smo<sup>6</sup>. Together, the studies of Sinha and Chen reveal that purmorphamine acts as an Hh signaling agonist by directly targeting Smo. Importantly, purmorphamine is structurally unrelated to the previously identified Smo agonist, thus establishing a new chemical class of Hh agonists.

Despite the importance of Smo as the intracellular initiator of Hh signal transduction, uncertainty surrounds the mechanisms by which Smo activation is regulated and coupled to downstream events. The availability of purmorphamine offers new opportunities to tackle this question. The similarity of Smo to seven-transmembrane receptors raises the possibility that a conformational change is associated with activation; perhaps purmorphamine stabilizes Smo in its active state. But how is this switch in activity normally controlled? A clue comes from the structure of Ptc, which has similarity to the resistance-nodulation division family of permeases, bacterial drug/toxin efflux pumps9. In addition, Ptc has a sterol-sensing domain (SSD), a cholesterolbinding motif that in a related protein regulates the movement of intracellular vesicles. In the absence of Hh, therefore, Ptc may control the distribution of an endogenous small molecule that acts as the natural agonist or antagonist of Smo. Conceivably purmorphamine could mimic the natural agonist or compete with the antagonist. Alternatively Ptc could affect the distribution of Smo such that it is transported into compartments containing endogenous antagonist or lacking agonist. In this connection it is interesting to note that recent findings provide evidence that Hh treatment induces translocation of Smo to cells' primary cilium<sup>10</sup>. Perhaps the cilium contains the endogenous agonist (or lacks the antagonist) responsible for Smo activation; alternatively, translocation may represent the response of Smo to activation, and the cilium is the location from which downstream events of Hh signaling take place. Examining the effect of purmorphamine on the subcellular localization of Smo may provide further insight and begin to fill what is a gaping hole in our knowledge of Hh signal transduction.

In addition to the mechanistic window opened by purmorphamine, recent evidence indicates positive effects from stimulating Hh signaling in disease models that range from terminal neurological conditions to male pattern baldness. The identification of purmorphamine as a new class of small molecules that activate Hh signaling further raises hopes that therapeutic applications can be developed to tackle these diseases.

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# Ion channels get flashy

#### Hagan Bayley

Ion channels have essential roles in the nervous system. An engineered ligand-gated channel with a photoactivated switch will be useful for addressing several issues in neuronal signaling.

Using a skillful combination of chemical synthesis, protein engineering and electrophysiology, a team at the University of California, Berkeley has demonstrated that a ligandgated ion channel can be controlled by light<sup>1</sup>. Remarkably, this has been achieved in living cells. Numerous applications of the work can be envisioned in areas ranging from fundamental neuroscience to nanotechnology.

Ion channels are of crucial importance in most types of cells. In neurons, they play critical roles in signaling by generating action potentials and participating in synaptic transmission. The Berkeley group has been investigating the photochemical control of ion channels important in the neurophysiology of higher organisms<sup>2</sup>. Their first success came with a light-switchable  $K^+$  channel<sup>3</sup>, and now they have turned their attention to GluR6, an ionotropic glutamate receptor (that is, an ion channel that is activated by the neurotransmitter glutamate)<sup>1</sup>.

Light-triggered (caged) proteins that can be turned on, but not off, have been made previously<sup>4</sup>: examples include channels and pores<sup>5,6</sup>. However, examples of engineered proteins that can be switched on and off are

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more rare<sup>4</sup>. In a farsighted endeavor, long before the days of membrane-protein structures and routine site-directed mutagenesis, Lester and coworkers attached a photoisomerizable agonist at a naturally occurring cysteine residue near the ligand-binding site of the nicotinic acetylcholine receptor and demonstrated photomodulation of the ionic current<sup>7</sup>. More recent examples of switchable ion channels using the more versatile targeted modification of genetically engineered polypeptides include the K<sup>+</sup> channel<sup>3</sup> and a mechanosensitive channel, MscL<sup>6</sup>. Few light-triggered or switchable proteins have been used in living cells, and in most cases these have been microinjected soluble proteins. For example, the action of cofilin on actin polymerization in tumor cells in culture was investigated with a caged derivative<sup>8</sup>. Dougherty, Lester and colleagues used suppressor tRNA technology to generate light-activated proteins in Xenopus laevis oocytes<sup>9</sup>, but this approach is not readily applicable to multicellular systems. Therefore, the ability to turn ion channels on and off with light in living cells with the generally applicable technology described in this issue is a significant breakthrough.

GluR6 comprises four subunits, each with an extracellular ligand-binding domain (LBD) coupled to a transmembrane domain. When glutamate binds to GluR6, the LBDs undergo a substantial conformational change that is transmitted to the transmembrane domains, which move apart to allow the movement of ions through a central pore. Making use of a three-dimensional structure of the LBD, Volgraf and colleagues designed and, in a multistep synthesis, made MAG, a GluR6 ligand connected through a photoisomerizable azobenzene linker to a cysteine-reactive maleimide group. Some conformational flexibility was built into MAG because of the uncertainties of relying on a single crystal structure for its design. The authors then examined mutants of GluR6 in which cysteine residues had been placed at eleven sites, each at slightly different distances from the mouth of the glutamatebinding cleft. The mutants were expressed individually in HEK293 cells and derivatized in situ with MAG, which is likely to act as an affinity label, thereby accentuating its specificity for GluR6 over endogenous proteins. Three of the modified mutants were activated by near-UV light in an imaging assay that measured Ca<sup>2+</sup> flux into the cells through GluR6.

The most active mutant was subjected to an extensive patch-clamp analysis in HEK cells, demonstrating several key features of the technology. Importantly, excellent control of activity was observed, and repeated stimulation was possible. GluR6 opened when irradiated at 380



**Figure 1** Schematic depiction of the photoactivation of engineered ion channels in cells in culture. Ion channels that can be switched on and off by light have many potential uses. For example, they might aid our understanding of multicellular neuronal networks by facilitating the stimulation of individual cells or combinations of cells. As shown here, scanning optical microscopy might be used to activate specific cells in a network (red) in a defined temporal sequence.

nm, which generates the cis form of the ligand, and closed at 500 nm, which regenerates the trans form. As expected, the response was fast, as azobenzenes isomerize rapidly after the absorption of a photon<sup>4</sup>. Even at high light fluxes, isomerization is unlikely to be the rate determining step in channel opening or closure. Thermal relaxation of the cis form of the azobenzene to the trans form was relatively slow, but the fraction of open channels could be controlled with the wavelength of the incident light because of the differential absorption of the cis and trans forms. Although the engineered channel does not quite open fully at 380 nm, it is fully closed at 500 nm, which is crucial for its use experimental neurobiology. Notably, the light-induced glutamate receptor activity could be tracked in many cells at once by using  $Ca^{2+}$  imaging.

What of the future? Ion channels that respond to light are expected to have applications in both basic science and technology. Opportunities in fundamental neuroscience might arise in several areas. Studies of the biophysics of ion channels will be aided by rapid synchronized activation. In addition, the electrical properties of individual cells might be further elucidated by making use of the exquisite control over specific channels. For example, there are about 75 voltage-gated K<sup>+</sup> channel genes in humans; it would be useful to know the role of each in various excitable cells. Activation might be pinpointed with micron resolution at defined areas of the cell membrane, as demonstrated for cofilin<sup>8</sup>. Photoswitchable ion channels will also be useful in understanding multicellular neuronal networks generated in tissue culture or dissected from transgenic animals. Here, investigation of the effects of

stimulating individual cells or combinations of cells would be greatly facilitated (Fig. 1). Such studies might be extended to tissues and whole animals; for example, zebrafish embryos would be especially amenable to manipulation. These studies of more complex systems will affect areas as diverse as the control of swimming or learning and memory. Photoswitchable ion channels might be used in conjunction with alternative photochemical techniques, such as the genetically targeted version of chromophoreassisted light inactivation (CALI), which has already been used to inactivate a specific Ca<sup>2+</sup> channel<sup>10</sup>. Interestingly, it is not necessary to express channels with their usual properties in the target cells<sup>3</sup>. Besides the cysteine required for modification, additional mutations might be made that allow subcellular targeting, alter ion selectivity, remove desensitization or inactivation and so on<sup>2</sup>.

Potential applications in nanotechnology are far more speculative. Nanodevices of the future might be expected to move, respond to their environment, take cargo on board and deliver it and perform computations<sup>11</sup>. Channels and pores that respond to light, which is in many ways the ideal external stimulus, could take part in these activities, for example, by controlling energy generation through the use of ion gradients, by allowing molecules to move into or out of devices or by permitting molecules to travel from one compartment to another within a device<sup>6</sup>. As the Berkeley group has suggested, photoswitchable channels might be used to perform simple 'computations' through the integration of multiple environmental inputs. For example, the engineered K<sup>+</sup> channel requires light and membrane depolarization for activation, whereas GluR6

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## Opening the pore hinges on proline

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Neurotransmitter binding to a member of the Cys-loop receptor superfamily elicits opening of an ion channel by inducing a change in the configuration of a prolyl imide bond. Engineering a series of mutant receptors to contain proline analogues with a range of *cis/trans* preferences demonstrates the molecular basis for channel gating.

Among the twenty naturally occurring amino acid building blocks that control protein structure and function, L-proline (pyrrolidine-2carboxylic acid) fills a unique niche. Proline (i) lacks a backbone NH group, (ii) is the only naturally occurring cyclic amino acid, and (iii) perhaps most importantly, it readily permits formation of the cis imide bond. The energy difference between the cis and trans geometries of the Xaa-proline imide bond (where Xaa is any amino acid) is significantly less than for corresponding non-proline containing peptide bonds. The proline-containing polypeptide therefore has the ability to populate two discrete conformations. This conformational switch can potentially serve as a precise regulator of biological function in folded proteins. A report by Lummis et al.1 published recently in Nature now provides evidence for a single proline residue that controls opening and closing of a neurotransmitter-gated ion channel.

For decades, model systems have been used to study *cis/trans* prolyl isomerization and the role of prolyl isomerization as a rate-limiting step in protein folding<sup>2–5</sup>. As illustrated in the work of Lummis *et al.*, prolyl isomerization is now beginning to emerge as a dynamic switch within already-folded, functional polypeptides. So far, only a small number of functional proline switches have been described, perhaps owing to difficulties associated with detection of prolyl isomerization in folded proteins. There are no specific reagents to reveal conformationally heterogeneous prolines in folded Closed Open

**Figure 1** The small conformational change induced by *trans* to *cis* prolyl isomerization at a hinge position in the transmembrane region of  $5\text{-HT}_3$  is accompanied by reorientation of a helix (represented by the door) that opens the ion channel pore. Lummis *et al.* provide compelling support for this model by generating mutant  $5\text{-HT}_3$  receptors that contain a series of nonnatural proline analogues that differ with respect to their intrinsic *cis/trans* preferences.

proteins. Moreover, the source of most protein structure information, X-ray crystallography, is likely to mask the dynamic exchange event by stabilizing one conformer at the expense of the other. In contrast, NMR spectroscopy is well suited for observation of prolineinduced conformational heterogeneity but is a technique for which only a limited number of protein systems are tractable. In the absence of direct detection methods, clues to the functional importance of specific proline residues are often provided by mutational approaches and sequence conservation. Lummis *et al.* have capitalized on precisely this latter type of information in elucidating the mechanism by which ion-channel gating occurs for the 5-hydroxytryptamine type 3 (5-HT<sub>3</sub>) receptor. A conserved and functionally essential proline residue (Pro8\*) is located at the top of the transmembrane domain of the 5-HT<sub>3</sub> receptor. Lummis *et al.* generated a series of mutant 5-HT<sub>3</sub> receptors that each contain a nonnatural analogue of proline at position 8\*. Receptor activation correlated with the propensity of each proline analog to adopt the *cis* imide bond. Receptors contain-

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