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Invited Paper

Deconstructing and constructing innate immune functions using molecular sensors and actuators

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Abstract

White blood cells such as neutrophils and macrophages are made competent for chemotaxis and phagocytosis – the dynamic cellular behaviors that are hallmarks of their innate immune functions – by the reorganization of complex biological circuits during differentiation. Conventional loss-of-function approaches have revealed that more than 100 genes participate in these cellular functions, and we have begun to understand the intricate signaling circuits that are built up from these gene products. We now appreciate: (1) that these circuits come in a variety of flavors – so that we can make a distinction between genetic circuits, metabolic circuits and signaling circuits; and (2) that they are usually so complex that the assumption of multiple feedback loops, as well as that of crosstalk between seemingly independent pathways, is now routine. It has not escaped our notice, however, that just as physicists and electrical engineers have long been able to disentangle complex electric circuits simply by repetitive cycles of probing and measuring electric currents using a voltmeter, we might similarly be able to dissect these intricate biological circuits by incorporating equivalent approaches in the fields of cell biology and bioengineering. Existing techniques in biology for probing individual circuit components are unfortunately lacking, so that the overarching goal of drawing an exact circuit diagram for the whole cell – complete with kinetic parameters for connections between individual circuit components – is not yet in near sight. My laboratory and others have thus begun the development of a new series of molecular tools that can measurably investigate the circuit connectivity inside living cells, as if we were doing so on a silicon board. In these proceedings, I will introduce some of these techniques, provide examples of their implementation, and offer a perspective on directions moving forward.

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Introduction

Chemotaxis involves the cellular detection of a minimal concentration gradient that has been established by a chemical outside the cell, followed by cellular migration in an appropriate direction based on this nominal information. It is a multistep process that progresses sequentially through: (1) anteroposterior polarization of the cell, (2) pseudopod formation at the cellular front, and (3) contraction of the cellular rear. The first step of polarization is the means by which a non-polar cell is induced, by a concentration gradient of chemical substances in the environment(1), to accumulate a series of signaling molecules (Rac, PI3K, RhoA, PTEN, etc.) on one of its sides. These molecules in their turn promote the polymerization-depolymerization of actin to enable the cell to form a pseudopod (provisional foot), which provides the driving force for the cell's forward motion. Finally, a contraction of the rear of the cell completes the chemotaxis process. Importantly, chemotaxis plays an important role in various physiological phenomena such as angiogenesis, neural circuit formation, immune reaction, wound healing and embryonic development; breakdown of this cellular process not only causes discrete physiological abnormalities, but also plays an active role in the development of diseases such as cancers and arthritis.

Neutrophils are somatic cells that have been widely used as a model system to study chemotaxis. They migrate faster than any other somatic cell type in humans. When an individual is infected by bacteria, neutrophils can quickly detect a concentration gradient of chemical substances that surrounds the bacterial cells from which they are released. Taking advantage of this property, the host neutrophils can then migrate to the site of infection and prey on the pathogenic targets. Crucial for this function of neutrophils is their ability to detect concentration gradients at a very high sensitivity. In fact, the degree of confidence with which they can distinguish between the presence of 100 molecules on one side of the cell and 99 molecules on the other (i.e., a 1% concentration gradient) is so high that the cell is capable of determining a direction for migration based solely on this information(2). Since the binding of a chemical substance to its receptor at the cell membrane fluctuates by more than 1% at equilibrium, it remains enigmatic exactly how a neutrophil is capable of detecting the signal gradient of a chemical substance, and of rigidly determining the direction of migration when noise is dominant.

From research that has taken the past few decades, a large number of signaling molecules that are involved in the control of cellular chemotaxis have been elucidated. Furthermore, via mathematical modeling, it has been shown that the information processing that forms the basis for cell migration decisions may be described in terms of positive and negative feedback between such signaling molecules. Yet, due to a lack of available experimental techniques, it remains unknown as to precisely how a group of molecules can detect a concentration gradient of chemicals and process this information to actuate migration in an appropriate direction. Since, during polarization and migration, neutrophils process environmental information quickly - on the order of minutes to seconds - it is necessary for an experimental perturbation to be able to control the activity of a candidate signaling molecule on timescales that are at least as fast. Unfortunately, because the time required for perturbations such as gene knockout, RNAi, and protein overexpression to take effect is slower than the speed of cellular signal transduction, these conventional genetic techniques are ill-suited for experimentally measuring the parameters of fast, nonlinear signaling circuits. In order to provide a solution for this problem, we developed a series of molecular actuators which can generate rapid perturbations based on protein-protein interactions, and which can be induced by an external signal such as a chemical. This technique is commonly termed Chemically Inducible Dimerization (CID)(3).

Development of molecular probes

CID is based on the use of a small molecule (rapamycin) which binds and induces the tight association of two distinct proteins (FKBP and FRB). As a basic principle, CID was developed by Stuart Schreiber, Gerald Crabtree, et al., over 20 years ago(4). We expanded and built upon this principle by adding multifaceted improvements, and constructed a technique that can control the activity of a protein of interest at a specific location within a living cell on the order of seconds(5,6). CID utilizes: (1) FRB, which is localized to a specific cellular location, such as the plasma membrane, and (2) a fusion of FKBP to any other protein of interest in the cell. With the addition of rapamycin, the association of FKBP and FRB is induced, and the FKBP fusion probe gets trapped at the cell membrane as a result. Importantly, by the simple addition of a chemical to the cells,

CID probes can mimic the plasma membrane concentration at which intracellular signaling molecules initiate information transfer upon receptor stimulation, as occurs during chemotaxis. Furthermore, since FRB may be targeted to different intracellular locations, CID probes can be directed to different organelles such as mitochondria or Golgi apparatus on the order of seconds(6-8). Since CID is a fast perturbation (<10 seconds) that can be achieved in living cells with highly modular – and therefore generalizable – features, a variety of proteins of interest can be swiftly actuated for function at different cellular locations. For example, by rapidly controlling the activity of low molecular weight G proteins (small GTPases) such as Ras, RhoA, Rac and Cdc42, as well as curvature-inducing domains known as BARs, we were able to show that these signaling molecules change the shape of a cell by directly or indirectly affecting the cytoskeleton(5,9). Also, by instantaneously changing the concentration of cell membrane phospholipids such as PI(4,5)P_2 and PI(3,4,5)P_3, we were able to elucidate the active control mechanism of ion channels(10), and actin polymerization(11).

Notably, we have been able to achieve several other sophisticated experimental outcomes by developing variants of the basic CID system in recent years. For example, we used optically-activated rapamycin so that molecular activity may be controlled only locally within a cell(12). We also established a new CID system which uses two proteins that bind specifically to a new derivative of the plant hormone gibberellin(13), and which is completely independent of the rapamycin-based system. Since these two CID systems function orthogonally, we can now control in the same cell, whether simultaneously or non-simultaneously, the activities of two distinct molecules, each of which can specifically move to a separate cellular location. An additional feature of the gibberellin-based CID system is that, unlike rapamycin, gibberellin does not inhibit mTOR, a homeostatic kinase that controls protein translation. It can thus be used to probe information transfer systems which involve mTOR and which are of great importance, such as those that accompany autophagy.

A feature of the CID system which cannot be overlooked is that, although association of the proteins is on the order of seconds, their dissociation in cells appears to be very slow(7). Although the reason for this is not known with certainty, we believe that two major causes contribute to the slowness of dissociation. One of these is the high

affinity of FKBP and FRB. A crystal structure analysis of the ternary complex revealed that although there is a tight coupling between rapamycin and FRB, direct hydrogen bonding between FKBP and FRB also prevails(14). Despite this, however, a complex of FKBP, rapamycin and FRB dissociates on the order of 10^{-2} sec^{-1}. Given this dissociation rate constant, there should be nearly 99% dissociation in about 10 minutes after rapamycin is withdrawn from the system. However, this was never observed in our hands, even with extensive washing of cells with rapamycin-deficient extracellular media(7). A second reason that contributes to for the slow dissociation of this ternary complex within the cell could be the low clearance of intracellular rapamycin(15). Since rapamycin is highly lipophilic, and likely adsorbs to various places both internal and external to the cell, it might be very difficult to be washed away, especially from within the cell. Further contributing to its low clearance from intracellular locations could be the fact that the cell probably lacks an efficient pump that is capable of ejecting such compounds.

In contrast, since all of the information transfer systems that occur *in vivo* are considered reversible, we realized that a system which allows reversible control of molecular activities would be a highly useful experimental technique, because it could provide a more physiological perturbation. We therefore sought to achieve reversible CID by integrating the two orthogonal CID systems we developed earlier: rapamycin-based and gibberellin-based. The reversible CID effect does not rely on the dissociation of a once-bound complex of FKBP, rapamycin and FRB, but rather moves the entire ternary complex away from the location of interest(7). For example, we regulated the activity of a target protein at the plasma membrane by changing its localization in a stepwise manner: the protein was first made to translocate to the plasma membrane where its activity was desired for a given time period, and then to a secondary location like the mitochondria which served as a way station where protein activity could be sequestered away from the plasma membrane. This principle of inter-organelle relocation was also utilized to minimize the background noise of the CID effect without compromising its dynamic range(8). I refer the reader to a more comprehensive review of the CID system(3).

Cytoskeleton and membrane lipids

The cytoskeleton and inositol phospholipids of the cell membrane are in a very close relationship. Importantly, their interaction is the focal point at which cellular chemotaxis is regulated. There are eight known inositol phospholipids, and their chemical structures are very similar; the difference is only in the number of phosphoric acid substituents with which the lipid is modified, and in the atomic positions at which these modifications take place. Importantly, these eight lipid entities are interconvertible, and an enzyme is responsible for each of the mutually dependent reactions. What this means is that when we vary the amount of any single one of these enzymes using conventional genetic techniques, we inevitably affect the concentrations of various lipids besides just the intended lipid of interest by the time the cell is being observed. Such an experimental constraint poses serious problems in the study of chemotaxis, and other processes that involve these membrane lipids.

Therefore, we sought to target our development of CID probes not just toward genetically encoded proteins, but also toward signaling factors that are not genetically encoded, such as the aforementioned membrane lipids. So far, we have been successful in developing two separate classes of CID probes – synthetic and liberative – each of which can rapidly adjust the membrane concentration of phospholipids such as PI(3,4,5)P_3 and PI(4,5)P_2. Notably, the application of these two probes toward the same phospholipid, PI(4,5)P_2, has provided great insight into the interdependence of biosynthetic reactions that have inositol phospholipids as substrates and products. For development of the first PI(4,5)P_2-targeted CID probe, we took advantage of the fact that the amount of PI(4,5)P_2 may generally be increased by overexpression of PI4P 5-kinase, which synthesizes PI(4,5)P_2 from PI4P. Therefore, when PI4P 5-kinase is incorporated into a membrane-targeted CID probe, membrane concentration of PI(4,5)P_2 may rapidly be increased at any user-defined time point(10). For development of our second, mechanistically distinct, PI(4,5)P_2-targeted CID probe, we leveraged the ability of our CID system to move a protein between two locations in the cell. Whereas during PI(4,5)P2 *in situ* synthesis via kinase, PI(4,5)P_2 concentration is increased while PI4P raw material is simultaneously consumed, our liberation-based PI(4,5)P_2 control tool makes use of a membrane-targeted CID probe to mask the membrane activity of PI(4,5)P_2. Then, at a user-defined time point, this probe is rapidly sent to a secondary location like the

mitochondrion, so that active $PI(4,5)P_2$ may be liberated to the membrane-based signaling platform – all without affecting the amount of PI4P. Excitedly, we obtained an incredible result upon comparing the effects of the two unique perturbation methods on cytoskeletal reconstitution: although both control systems produce the same degree of $PI(4,5)P_2$ enrichment, they each had a different effect on the actin cytoskeleton(11). The *in situ* synthesis method that consumes PI4P raw material produced actin comets, whereas the liberation method that does not affect PI4P levels produced membrane ruffles. Therefore, this study established $PI(4,5)P_2$ as a multitasker whose precise cellular function is determined by an accompanying molecule.

Towards reconstituting cell chemotaxis without chemoattractant

As PI 3-kinase plays a critical role in cell chemotaxis, we first tested if activating PI 3-kinase via CID was sufficient for reproducing directed cell migration. Upon rapamycin addition, neutrophil-like HL60 cells that had been transfected with CID probes became polarized and started to move in one direction(16), just as if they had been stimulated with a chemoattractant such as fMLP. Furthermore, we quantified the amount of $PI(3,4,5)P_3$ (a reaction product of PI 3-kinase which is positively fed back into the biological circuit) that was produced upon rapamycin stimulation in real time, just as if we had introduced a current into an electric circuit and were measuring the output current simultaneously. A subsequent series of experiments demonstrated that the PI 3-kinase positive feedback loop is described by an AND gate in Boolean logic(16), and that H-Ras is a key component of this AND gate(17).

We next attempted to make non-chemotactic cells chemotax along a gradient of synthetic chemical by using a microfluidic device that was engineered to establish an arbitrary concentration gradient of rapamycin along the flow path. When a cell was confined to the flow path, the Rac protein that is important for pseudopod formation was activated and developed an intracellular gradient that was informed by the external rapamycin gradient. Astonishingly, the cell migrated towards the higher concentration of rapamycin as a result of this asymmetric Rac activation(18). Thus, we succeeded in imparting a new feature (chemotaxis) to a functionally inert cell (the HeLa cell, which is not specialized for chemotaxis) via a minimal external perturbation. We hope that more

such systems that can artificially remote-control cellular functions via minimal perturbations will be developed in the future.

A synthetic cell that can chemotax: the next frontier?

The rise and spread of fluorescence imaging has undoubtedly allowed our understanding of intracellular signal transduction events to deepen. We now know, for example, that activation of signal circuitry does not proceed simply in a linear manner. Instead, feedback, crosstalk, and spatiotemporal precision have emerged as crucial features of a higher-order signal transduction network that is highly complex and non-linear. However, the development of perturbation tools that can validate these complex networks has thus far remained a bottleneck. The introduction of technologies such as CID and optogenetics, both of whose development has required the integration of knowledge from a variety of academic fields (organic chemistry, genetics, chemical biology, molecular biology, cell biology, bioengineering, optics, etc.), is already proving essential for the scrutiny of this complex signaling circuitry. Since we were able to identify actin polymerization by a gradient of active Rac as the minimal signal circuitry that is necessary for chemotaxis, we now aim to reconstitute this cellular function in a test tube. As far as we are aware, there are only a few attempts at reconstituting directed cell migration that have been published. In 2008, we reported the CID-based induction of neutrophil migration without receptor stimulation at the cell membrane(16). In 2000 and 2009, the movement of the nerve growth cone was freely manipulated in cells by controlling the IP_3 and calcium concentration in the cell via light(19,20). Three other papers also similarly reported light-guided cell migration using optogenetics tools in 2009 and 2010(21-23). In all of these cases, cell migration was actuated by directly controlling molecular activity in live cells using light or chemical. I refer the reader to our recent review article, which summarizes several studies that aim at reconstituting complex cellular functions(24).

My group now aims to rebuild chemotaxis in a more artificial system such as a giant unilamellar vesicle (GUV), which is essentially a liposome whose size is comparable to that of a cell. GUVs are built from a lipid bilayer, and have been a useful model for reviewing the impact of proteins and lipids on membranes(25); in particular, they are

frequently used to study structural changes in the membrane(26), including our recent work on bacterial proteins(27). Techniques for varying the lipid composition of the artificial membrane have already been established, as have techniques for the introduction of purified proteins into the bilayer or lumen of the lipid vesicle. Thus, we now aim at introducing two functional modules into GUVs: a directional compass and a motility machine. The compass module relies on CID to sense an external chemical gradient and relay this information to the motility module. The motility module in turn consists of actin and its regulators, which provide the force necessary for chemotaxis. Thus, we are in the process of making a cell-like device that can sense, process and actuate a complex cellular function – in this case, chemotaxis.

Other innate immune functions

Another direction in my group is to reconstitute other innate immune functions. After chasing down pathogens, neutrophils and macrophages engulf the target and kill them via a complex cellular process termed phagocytosis. We have recently demonstrated that phagocytosis can be accomplished in phagocytosis-poor cells by the introduction of two molecular modules: one for target recognition by the host cell, and the other for Rac-mediated deformation of the host cell membrane(28). However, it must be noted that innate immune functions are not limited to chemotaxis and phagocytosis alone. Cellular responses that seek to directly damage pathogens can also rely on chemical entities such as perforins, reactive oxygen species and complement proteins. Additionally, proteins like cytokines are crucial for the catalysis of systemic immune responses against the enemy targets. Based on various motivations, these cellular functions are also subject to complex high-order control within the immune system, and are fields of intense study.

Perspectives

Recent advances in genetics, biochemistry and fluorescence imaging have revealed that just a few multitasking signaling molecules can be responsible for many diverse cellular functions. Important such molecules include Rac, Ras, $PI(4,5)P_2$, and $PI(3,4,5)P_3$, to name a few. In order to survive, a cell must select a function that is most appropriate for it to adapt to an ever-changing environment. Undoubtedly, the

combination of these fluctuating environmental factors – which can include temperature, pH, light, and hundreds of chemicals – is often an enormous number. Thus, the cell comprises a sophisticated device that can process a huge array of inputs, using a limited number of elements in a highly confined space, to generate an appropriate output such as directed cell migration. Having said this, the fact that the molecules which transmit and process this complex information are generally only binary switches that can take either of two states – active or inactive – creates an enormous "signaling paradox" which poses a fundamental problem to modern biology. Technologies like CID, which allow biologists to manipulate signaling in live cells at will, are expected to be crucial for solving the signaling paradox.

Furthermore, CID can provide a platform for answering other related questions, such as: (1) how do cells create non-linear feedback signals?; (2) how do they know which response is appropriate in a variety of different environments; and (3) is the signaling style observed during cell migration generalizable to different cellular functions? If not, how many different signaling styles exist? Since chemotaxis is not the only cellular function that occurs with spatiotemporal precision, the same technique may thus be useful for the study of other complex processes such as cell division, cell fusion, and cell transport. CID techniques, and the knowledge they have thus far yielded, will also undoubtedly help greatly in the development of treatment strategies for human conditions such as immune diseases or cancers – especially since both of these are associated with complex cellular processes (like cell migration) that have gone awry.

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