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Review Article

Chemical Approaches to the Investigation of Cellular Systems

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Abstract—Biochemistry in the context of a living cell or organism is complicated by many variables such as supramolecular organization, cytoplasmic viscosity, and substrate heterogeneity. While these variables are easily excluded or avoided in reconstituted systems, they must be dealt with in cellular environments. New developments have allowed researchers to begin probing the inner workings of the cell to gain new insight into cell function and metabolism. Advances in cellular imaging and in small molecule-controlled gene expression, signal transduction and cell surface modification are discussed in this review. These techniques have permitted the study of molecular components within the context of living cells.

Contents

Introduction

For decades, biochemists have been attempting to understand the molecular underpinnings of life by purifying, reconstituting, and studying homogeneous populations of molecules in vitro. These efforts have resulted in a wealth of information about basic biochemical events such as enzyme catalysis and protein–ligand interactions. However, it is becoming apparent that biochemistry studied outside of a cellular environment will not yield a complete understanding of the chemical processes that ultimately contribute to life. In the context of a cell, the biochemical questions of quantity, timing, and location become as important as knowing the molecular targets of every enzyme or receptor. Understanding biochemistry in a cellular environment may also lead to the evolution of novel therapies that are not apparent when one dismisses the

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molecular landscape of the cell in favor of a solitary enzyme or receptor. The examination of biochemical processes within living cells is therefore necessary for both obtaining a basic chemical description of life and reaping the therapeutic rewards associated with such knowledge.

The prospect of monitoring or controlling specific molecular processes within a cell has been made possible by two central and related concepts, chemical orthogonality and chemoselectivity. Introduction of a unique probe into the molecule under investigation allows one to distinguish it from a myriad of related species; fluorescent probes of both chemical and genetic origin have been paramount in this regard. Chemical entities that can react covalently or non-covalently with one cellular component among all others have also been pivotal for probing intracellular processes. Such reagents have been used to monitor biochemical reactions, stimulate signaling cascades or gene expression, and engineer unique reactivity on living cells. The information gained through the use of these tools complements the vast amount of knowledge garnered from traditional biochemical approaches involving in vitro reconstitution. This review focuses on some recent advances in the development of methods for studying biochemistry in living systems.

Cellular Imaging

Understanding how chemical events within a cell are integrated requires real-time observation of molecules inside cells. The method of detection must sense both localization and chemical or physical changes of the molecules involved. Because of its flexibility in implementation, noninvasiveness, and high sensitivity, fluorescence has emerged as the key tool for probing cellular biochemistry.[1]

The flux of important ions such as Ca^{2+} within the cell mediates complex signaling cascades involved in neuronal and muscular functioning. The visualization of these ions in real time through the use of fluorescent calcium-sensitive molecules has provided a better understanding of the roles played by Ca^{2+}. For some applications, however, it is desirable to localize the ion sensor to a specific region or molecule of a cell.[2] For example, Aequorin, which exhibits calcium-dependent fluorescence, has been targeted to gap junctions via the creation of a fusion protein with connexin, a constitutive gap junction protein.[3] Since gap junction proteins mediate the flow of ions such as Ca^{2+} between cells, the Aequorin is situated to bind calcium and change its fluorescence, allowing the imaging of calcium flux between cells.

The ubiquitous use of Aequorin as a calcium sensor is somewhat limited by its inefficient fluorescence emission, cofactor requirement, and the irreversibility of Ca^{2+}-mediated fluorescence modulation. A more generally useful, genetically encoded fluorescence probe, Green Fluorescent Protein (GFP), requires no cofactors and possesses photophysical properties superior to Aequorin.[4–6] More recently, a red fluorescent protein (dsRed), isolated from the coral *discosoma,* has been biochemically characterized and studied as a possible alternative in cellular applications. DsRed possesses many attractive qualities such as resistance to pH changes and photobleaching, but due to its slow maturation and tendency to oligomerize, further work remains before it can be broadly used in cellular applications.[7–9] Mutagenesis of GFP has yielded several variants that have overlapping emission and excitation spectra allowing properly matched variants to function as fluorescence resonance energy transfer (FRET) donor–acceptor pairs. GFP-based Ca^{2+} sensors have been created which consist of two GFP variants that function as a FRET donor/acceptor pair linked together by a spacer consisting of calmodulin and the calmodulin binding peptide M13 (Fig. 1A).[10,11] Calmodulin's binding of calcium results in a conformational change in the fusion protein, bringing the GFP-based donor and acceptor into closer proximity and increasing the FRET observed. These 'cameleons' have been used to monitor changes in calcium concentration associated with cellular signaling pathways; of particular note is a study that probed changes in Ca^{2+} levels during stomatal closure of *Arabidopsis* guard cells.[12,13] Additionally, cameleon proteins were expressed in both neuronal cells and pharyngeal muscle cells of *C. elegans* to detect calcium transients and to study the role of calcium channel proteins.[14]

FRET has also been used to study protein–protein interactions in cellular environments. The FRET acceptor and donor are attached to biological molecules of interest, and when the two molecules interact, FRET is observed. The resultant change in fluorescence provides a real-time indication of both the cellular localization and the extent of interaction. This technique has been successfully applied to the EGF-Grb2 interaction, nuclear receptor and coactivator interactions, and Fas receptor complex formation.[15–17]

Other GFP mutants that exhibit pH-sensitive fluorescence have been utilized to measure the pH of intracellular compartments. Fusion proteins have been constructed between these pH-sensitive GFP mutants and known protein sequences to target specific cellular locations. Once localized in the desired compartment, such fluorescent probes reveal information about the pH of that organelle. Using this technique, the pHs of the medial/*trans*-Golgi, the mitochondrial matrix,[18] and both secretory and synaptic vesicles[19] have been determined. In another study, a cell-permeable, biotin-tagged fluorescein molecule was captured by an avidin chimera that had been targeted to the ER and Golgi compartments of HeLa cells. The fluorescence of the localized, pH-dependent fluorescein dye allowed for a precise pH measurement of those organelles.[20]

In a relatively short period of time, GFP fusions with proteins of interest have become among the most frequently utilized tools in cell biology. However, there are limitations to using GFP-based sensors to monitor aspects of cellular biochemistry, such as a potentially

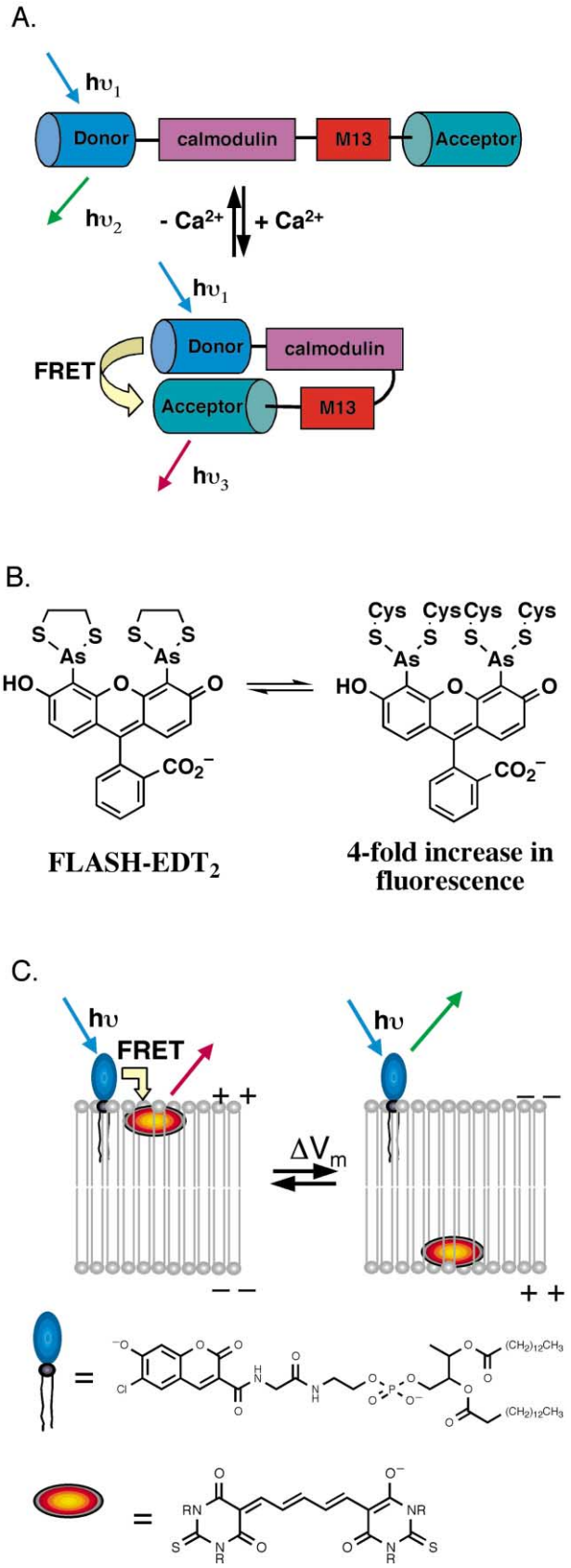

Figure 1. (A) Schematic diagram demonstrating the Ca^{2+}-induced conformational change resulting in increased FRET between acceptor and donor. (B) FLASH-EDT_2 binds selectively to a solvent-exposed tetracysteine motif on a protein of interest. (C) The movement of macromolecules in cell membranes can be monitored by FRET between acceptor and donor.

large perturbation to the protein of interest (GFP consists of 249 amino acids), limited points of attachment, and a small spectral range. Small molecule fluorophores do not suffer from these problems; however, because they are not under genetic control, they lack the possibility for subcellular localization which has proven so effective with GFP. To solve this problem, a clever approach to labeling a specific protein with a synthetic fluorophore inside a cell has been described.[21] The molecule used for this purpose, termed FLASH (Fig. 1B), uses 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein, a cell-permeable, non-fluorescent molecule which binds specifically to recombinant proteins displaying a tetracysteine motif (i, $i+1$, $i+4$, $i+5$). Upon covalent reaction with a target protein, the fluorescence intensity of the molecule increased 4-fold, allowing imaging of the protein of interest. The potential toxicity of the organoarsenate compound was alleviated by using a 10-fold excess of ethanedithiol during the labeling step. This large excess did not affect binding to the target protein, which remained favorable due to the entropic advantage of chelation by the tetracysteine moiety compared to the divalent binding of ethanedithiol.

Small molecule fluorescence has been used effectively in a FRET-based system to image changes in electrical potential across plasma membranes of neurons. A two-component FRET sensor has been designed that utilizes fluorescent phospholipid donors bound on one side of the membrane and acceptor molecules which are sensitive to membrane potential.[22] At normal negative resting potentials, FRET occurs between the phospholipid donor and the oxonol acceptor (Fig. 1C). Upon depolarization, the acceptor electrophoreses across the membrane and a change in fluorescence due to an interruption in FRET is observed. This system improves on other systems of potentiometric fluorescence probes as it is more sensitive and the response time is faster than the duration of the action potential.

Cellular fluorescence imaging has been extended to monitor nucleic acid movements in a cell. Individual chromosomes are not normally visible during interphase in most somatic cells. However, it has been shown that the enzymes involved in DNA biosynthesis tolerate the incorporation of the unnatural fluorescent base Cy5-dUTP. Introduction of this fluorescent base allows for monitoring of the DNA throughout the cell cycle.[23] Using this technique, fluorescent DNA strands have been visualized in real time as they assembled into chromosomes during mitosis.

Caged Signaling Molecules

The ability to unleash the bioactivity of a molecule of interest in a time-resolved, dose-dependent, and spatially localized manner has proven highly useful for intracellular studies. Recent progress towards this goal has involved caging molecules with a photoreactive functional group.[24] The caged molecules diffuse through the plasma membrane into the interior of the cell. Irradiation of the cell with the appropriate wavelength of

light serves to uncage the molecule of interest, allowing a time- and dose-dependent effect of that molecule to be observed. For example, Figure 2A depicts a caged analog of inositol triphosphate (IP_3), an important secondary signaling molecule in cells. The caged compound passes through the cell membrane and accumulates in the cytosol once the ester protecting groups are removed by cytosolic esterases. Irradiation of the cell with UV light removes the photolabile dimethoxynitrobenzyl (DMNB) protecting group at the desired time, releasing a potent analog of IP_3 which stimulates increases the concentration of Ca^{2+} and leads to a measurable increase in gene expression. It was found that a pulsed release of the IP_3 analog, thought to simulate a more native neuronal message, induces a greater level of gene expression than that produced by sustained release during constant illumination.[25]

Given the ubiquitous involvement of Ca^{2+} in biological signaling, caged chelators of calcium have been another important development.[26] While many different photoreleasable calcium cages have been described,[27] most suffer from a lack of sensitivity derived from a relatively modest absorption and a low quantum yield for photolysis. A novel calcium cage termed azid-1, shown in Figure 2B, has been developed based on the calcium indicator fura-2.[28] Upon irradiation with UV light, the aryl azide undergoes decomposition which reduces the binding affinity of the chelator for Ca^{2+}. Using longer wavelengths of light, this cage has been shown to release calcium with complete quantum efficiency. The photoinitiated release of Ca^{2+} was used to simulate normal calcium release in neuronal cells.

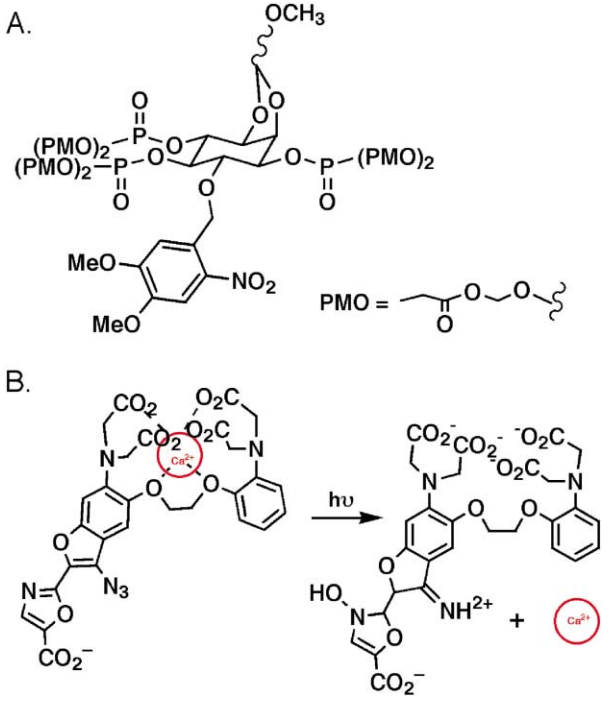

Figure 2. (A) A photo-releasable analogue of IP_3; (B) upon irradiation with UV light, azid-1 releases Ca^{2+}.

Controlling Gene Expression

Since the activities of many gene products are regulated at the level of transcription, small molecule control of gene expression can provide control of protein activity within cells. Such control would allow determination of the effects of temporal activation or repression of a gene product on cellular processes.[29]

A molecule that can recognize and bind to a specific sequence of DNA can prevent transcription (and subsequent translation) of that gene sequence. This has been accomplished by Dervan and coworkers through the use of pyrrole-imidazole polyamides that are designed based on the known specificity and affinity of the pyrrole and imidazole building blocks for specific DNA base pairs.[30] The culmination of this work has resulted in a code which allows one to design a sequence of pyrrole-imidazole polyamides to target any given DNA sequence. This polypyrrole-imidazole oligomer will bind to the chosen DNA sequence and inhibit transcription of that specific gene product. Indeed, the approach has been used successfully to block HIV-1 RNA synthesis in cells, leading to an inhibition of viral replication of greater than 99%.[31] Laemmli and coworkers have used these polyamides in *Drosophila melanogaster* to bind DNA satellites and to cause both gain and loss of function phenotypes due to changes in chromatin accessibility.[32] For an in-depth analysis of DNA recognition by small molecules, please see the recent review by Peter Dervan.[33]

Another approach to controlling gene expression involves small molecule-mediated recruitment of transcriptional machinery to a gene sequence to activate transcription. During the initiation of eukaryotic transcription, sequence-specific DNA binding proteins in collaboration with coactivator proteins bind to transcriptional machinery to transcribe a gene. The introduction of exogenous small molecules that function as transcriptional coactivators in an engineered system has been used to stimulate gene expression in vivo.[34] Verdine and coworkers linked a derivative of the natural product FK506 to a peptide sequence derived from a known transcriptional coactivator (Fig. 3A). A fusion protein consisting of the DNA binding protein GAL4 fused to the FK506 binding protein FKBP12 was expressed in cells containing a plasmid with the GAL4 binding sequence upstream of a reporter gene. Upon administration of the small molecule coactivator, the GAL4 fusion protein bound to the GAL4 binding site on the DNA and recruited the FK506 coactivator, which bound to the transcriptional machinery and resulted in transcription of the reporter gene.

Gene expression has also been altered with small molecules at the level of translation. Many techniques exist for sequestering the mRNA transcribed from DNA via hybridization with a complementary base sequence. Such sequestering molecules can comprise peptide nucleic acids (PNA) or triple helix-forming DNA strands, and have been shown to prevent RNA translation in vitro.[29,35] However, the poor membrane

permeability of these molecules renders them of limited value in a cellular context. Recently, it was shown that translation could be inhibited by aptamer–small molecule complexes.[36] An RNA aptamer that had been selected to bind to the small molecule H33342 was expressed as part of the 5′-untranslated region of an mRNA sequence in Chinese hamster ovary (CHO) cells (Fig. 3B). Translation of the whole sequence was found to be disrupted upon introduction of H33342, which is bound by the aptamer sequence. It was proposed that the system functioned similarly to naturally occurring protein inhibitors of RNA translation by blocking the translational machinery from binding to the start codon.

Protein engineering approaches have also been used in attempts to create orthogonal systems of transcriptional control within a cell. The nuclear membrane retinoid X receptor (RXR) activates transcription upon binding to 9-cis retinoic acid. However, it is possible to increase the specificity of the RXR for various unnatural synthetic ligands (Fig. 4A), while preserving the ability to activate transcription by structure-based mutagenesis of the retinoic acid binding site.[37] By making two mutations to the RXR ligand binding site, two new classes of RXR were created. One exhibited preferential activation by synthetic ligands. The other stimulated transcription only in the presence of the natural ligand. Structure-based engineering of nuclear hormone receptors has been extended to the retinoic acid receptor-γ which is naturally activated by all-*trans* retinoic acid.[38] Koh and co-workers found that by reversing the polarity of the receptor-ligand pair, a mutant nuclear hormone receptor could be created which responded solely to a positively-charged retinal analogue. These results suggest that nuclear hormone receptors are versatile scaffolds for structure-based engineering, and highlight their promise as vehicles for small molecule control of gene expression.

Recently, Rivera and coworkers described a method of controlling the secretion of proteins using an exogenous

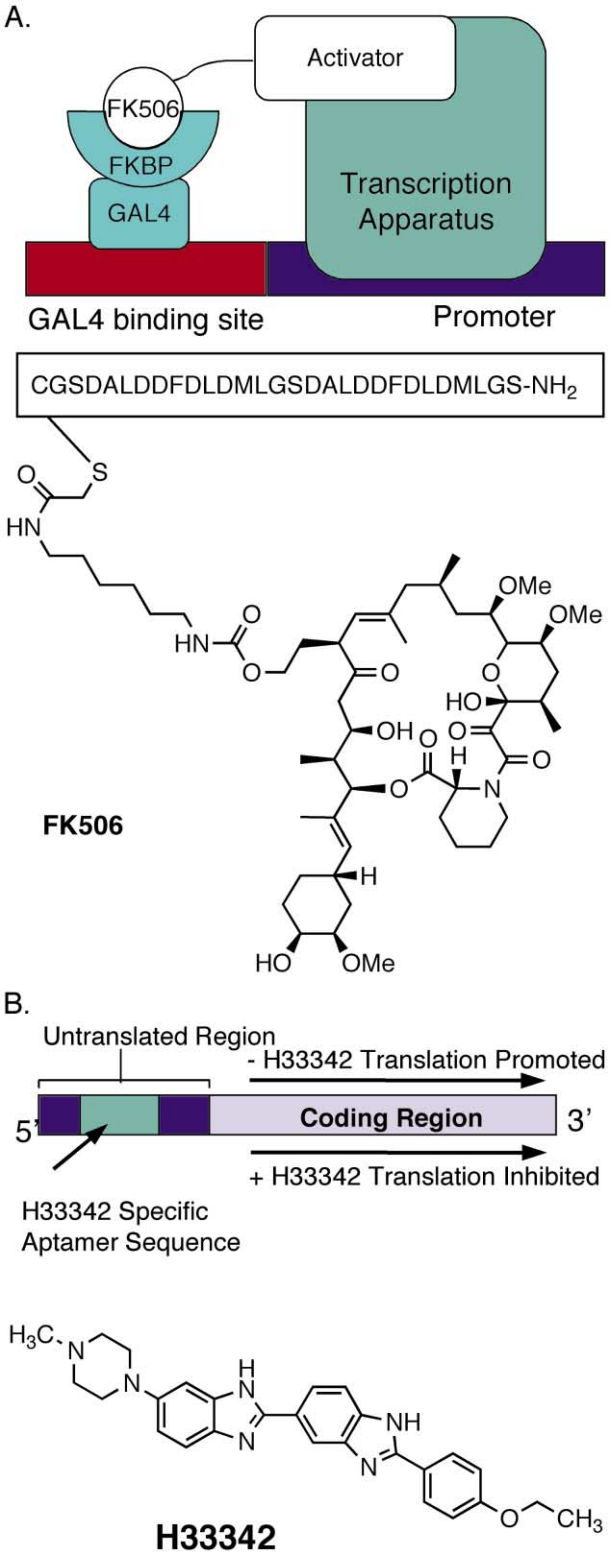

Figure 3. (A) FK506-transcriptional activator binds to the GAL4-FKBP fusion protein to activate transcription. (B) Linking of a specific H33342-binding aptamer to the coding region of a reporter gene allows control of transcription by addition of H3342.

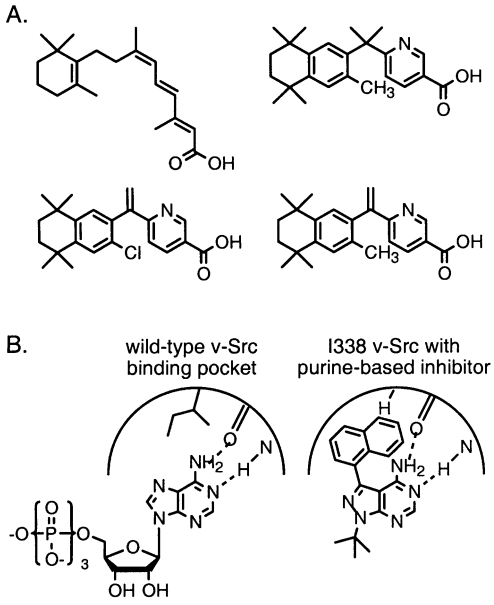

Figure 4. (A) Panel of unnatural retinoic acid derivatives used to activate RXR receptor. (B) Site-specific mutation of kinase active sites allows for use of orthogonal ATP analogues to selectively target the mutant kinases.

small molecule.[39] They designed a fusion protein which contained a conditional aggregation domain (CAD), a furin cleavage sequence, and their protein of interest, either proinsulin or growth hormone. Then expressed in cells, the fusion protein traveled to the ER where it aggregates due to the CAD. However, upon addition of a small molecule that bound to and blocked the CAD, aggregation was disrupted, and furin, a common protease in the *trans*-Golgi, cleaved the fusion protein as it transited the secretory system, resulting in secretion of the free protein of interest. This system was shown to be both robust and versatile. Protein secretion could be triggered multiple times by addition of the specific small molecule, making this system potentially useful for delivery of proteins normally administered or secreted in sporadic doses.

Engineering Signal Transduction Pathways

Deconvolution of signal transduction pathways is an area of very active research. Kinases are an abundant class of enzymes involved in signal transduction that display overlapping substrate specificities and biochemical activities in vitro, leading to confusion as to their individual roles in vivo. An interesting solution to this problem, presented by Shokat and coworkers, involved engineering kinases with unique ATP analogue specifities.[40,41] Based on X-ray structural information, the active site of the desired kinase was mutated to create a cavity or 'hole' in the ATP binding pocket. The mutant had reduced affinity for ATP, but greatly increased affinity for an ATP analogue displaying a complementary appendage, or 'bump', which fit into the engineered cavity. Thus, the kinase and ATP analogue were made orthogonal so that their activity with each other could be monitored in the presence of numerous similar enzymes and substrates. Using this system, determination of the enzyme's protein substrates could be made in vivo.[42]

Inhibitors have also been designed to take advantage of orthogonally mutated kinases (Fig. 4B). These inhibitors specifically target the mutant enzyme, allowing the researchers to study the effects of knocking out that particular kinase activity within cells.[43] For example, fibroblasts transformed with a mutant form of viral Src kinase exhibited a reversion to the non-transformed phenotype upon treatment with an inhibitor specific for the mutant kinase.[44] The generality of this approach was further demonstrated by its successful application in the study of cell-cycle arrest caused by inhibition of a cdc28 mutant kinase sensitive to a specific inhibitor.[45] This method allows for the otherwise daunting task of unraveling kinase signaling pathways in live cells.

An interesting and powerful extension of this 'bump and hole' approach, demonstrated by Schultz and coworkers, is the control of protein–protein interactions that stimulate signal transduction. A cavity was created at the interface of the human growth hormone (hGH) and the hGH receptor by mutagenesis of both components. A library of small molecules was then screened to find binders that complemented that empty pocket. Compounds were identified that could restore the lost affinity caused by the mutations to hGH and its receptor, thus placing hGH signaling under external control by simple addition of a small molecule. This approach could be applied to control many other biological interactions as well.[46]

Signal transduction has also been modulated in cells by targeting the dimerization of cell-surface receptors, an event usually triggered by ligand binding and which often induces subsequent kinase activation pathways.[47–49] The general scheme (Fig. 5) involves fusion of a protein of interest to an immunophilin, a protein that binds to cell permeable immunosuppressive natural products such as rapamycin, FK506, cyclosporin A or their synthetic derivatives.[50,51] By administering synthetic homo- or heterodimers of these natural products to cells transfected with the corresponding fusion proteins, biological effects of homo- and heterodimerization of proteins of interest in the cytosol or plasma membrane can be investigated. For example, these chemical inducers of dimerization (CIDs) have been used elucidate the role of oligomerization in tumor derived growth factor-β (TGF-β)-mediated signaling.[52] It was shown that

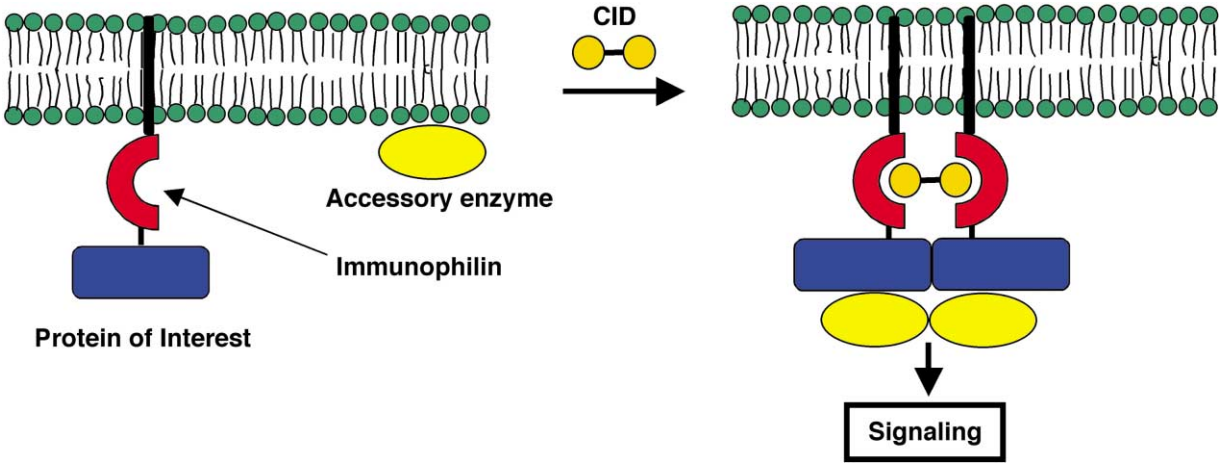

Figure 5. Introduction of chemical inducers of dimerization (CIDs) causes dimerization of proteins, leading to signaling cascades.

homotypic interactions involving the cytoplasmic domain of TGF-β receptor I were sufficient to promote signaling normally associated with TGF-β. This system has proven to be quite general and has been used in many circumstances, such as to probe platelet-derived growth factor signaling pathways.[53]

Unnatural Amino Acid Incorporation into Cellular Proteins

In most eukaryotic systems, the chemical functionality observed in proteins is dictated by the 20 naturally occurring amino acid side chains. The incorporation of unnatural amino acids at specific sites of a protein would provide a precise and subtle means for perturbing biological systems within cells.[54] The nonsense suppression technique initially reported by Noren et al.[55] for unnatural amino acid mutagenesis in vitro has since been extended to cellular systems such as the *Xenopus* oocyte.[56,57] Recently, Wang et al. described the engineering of an *Escherichia coli* system that uses an amber nonsense codon to incorporate an unnatural amino acid into recombinant proteins in vivo.[58]

Unnatural amino acid mutagenesis within eukaryotic cells has been applied most extensively to studies of the nicotinic acetylcholine receptor (nAChR). England and coworkers[59] have incorporated the unnatural amino acid (2-nitrophenyl)glycine (Npg) into ion channels expressed in *Xenopus* oocytes. Irradiation of the oocyte with 360 nm light resulted in the specific cleavage of the peptide chain at the site of that amino acid residue (Fig. 6A). The technique was used to examine the functional importance of the backbone conformation of the M2 domain and the extracellular disulfide loop of the α subunit of nAChR.[59,60] More generally, this technique represents a novel approach to site-specific proteolysis of a functionally active multi-subunit protein within a cell.

In another study, *ortho*-nitrobenzyl ethers of tyrosine were incorporated into three different sites within the α subunit of nAChR expressed in *Xenopus* oocytes, rendering the channel non-functional.[61] Irradiation of voltage-clamped oocytes expressing these mutant nAChRs with millisecond pulses of 300–350 nm laser light served to abruptly decage the tyrosine residues (Fig. 6B) within the nAChR. The photochemical decaging of the tyrosine residues led to a time-resolved increase in the conductance of the channel in the presence of acetylcholine. The same technique could also be applied to decaging of residues that are candidates for post-translational modification within intact cells, thereby allowing one to enable such modifications in a termporally-controlled fashion.

In addition to initiating or inhibiting protein function, unnatural amino acid mutagenesis has been used to study physical aspects protein structure and ligand binding. Zhong and coworkers have probed for the presence of a cation–π interaction within the proposed acetylcholine binding region of the α subunit of nAChR through the incorporation of unnatural tryptophan derivatives (Fig. 6C).[62] The acetylcholine binding affinity was found to vary with modifications to Trp149, whereas modifications to three other Trp residues had no effect on ligand binding. In addition, the authors observed an inverse linear correlation between the calculated cation–π binding energy of the fluorinated indoles and the EC_{50} value of each Trp149 mutant, supporting a direct interaction between Trp149 and the quaternary ammonium cation of acetylcholine (Fig. 6D). Further evidence for this interaction was obtained when the authors mutated Trp149 to an unnatural tyrosine derivative bearing a quaternary ammonium group (Fig. 6E), mimicking a permanent cation–π interaction. This mutation resulted in a constitutively active nAChR. The results of this study underscore how specific and subtle chemical perturbations to a protein can be applied to study its properties within a cell.

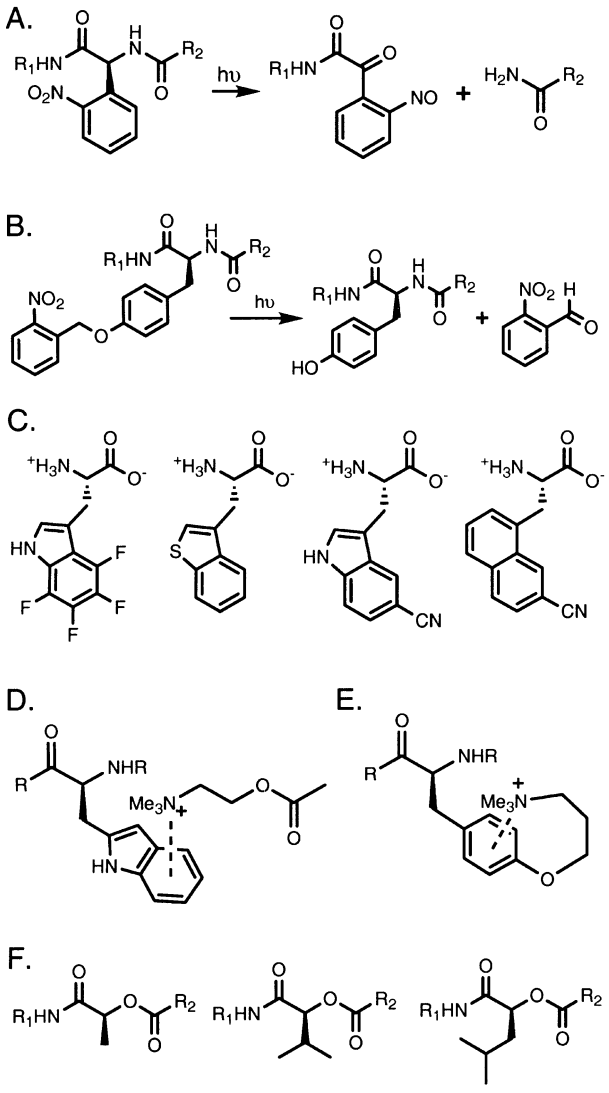

Figure 6. (A) Irradiation of an unnatural amino acid in a protein causes site-specific proteolysis. (B) A caged tyrosine residue can be deprotected with UV irradiation. (C) Unnatural Trp derivatives used to study cation–π interactions. (D) The cation–π interaction of acetylcholine with Trp149. (E) Unnatural Trp149 that produces a constitutively active receptor. (F) α-Hydroxy acids which lack backbone hydrogen bond donors.

In order to probe the structural role of backbone amide hydrogen bonding, England et al. substituted esters in place of amides in the nAChR via the incorporation of α-hydroxy acids (Fig. 6F).[60] Lacking hydrogen bond donor capabilities, these esters disrupt backbone hydrogen bonds at the point of mutation. An α-hydroxy acid residue was substituted for a proline residue which is conserved at position α221 in all ligand-gated ion channels. Since proline also lacks a hydrogen bond donor, the resulting mutant nAChR functioned similarly to the wild-type protein. However, mutation of αPro221 to amino acids such as Gly, Thr, Phe produced mutants that were less sensitive to acetylcholine stimulation. In addition, the conformational dependency of the M2 domain of nAChR was probed with amide to ester mutations, yielding some mutant receptors that were even more sensitive to agonist binding. Based on these results, the authors proposed a model that includes significant conformational changes within the M2 domain that allow gating to occur.

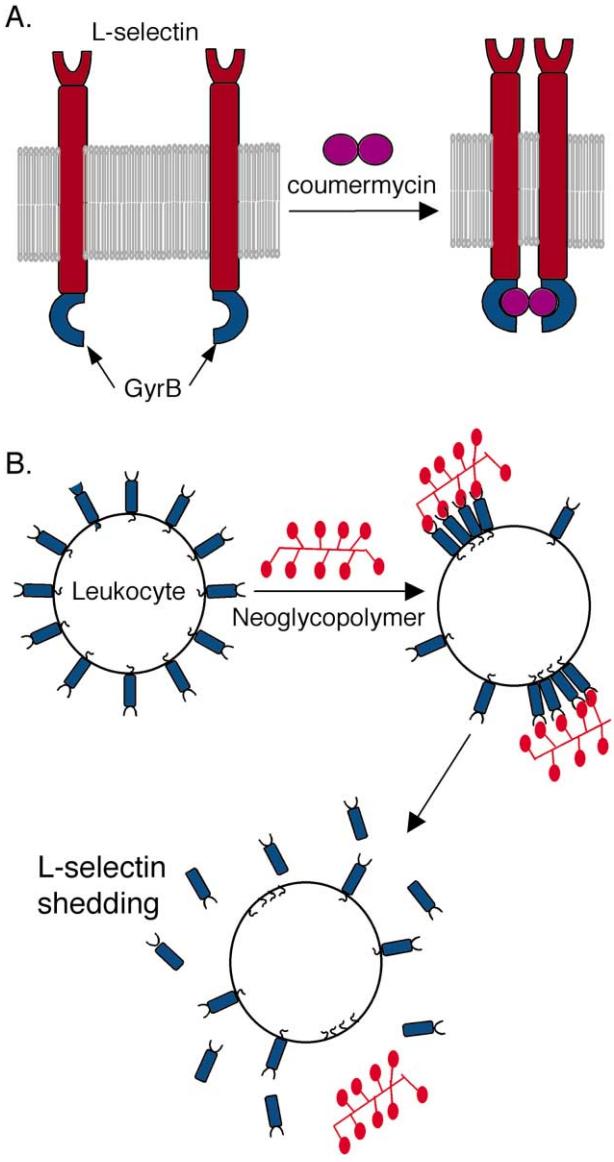

Figure 7. (A) Coumermycin can be used to dimerize GyrB-L-selectin fusions to increase L-selectin avidity. (B) A synthetic neoglycopolymer clusters L-selectin on leukocyte cell surfaces and induces proteolytic L-selectin shedding.

Modulating Cell Surface Architecture Using Chemical Tools

The interactions that take place at the surface of a cell are of critical importance to the cell cycle and to the communication of cells within complex tissues. Molecular alteration of the cell surface would therefore change the presentation of the cell to the outside world and affect processes including cell–cell adhesion and virus–cell interactions.[63] Thus, there is tremendous interest in chemical methods that modulate cell-surface molecules for probing their function in the context of intercellular communication.

L-Selectin is a cell-surface adhesion molecule involved in leukocyte homing and recruitment to inflamed tissues. Its binding affinity for carbohydrate-based ligands on endothelial cells is enhanced following leukocyte activation by chemokines or stimulation through antigen receptors. It has been proposed that the avidity of L-selectin binding is enhanced through clustering of L-selectin through its cytoplasmic domains. To study the effect of dimerization on adhesion, Farrar et al. fused L-selectin at its cytoplasmic tail to the B subunit of bacterial DNA gyrase (GyrB) which binds the dimeric antibiotic coumermycin (Fig. 7A) and had been shown previously to mediate the dimerization of other protein–GyrB fusions within cells.[64] Indeed, treatment of cells expressing the L-selectin–GyrB fusion protein with coumermycin increased the strength of L-selectin-mediated cell adhesion similar to the effects of leukocyte activation. Furthermore, the effect was readily reversed upon treatment of the leukocytes with novobyocin, a monomeric competitor of coumermycin. The authors suggested that L-selectin dimerization could account for increased endothelial–leukocyte interactions observed following naturally induced leukocyte activation. Moreover, this work offers a potentially general method for artificially inducing clustering of cell-surface molecules in order to engender cell adhesion.[65]

L-Selectin-mediated cell adhesion is also thought to be regulated in part through shedding via proteolysis. Kiessling and coworkers designed neoglycopolymers bearing L-selectin binding sugars to probe the effect of clustering on proteolytic release of L-selectin from the leukocyte surfaces (Fig. 7B).[66,67] The polymers comprised approximately 15 sulfated LewisXtrisaccharides designed to mimic the multivalent glycoproteins that bind to L-selectin in vivo. The authors observed a dose-dependent decrease in anti-L-selectin antibody binding to the surfaces of leukocytes treated with the neoglycopolymers. Thus, the presentation of a cell-surface receptor paramount to the inflammatory response can be modulated by exposure to a synthetic oligomer.

The cell surface is also a medium for recognition of foreign bodies by the immune system. It is often desirable to mask the immunological properties of a cell

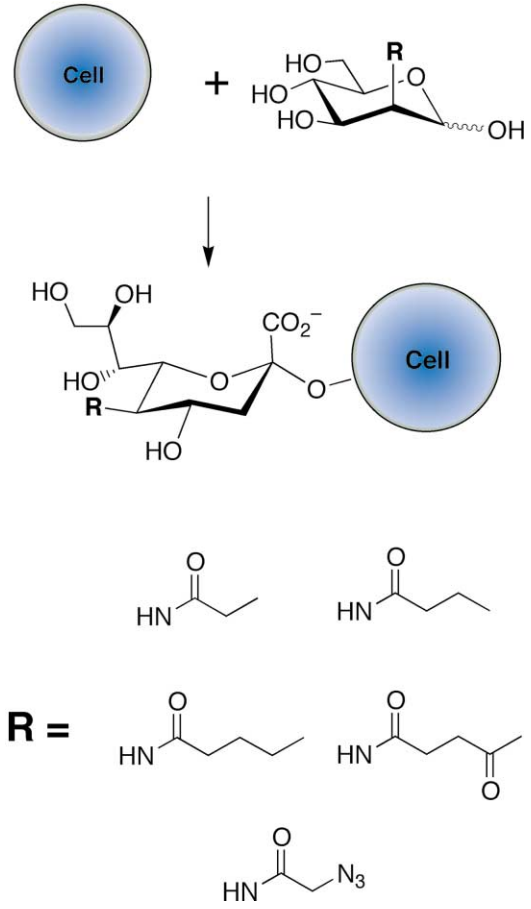

Figure 8. Treatment of cells with unnatural *N*-acyl mannosamines results in the corresponding cell surface *N*-acyl sialic acids.

during such medical procedures as tissue transplantation, the most common of which being red blood cell transfusion. While immune system rejection of transplanted blood can be minimized by cell-surface blood group matching, problems of allosensitization to minor blood groups exist in chronically transfused patients. In such occasions, it would be desirable to transfuse patients with immunologically silent red blood cells. The surface of red blood cells has been modified by covalently coupling methoxy-polyethylene glycol (mPEG) onto cell surface-exposed lysine residues.[68] Scott et al. found that mPEG-derivatized red blood cells exhibited a striking decrease in immunogenicity. They showed both minimal reactivity with anti-A serum as well a dramatic resistance to phagocytic uptake by human peripheral monocytes. Fluorescently-labeled, mPEG-modified human red blood cells injected into mice exhibited circulation lifetimes that were similar to those of endogenous mouse cells. The derivatization of red blood cells with mPEG was found to have very little effect on cell structure or function.[69] The authors suggest that attachment of mPEG to red blood cells may be a viable solution to problem of allosensitization that afflicts chronically transfused patients.

Alteration of cell-surface recognition properties has also been accomplished through metabolic methods. In contrast to many biosynthetic processes, the sialic acid biosynthetic pathway has been found permissive for a number of substrates possessing unnatural *N*-acyl side-chain modifications (Fig. 8).[70,71] By culturing cells in the presence mannosamines containing unnatural *N*-acyl side-chains, cell surfaces can be coated with the corresponding *N*-acyl sialic acids.

Modification of the cell surfaces by incorporation of unnatural sialic acid moieties containing *N*-propanoyl, -butanoyl, or -pentanoyl side chains has been shown to modulate their infection by viruses that utilize sialic acid as a receptor to initiate cellular infection. Treatment of MDCK II cells with unnatural cell-surface sialic acid precursors inhibited influenza A virion binding and infection of the cells.[72] Modeling studies showed that the influenza A haemagglutinin binding pocket, which recognizes *N*-acetyl sialic acid, could not accommodate the lengthened *N*-acyl side chain of the biosynthetically modified derivatives. It was determined that replacement of 18–23% of normal sialosides with the unnatural *N*-butanoyl and -pentanoyl analogues was sufficient to inhibit influenza A infection by up to 80%. Cultured mouse NIH-3T6 cells have also been shown to incorporate unnatural elongated *N*-acyl sialic acids into cell-surface glycoconjugates. The presence of *N*-propanoyl side chains dramatically reduces murine polyoma virus binding and infection, another sialic acid-dependent process.[73]

Metabolic engineering has also been used to specifically target antibodies to tumor cells. Liu and Jennings treated cells with *N*-propanoyl mannosamine, which was metabolized and converted to *N*-propanoyl poly-α2,8-sialic acid, an unnatural variant of a tumor-specific antigen.[74] The cells expressing this unusual motif were then selectively killed by treatment with mAb 13D9, an antibody specific for the unnatural epitope, in the presence of serum complement. This procedure was shown to significantly reduce tumor metastasis in mice.

Interestingly, *N*-butanoyl mannosamine was also found to be a metabolic inhibitor of poly-α2,8-sialic acid biosynthesis in neurons and tumor cells (Fig. 9).[75] Inhibition occurs through incorporation of a few residues of the unnatural sialoside into the polymer, which then terminates further elongation. Thus, the cell surface of poly-α2,8-sialic acid expression can be inhibited reversibly with a small molecule, a power tool for studying the role of the epitope in neuronal development and tumor-related processes.

Schmidt and coworkers have shown that neuronal cells incorporate unnatural sialic acids more broadly into cell-surface glycoconjugates.[76] They discovered that treating neural elements from neonatal rat brain with unnatural sialoside precursors induced the proliferation of certain types of neural cells. Unnatural sialic acid expression also correlated with the expression of specific neural developmental markers in a cell-dependent manner.

Metabolic processes can also be used to incorporate orthogonal, reactive functionality into cell-surface

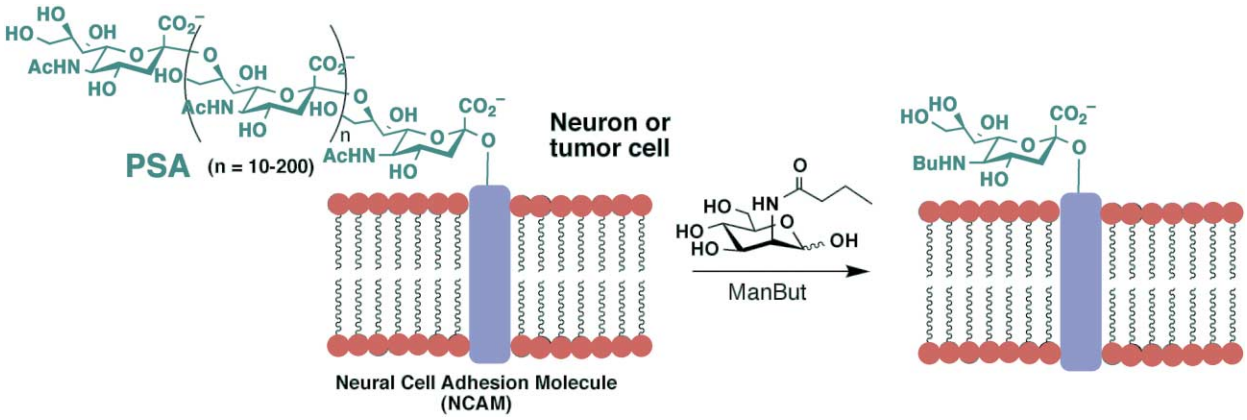

Figure 9. *N*-Butanoylmannosamine (ManBut) inhibits poly-α2,8-sialic acid (PSA) expression by a metabolic mechanism.

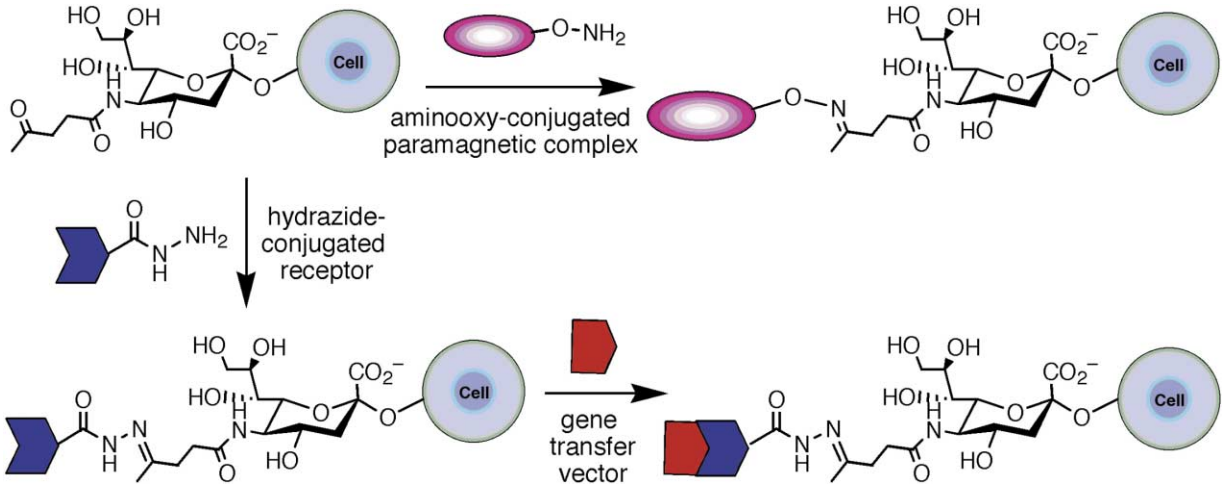

Figure 10. Ketones expressed on cell surfaces can be used to target MRI contrast agents or attach artificial receptors that facilitate adenoviral infection.

glycoproteins. A mannosamine analogue derivatized with a levulinoyl side chain (ManLev) was transformed into the corresponding cell-surface sialoside in human cells.[70] The ketone group is foreign to the cell surface and will readily undergo selective reaction with aminooxy, hydrazide or related functional groups to form stable covalent adducts. As a consequence, cell surfaces can be covalently modified with an epitope of interest generated by chemical synthesis. At least 10^7 ketones on a single cell surface can be achieved, offering significant potential for remodeling cells for specific cell-surface interactions.[77]

An alternative chemistry for chemical cell-surface remodeling is the Staudinger ligation. This reaction occurs between two abiotic functional groups, a specifically derivatized phosphine and an azide, to produce an amide-linked adduct.[71] Azido groups, like the ketone, can be delivered to cell surfaces via carbohydrate metabolism and subsequently reacted with phosphine probes. The reaction is so selective that it may also be performed within a cell's interior, offering the potential for specific protein tagging with fluorescent probes.

Cell-surface sialic acid expression levels often vary as cells transform to a malignant state. It is possible to exploit differences in sialoside expression for the selective targeting of magnetic resonance imaging (MRI) contrast reagents to cell surfaces[78] (Fig. 10). Cells varying in cell-surface sialic acid levels were treated with ManLev, and their differences in ketone numbers reflected their various sialic acid expression levels. Contrast reagents comprising chelates of Gd^{3+} conjugated to an aminooxy group localized more abundantly on cells with the higher sialic acid (and ketone) levels. The ability to image cells on the basis of sialic acid expression levels may have applications in the early diagnosis of cancers.

Metabolically incorporated cell-surface ketones have also been utilized to construct artificial adenoviral receptors on cells that had been previously refractory to gene delivery (Fig. 9).[79] NIH-3T3 cells which lack native adenoviral receptors were grown in the presence of ManLev and derivatized with biotin via reaction with biotin hydrazide. The biotinylated cells were then treated with neutravidin conjugated to an anti-adenovirus

antibody. The artificial receptor was found to mediate a 50-fold increase in infection when the cells were treated with adenovirus.

Conclusions

The chemical details of cellular metabolic pathways and signaling events have been elusive due to the complexities of cells. In the past, most biochemical experiments removed biomolecules from their cellular context, focusing instead on their more accessible in vitro behaviors. However, the advanced highlighted here have made it possible to witness biochemical processes within a cellular context. One can now monitor biomolecules in their native habitat and watch biochemical reactions take place. These advances have further emboldened scientists to design methods for controlling cellular events. Caged molecules or externally administered small molecules are now available to influence, disrupt, or trigger biological processes. The information gained, teamed with the genetic data from sequencing efforts, sets the stage for exciting new discoveries and a deeper understanding of the cell.

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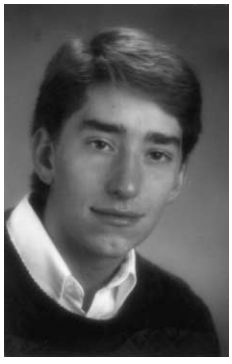

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