Challenges and Approaches for Assay Development of Membrane and Membrane-Associated Proteins in Drug Discovery

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In addition to its role as a barrier between the cytoplasm and the extracellular milieu, the cell membrane is a scaffold for a diverse collection of receptors and enzymes. The organization afforded by this scaffold serves to ensure an efficient interaction between the components of the membrane. The desire to maintain this organization in solution is a challenge for the appropriate interrogation of these biochemical components. This chapter will discuss strategies that allow biochemical analysis of membrane-associated enzymes within standard biochemical reactions. The advantages of these screening strategies in identifying valuable compounds from compound libraries and in understanding the intricacies of complex multiprotein complexes (i.e., chemotaxis) will be discussed.
I. Introduction

A diverse group of transmembrane receptors drive cellular responses to external stimulants via a multitude of signal transduction pathways. Transmembrane signaling is driven by the binding of an extracellular ligand to a transmembrane receptor which then typically activates the receptor, resulting in the recruitment of multiple proteins to the plasma membrane. Subsequent activation of the recruited protein signaling agents leads to the propagation of a multitude of specific signals throughout the cell in order to elicit a specific response to the initial stimulus. This dynamic, yet intricate, network relies primarily on the 2D nature of the plasma membrane that promotes the arrangement of these functional protein assemblies. Complex protein assemblies are not unique to the plasma membrane and have been observed on the ER, nuclear, and the Golgi membranes as well. While this discussion will largely focus on membrane-associated assemblies, we will also address their similarities with signaling complexes formed on protein scaffolds later in the discussion. Other proteins, which may or may not have membrane localization domains (MLDs), can also be involved in the localization of critical signaling proteins. Such adaptor proteins often have multiple protein recognition domains which enable temporal regulation of the recruitment of cytoplasmic proteins via bridging interactions between the activated membrane protein and their cytoplasmic signaling partners. These cytoplasmic partners may be recruited for activation or simply for colocalization with a low-affinity substrate. All-in-all, all organisms use their membranes as conduits to drive all their cell functions—it is the interrogation of these networks using a novel templating technique that will be the focus of this chapter. As will be discussed, while the membrane is an integral part of the signaling networks found in any cell, it is also the major hurdle that needs to be overcome in order to study these networks.

II. Strategies Used by Proteins to Target the Membrane

Proteins have adopted many strategies to establish associations with the cell membrane. The most stable interactions occur by actual transit of the peptide chain through the membrane, and range from conventional single-pass proteins to seven transmembrane protein receptors (7TM). Of more interest to this discussion is the fact that a number of proteins interact with the membrane scaffold via a targeting domain—domains that may serve as interesting drug targets. Such membrane targeting may involve direct interaction with the cell membrane (myristoylation or palmitoylation; Ref. 2) or via other types of targeting domains (see Table 1). Examples of these proteins include the receptor tyrosine kinases (RTKs; single transit proteins; e.g., insulin receptor and Epidermal Growth Factor (EGF) receptor), G-protein coupled receptors (GPCRs; 7TM, cytochrome p450), posttranslationally modified targets which are myristoylated or palmitoylated...
(Src-family kinases), those associated by lipid-binding domains such as the pleckstrin homology domain (BTK), and many others. Many proteins have more than one MLD to ensure colocalization with their respective binding partners.

For many of these proteins (e.g., the 7TM receptors), an association with the cell membrane is required for biochemical activity. Thus, assay methods using membrane fragments or whole cells have been developed to study these targets. For other proteins, molecular biological techniques can be employed to interrogate at least part of the biology of these targets. Thus, many membrane-associated protein kinases are evaluated as protein fragments in which the soluble catalytic domain is retained but the targeting domain is engineered away. This strategy has proved successful in the identification of orthosteric inhibitors that block the interaction with the enzyme’s active site. However, this strategy assumes that the conformation of the catalytic domain is not affected by the targeting domain, a situation that may or may not be true. In our search for strategies to develop better therapeutic compounds, strategies that permit interrogation of the entire kinase protein are desired.

### III. NanoDisc Technology

NanoDisc technology, developed by Sligar and coworkers, allows the researcher to use intact receptors rather than engineered fragments.\textsuperscript{12–14} This method has shown much promise in the study of small numbers of transmembrane proteins and GPCRs, which are a large target class for drug discovery.\textsuperscript{15}
The use of full proteins is the principle advantage in the use of NanoDisc technology; however, the methodology is significantly more laborious than the use of template-directed assembly (TDA) technology (see below) and requires special skills and equipment.\(^\text{16}\) Additionally, the surface area on TDA liposomes allows for dynamic exchange between a large population of receptors, which occurs at the cell surface (and on TDA 2.0), while NanoDiscs only allow for a few complexes to form due to a smaller surface area.\(^\text{12}\) NanoDisc technology (described in Fig. 1) is an outstanding method for the study of GPCR receptors as evidenced by a recent review by Ritchie et al. The authors describe the reconstitution of bacteriorhodopsin, rhodopsin, and other systems by this technique.\(^\text{17}\) Lastly, Morrissey and coworkers employed both TDA 2.0 and NanoDiscs to study tissue factor (TF) complex assemblies and found extremely similar activity and kinetic results with both approaches.\(^\text{18}\)

**IV. TDA Technology**

TDA technology was developed and patented based on work by Weis and coworkers; it was initially brought to market by P.A. Technologies, LLC, and is now under the exclusive license of Blue Sky BioProducts. TDA technology provides a simple method that facilitates the interrogation of membrane-associated proteins and more complex protein assemblies that form at the membrane surface—a common feature among all organisms. TDA makes use of the ubiquitous histidine tag, which has gained popularity in protein purification and structural studies over the last two decades.\(^\text{19–22}\) Briefly, the TDA platform comprises stable liposomes that are decorated with Ni-NTA head groups. When these liposomes are combined with the His-tagged receptor fragments, the proteins reversibly anchor to the surface and the two-dimensional (2D) organization that normally occurs in the cell is imparted to these protein fragments. Proper enzymatic function and the ability of these proteins to form
signaling complexes, attributes of proteins that are normally found at the membrane surface of the cell, can now be observed. Specifically, TDA technology regenerates the special features of the membrane environment, including (i) restriction of motion to two dimensions, (ii) the increased local concentration that accompanies such reduction in dimensionality, and (iii) facilitation of scaffolding interactions that lead to the formation of protein signaling complexes.

While the method is straightforward in concept, there are procedural and engineering concerns which must be addressed in order to obtain the maximum benefit. Chemical considerations include carefully developed buffer systems free from agents such as EDTA and large concentrations of detergents, as these disrupt the Ni-NTA-Ni\(^{2+}\)—protein interactions and destroy protein–protein interactions and membrane structure, respectively. Additionally, the engineering of membrane-associated proteins must be cautiously executed as these proteins are frequently sensitive to changes in their juxtamembrane domains and may need to be engineered specifically to function on the TDA platform. Lastly, protein cytoplasmic domains should be N-terminally His-tagged for proper orientation (C-terminal tag for recombinant ectodomain fragments). Thus, care must be taken when designing proteins and reagents for use in the TDA platform. The end result, however, is a more biologically relevant system where mobile arrays of cytoplasmic domains can interact as they do in the cell. Additional experimental benefits include lower concentrations of metal ions and the fact that researchers do not need to employ aggregating agents to stimulate enzymatic activity. Essentially, the TDA technology combines the ease of using engineered fragments with the ability to effectively interrogate cell-like arrays of receptors and protein complexes that exhibit increased function, activity, and cell-like behavior. See Fig. 2 for a scheme that depicts how this lipid-based nanosphere enables assembly of proteins.

### A. TDA—Simple Solution for RTKs

RTKs have become important targets for drug discovery because of their roles in cancer and other diseases.\(^{23,24}\) One significant challenge that researchers and drug developers face is the involvement of RTKs in multiple, overlapping, and branched signaling pathways, which makes it difficult to identify individual functions.\(^{25–27}\) Furthermore, these proteins are intimately associated with the cell membrane which makes their isolation and study difficult. While practiced throughout the field, the removal of these proteins from the organizing environment of the cell membrane often results in loss of activity and an inability to form complexes with other proteins that are important for proper function. Recent data provides evidence for the formation of large clusters of proteins and suggests that these interactions contribute to the mechanism of activation for many RTKs, such as signal propagation and amplification.\(^{28–31}\) For example, Ichinose and coworkers have demonstrated
that EGF receptors respond to ligand binding by dynamic clustering, which functions to amplify the signal initiated by ligand binding. Assessing cooperative interactions between soluble receptor fragments of this class of proteins has not been possible using conventional enzyme \textit{in vitro} assays. As RTKs and other membrane-associated proteins utilize the membrane as a scaffold to promote protein–protein interactions; these interactions are too weak to occur in solution and this was realized when engineered fragments demonstrated low or no activity in solution without the use of agents that promote close associations, similar to those that can be exhibited on the cell membrane surface. When the motion of molecules is limited to two dimensions, as it is in the TDA environment, or in the presence of the cell membrane, the increased local concentration and orientation promote clustering interactions to a degree that is not observed in solution, even at a comparable bulk concentration.

\textbf{Fig. 2.} Commercially available histidine tagged proteins bind to the template and are free to interact as they do in the cell, because of the fluid nature of the template. Below—the lipids used in early generations of template development.
The TDA platform acts like the cell membrane in reducing the entropy of the system substantially; this reduction in entropy allows weak protein–protein interactions to occur.\textsuperscript{32}

Drug discovery efforts to find specific small molecule inhibitors has focused almost exclusively on inhibitors that target the ATP-binding site, the substrate-binding site, or both; these sites reside in the cytoplasmic tyrosine kinase domain. As mentioned earlier, recombinant kinase domains are easily handled and are amenable to high throughput screening for specific inhibitors of enzyme activity; however, the soluble fragments lack the full function of intact receptors for a large part because they are no longer associated with the membrane and, therefore, do not form the interactions necessary for complete assembly and function. This issue is known industry-wide. Also, it has been noted that the use of soluble fragments frequently produces misleading screening results.\textsuperscript{33} Our own work has demonstrated a difference in substrate specificity with and without the TDA platform. In spite of these concerns, soluble catalytic domains derived from RTKs are still widely used in research and drug discovery. Therefore, methods to improve the functional properties of these protein fragments for research and drug discovery are required.

As a means to reconstitute large portions of signaling pathways, TDA was first brought to practice by Shrout et al. for signaling proteins purified from the bacterial chemotaxis system.\textsuperscript{34–39} TDA has proven effective for assembling the "receptosome" from the bacterial chemotaxis system which consists of four proteins: the cytoplasmic domain from the aspartate receptor (Tar-CD), the histidine kinase, CheA, the adaptor protein, CheW, and the response regulator protein, CheY.\textsuperscript{34,35} Electron microscopic images of the surface-assembled complexes of Tar-CD, CheW, and CheA resemble the receptor arrays that occur in cells.\textsuperscript{35} With the completion of this work, TDA has been successfully used to restore the biochemical activity in a variety of systems discussed in this chapter.

B. Template-Directed Assembly Regenerates Functional Interactions

In the cell, RTKs are confined to two dimensions, where the large local concentration of receptors promotes clustering. The interaction strengths among receptors are “tuned” to this environment such that intact receptors can engage each other and form higher order oligomers that are relevant for signaling.\textsuperscript{30,40} Also, receptor clusters in both prokaryotes and eukaryotes act as scaffolds for adaptor proteins and downstream signaling proteins; these membrane-associated complexes that so assemble can exhibit cooperative and allosteric phenomena. Figure 3 illustrates how TDA facilitates functional interactions among the RTK domains that are not present in the conventional assay format. In the cell, the orientation and spatial arrangement of the kinase domains are
regulated by the membrane (Fig. 3A). For example, regulated interactions among dimers of cytoplasmic domain are necessary for their transphosphorylation and stimulated substrate activity. In some cases, signal enhancement may occur via higher order oligomerization. These interactions, among TK domains, do not occur without the template (Fig. 3B) and interactions with adaptor proteins and downstream signaling elements are similarly ineffective. Figure 3C depicts the patented approach used by Blue Sky Bioproducts to assemble TK domains in an environment that imitates the membrane surface. The TDA technology can also be used to assemble downstream signaling proteins.

We now understand that all membrane-based signaling systems have the same organizing principles regardless of the origin of the cell, so it is plausible to expect that TDA will improve the activity and function of eukaryotic signaling proteins.
V. Lipoparticle Approach

Another approach of interest for studying membrane-associated proteins is the use of retroviral pseudotypes. These virus-like particles (VLPs) consist of a protein core surrounded by cell membrane with intact integral membrane proteins. They are produced by co-expressing a target membrane-associated protein with a retroviral core protein (Gag) in mammalian cells. The viral Gag protein self assembles and buds from the infected cell surrounded by the host lipid-membrane and its integral proteins, within which the transmembrane proteins retain proper orientation. The resulting noninfectious VLPs are ~150 nm in size, are relatively easy to isolate, and the enriched membrane proteins retain their function. VLPs have been utilized in biosensor assays as either attached to the surface or as a soluble component.

A. Retention of Function

VLPs expressing the cellular receptors for the Rous sarcoma virus (Tva) or the murine leukemia virus (MCAT-1) were used to determine whether the expressed viral receptors could target infected cells by presenting the appropriate viral glycoproteins. Tva and MCAT-1 were chosen because they do not require additional cofactors for recognition or subsequent infection. Additionally, Tva and MCAT-1 represent Type I and multipass transmembrane receptors, respectively. Following isolation of the Tva-VLPs and confirmation of Tva expression, the Tva-VLPs were added to chronically infected cells, which expressed the RSV-A envelope glycoprotein (EnvA), and to uninfected cells. In uninfected cells, Tva-VLPs were not incorporated, however, infected cells that expressed EnvA were readily infected by the Tva-VLPs. Similarly, cells transfected with an EnvA vector encoding a functional EnvA were also efficiently infected with the Tva-VLPs. EnvA mutants with known entry defects were not infected or poorly infected in control experiments.

The multipass TM viral receptor MCAT-1 was also isolated as a VLP and assayed for function in cells expressing either the full-length MLV envelope protein (MLV env) or MLV env with a C-terminal deletion that has been reported to increase fusogenicity. In these experiments, cells expressing the C-term deletion were 100 to 1000 times more susceptible to MCAT-VLP infection than the cells expressing the wild-type protein, demonstrating, not only receptor specificity but also biologically relevant function.

B. Attachment to Optical Sensor

Functionalization of surfaces with intact, properly oriented TM proteins has long been desirable for the purpose of identifying binding partners and/or inhibitors of binding partners, as well as for the kinetic study of such interactions. Utilizing VLPs, Hoffman et al. have defined a general method by which
intact TM proteins, in their native lipid environment, can be attached to optical biosensors. Utilizing standard Biacore chips (C1 or F1), the researchers could successfully attach VLPs containing a number of overexpressed chemokine receptors for both the Type I and 7TM families. Importantly, the VLP-coated chips could be regenerated several times and the individual receptors retained their ability to bind antibodies and the native ligand. To demonstrate the utility of this technique, the authors examined interactions between the HIV-1 envelope protein (EnvH) and its 7TM coreceptors and between collapsin-1 and a single-pass receptor, neuropilin-1 (NP1).

In addition to binding CD4, different HIV viral strains bind to unique coreceptors on the host cell surface to gain entry in to the cell. EnvH-coreceptor affinity, for some viral strains, has been difficult to measure using the conventional cell-surface binding assays. The improved ability to measure these binding events and inhibit this binding offer tremendous therapeutic potential. To achieve this aim, an EnvH gp120 subunit responsible for CD4-independent coreceptor binding (8xgp120) was used as a capture ligand for surface-attached VLPs expressing the CXCR4 coreceptor. After determining appropriate conditions for specific binding and demonstrating antibody-specific inhibition, a dose–response experiment was performed to calculate $K_d$. The calculated $K_d$ of $506 \pm 101$ nM justifies the authors’ difficulty in determining this value using standard cell-surface binding assays, because most of the ligand is released before completion of the wash steps. In a subsequent experiment, CD4-dependant gp120 was utilized and, appropriately, binding of gp120 to CXCR4 was not observed without addition of CD4 to the running buffer.

NP1 is a Type I TM protein receptor involved in axonal guidance, and the binding of collapsin to NP1 triggers repulsion and redirection. VLPs generated from cells overexpressing NP1 were attached to biosensor chips, and collapsin-1 was shown to bind specifically to the surface-anchored VLPs. These results suggest that a diverse group of receptors can not only be incorporated into VLPs, but can also retain their cellular structure and functional capacity.

In an alternate approach, Willis et al. have attached an antibody to the Biacore chips and demonstrated the ability to quantitatively define antibody–receptor interactions. VLPs expressing seven different 7TM receptors, a type I receptor, and a Type II receptor were assayed against surface-anchored antibodies to their respective receptors. In each experiment, only the VLPs with the cognate receptors were specifically captured by the anchored antibodies. Then, using VLPs generated from cells over expressing different CCR5 receptor constructs, this group screened a dozen commercially available CCR5 antibodies and quantified the relative binding efficiency of each antibody. One of the commercial antibodies, CTC5, is known to interact with an epitope present on the intact N-terminus of CCR5. When an N-terminal HA-tagged CCR5 was incorporated into VLPs, the VLPs could not be captured by the immobilized CTC5 antibody, thus demonstrating retention of epitope specificity.
Taken together, these results demonstrate that over expressed receptors in VLPs represent a more facile method of studying binding interactions with TM receptors. It will be interesting to see whether the protein core in the VLPs can limit protein mobility and the ability to form functional oligomers such as those formed by the TF complexes. Additionally, it will be of tremendous value if these VLPs can be used to generate functional assays which are amenable to the more common, high throughput screening methods.

Many systems have now been studied using the TDA approach and we describe the breadth of utility that has been recently demonstrated by several different research groups. A future use of the technology may be to assemble biologically relevant signaling complexes as targets for compound screening. Such complexes may better reflect the biology of a particular target of therapeutic interest, and this approach may be better suited to find the most relevant candidate compounds for medicinal chemistry. The potential of this approach is seen in the work that investigated a complex chemomechanistic system that drives a bacterial response to environmental cues that are critical for the organism’s survival—the bacterial chemotaxis system.

VI. Brief Review of the Chemotaxis System—Pioneering Work

The two main requirements for an organism’s survival are, at least on the surface, quite simple: successfully seek nutrients and move away from harmful agents. Members of the Enterobacteriaceae family of bacteria, including *Escherichia coli*, *Salmonella*, *Shigella*, and many others, possess a two-component sensory system that allows the cell to constantly monitor a wide range of chemical cues in their immediate environment and adjust their swimming behavior accordingly.\(^53\) In fact, nearly all motile prokaryotes possess a two-component system that allows strict control of motor activity.\(^54\) This behavior, termed chemotaxis, was first observed by Englemann and Pfeffer in the 1880s. The modern study of chemotaxis, via genetics, was initiated by Julius Adler in the mid 1960s.\(^55–58\) These pioneers observed that bacteria could move away or toward chemicals by a mechanism that is now known to involve a biased random walk. Today, through a series of biochemical, genetic, and experimental advances, the two-component chemotaxis signaling systems of *E. coli* and *Salmonella typhimurium* are the most thoroughly studied of all biological signaling systems and is, in part, due to the relatively few proteins involved. Most importantly, these systems exhibit many of the same fundamental processes for signal transduction found in more complex systems.\(^54,59–66\) More to the point, the kinase responsible for signal generation in *E. coli* shares some of the same protein architecture of proteins found in human signaling, like the SH3 domain.\(^67,68\) While this system seems simple, it possesses and displays
many remarkable properties such as extremely high sensitivity to stimuli, signal amplification, and also the ability to assess and process many different environmental stimuli simultaneously, the same attributes and functions as human signaling systems. The cell’s ability to sense and respond to extremely low concentrations of chemical stimuli (\(\sim 10^{-7}\) M) and also adapt to the presence of high concentrations requires further investigation into how the signaling proteins in this system interact and how these complex interactions affect the cell’s ability to behave and respond in such a manner.

While an exhaustive explanation of the chemosensory system in bacteria is inappropriate for this publication, the authors do wish to provide a basic overview of the system in order to establish the driving-force for development of the TDA technology, which is the central focus of this article.

A. The Two-Component System—Brief Overview

Attractants, for example amino acids and sugars, and repellents, for example metal ions and acids, are sensed by one of four methylatable chemotaxis proteins (MCPs) which are \(\sim 60\) kDa monomers when initially expressed. In \(E.\ coli\) these are: serine (Tsr), aspartate, ribose and galactose (Trg), and the dipeptide receptor (Tap). These transmembrane receptors are expressed as monomeric units, however many studies show that they are functional only in a dimeric state. Tsr, Tar, Trg, and Tap are also \(\sim 85\%\) homologous (pair-wise) in the cytoplasmic region of the protein and have essentially the same topology. Thus, it is not surprising that they all interact with the same collection of cytosolic signaling proteins. Evidences for the dimeric state of the receptor include crystallographic determinations of the periplasmic domain (ligand-binding region of the receptor) both in the presence and absence of the ligand. This domain was organized as a dimer with the binding pocket at the interface of the two monomers. Furthermore, biochemical evidence, including cysteine cross-linking analysis showing that the receptors are primarily dimers, has demonstrated the dimeric state of the receptors, does not form heterodimers, and is stably associated even in the presence of detergents. Perhaps the most important evidence over the last decade was the demonstration that these receptors and associated cytosolic proteins are cooperatively linked—acting in concert while at the cell membrane.

B. The Two-Component System—Set in Motion

The two components of the chemotaxis system in \(E.\ coli\) and related bacteria are the transmitters of the signal CheA, a histidine kinase, and the response regulator/receiver CheY, an aspartate kinase. The chemosensory system is regulated by a ternary complex consisting of CheA (142 kDa, dimer), CheW (18 kDa, scaffold protein), and a dimeric MCP, as depicted in Fig. 4. All of these proteins are complexed to the receptors at the inner leaflet of the cell membrane. When neither an attractant nor a repellant are present, CheA is
constantly involved in the phosphorylation of the regulator protein, CheY. CheY-phosphate then diffuses through the cytoplasm to interact with the 37 kDa flagellar motor switch, fliM.\(^{86,87}\) The flagella are long, left handed protein structures that, when rotated CCW, bundle and propel the cell forward. Consequently, when the direction of the motor switches, these unwind briefly and cause a tumble, leading to a random change in the direction of movement.\(^{88}\)

To adapt to the ever-changing stimuli in the environment, the chemotaxis system has developed a mechanism of adaptation which is analogous to how our own eyes adapt to a darkened room.\(^{53,66,89,90}\) The adaptation branch is mainly controlled by CheR, a 32 kDa methyltransferase, and CheB, a 35 kDa methylesterase.\(^{91-93}\) Prior to the addition of an attractant the tumble frequency, and

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**Fig. 4.** Chemosensory circuit diagram, with single letter designations for all cytosolic proteins involved in the pathway presented.
therefore the CheY-phosphate concentration, is determined by the kinase activity of the adapted state. When a stimulus is added, an immediate decrease in kinase activity occurs, and this results in a decrease in the concentration of CheY-phosphate and CheB-phosphate (the active form of the esterase). Additionally, the receptors become better substrates for methylation via CheR. Increased methylation, over time, restores the kinase activity and increases tumble frequency to prestimulus levels, thereby allowing the cell to adapt to the current conditions, continue its progress up an attractant gradient, and detect further changes in the environment. Repellents, or removal of attractants, have the opposite effect on the system, leading to increased kinase activity and therefore an increase in tumble frequency and CheB-Pi concentrations, which serves to remove the methyl groups from the MCPs. Ultimately, the relative rates of CheR and CheB determine the steady-state methylation level of the system and allow the cell to move toward attractants or away from repellents. Figure 4, below, illustrates the proteins involved in the chemosensory system of E. coli.

C. Signal Propagation—Conformational Changes and Clusters

Subtle conformational changes in the ligand-binding subunits have been thought to be most responsible for the propagation of a signal. Careful study of the crystal structures of the ligand-binding domain, with and without aspartate, has revealed a subtle rotation between subunits and a piston-type motion. The use of spin labels to measure distance changes upon ligand binding using EPR showed that ligand binding generated a \( \sim 1 \) Å intrasubunit piston motion or shift within the receptor dimer; this is consistent with other experimental evidences of small structural perturbations that were earlier observed. How these subtle changes are relayed from the periplasmic portion of the receptor down through the membrane to the kinase, which is approximately 300 Å away, is not yet understood.

Over the last two decades, mounting evidence shows that receptor clustering plays a significant role in signal propagation, adaptation, and amplification of signaling in the chemosensory pathway. Specific evidence for the existence of receptor clusters was first provided by a number of electron microscopy studies that showed receptors localized at the cell poles. Allosteric interactions among the dimers are now thought to significantly influence how information is sensed and processed, by propagating conformational changes to neighboring dimers within the patch. Evidence for such communication between neighboring receptors has been collected through the use of mutations in the serine receptor that were suppressed by mutations in the aspartate receptor and also by employing synthetic multivalent ligands.
that specifically bound to the Trg receptor but enhanced the response to serine.\textsuperscript{110–113} Additionally, it has been demonstrated that fragments of the Tar receptor, engineered with leucine zipper motifs, spontaneously associate, in solution, with a well-defined number of subunits involved to form large kinase-activating complexes.\textsuperscript{104,114–116}

Throughout the decades of research on this seemingly simple system, there has been a need for simple reconstitution of the main protein players. While the molecular puzzle pieces of the system have all been identified there had not yet existed a simple experimental procedure to assemble the system in a way that enabled easy characterization. Recent studies from the Weis lab and from others have indicated that clustering gives rise to kinase activation via a highly cooperative process suggesting that teams of receptors act together to bind and activate the kinase.\textsuperscript{63,64,81,105}

Empirical observations and a number of theoretical endeavors have been completed on the composition of receptor clusters and how these receptors communicate and respond within the context of a cluster or a patch.\textsuperscript{109,110,117–124} Collectively, these data, along with \textit{in vivo} observations demonstrating that receptors localize at the cell poles, have provided a driving-force for a method that can reconstitute well-defined samples of clustered receptors. Subtle changes in structure within a dimer upon ligand binding are most likely propagated via clustering, thus providing lateral communication between dimers and clusters of dimers. Therefore, reconstituting arrays of receptors rapidly and in a defined manner is a worthy effort.

D. Surface Templating Approach

\textit{In vitro} systems have been used to study the behavior of heterogeneous protein complexes of bacteria for many years. Purified \textit{E. coli} inner membranes containing overexpressed MCPs have been shown to activate the kinase in the presence of CheW.\textsuperscript{81,125–129} However, purified inner membranes cannot be easily used for experiments that require the manipulation of receptor surface density, that is, degree of clustering, or experiments that require stoichiometric control over protein reagents. Furthermore, in traditional membranous samples, it is often difficult to characterize the accessibility to CheA and CheW, and perhaps most importantly, the vectoral orientation of the receptor.\textsuperscript{130,131} These challenges are common to all transmembrane signaling systems because of the 2D organization of receptors and the complexes formed between them and a number of scaffolding and regulatory proteins.

TDA was developed by Weis and coworkers in 2003 as a means of providing an experimental system that can recreate the 2D organization of transmembrane signaling systems while overcoming many of the difficulties normally associated with membrane receptor samples.\textsuperscript{34} NTA-DOGS-Ni\textsuperscript{2+} and DOPC lipids were used to form vesicles which can anchor a His-tagged cytoplasmic
domain fragment, CF, which can also be identified by its level of modification at the major sites of methylation: CF$_{EEEE}$, CF$_{QEEE}$, and CF$_{QQQQ}$, where Q = glutamine and E = glutamate. This Ni-NTA lipid was commercially available and was originally developed for applications in the 2D protein crystallization and biosensor development.

Addition of CheA and CheW to the surface-assembled fragments allowed the formation of active ternary complexes (see Fig. 5). It was demonstrated that the kinase, CheA, was bound to the surface-assembled fragments along with CheW; and ultimately the level of covalent modification led to stark differences in complex stability. Additionally, it was observed that the templated CFs also served as a suitable substrate for the methyltransferase, CheR, thereby generating methylated receptor fragments that demonstrated a close association with fragments on the surface as it is already known that methylation activity is an interdimer event.

E. Continued TDA Success

After the first publication in 2003, Weis and coworkers have furthered the utility of the TDA system significantly. For example, known signaling mutations located in the receptor were employed to determine how competitive and cooperative interactions between receptors contributed to the control of the kinase, CheA. These experiments highlight a major advantage of TDA—its ability to prepare controlled samples of heterogeneous receptor fragments on the surface. Fragments of the CF that were known to not stimulate kinase activity exhibited the same behavior with the TDA platform. When mixtures of nonactivating fragments were titrated with kinase stimulating fragments (wt) the TDA platform allowed the determination of how kinase activity was diminished. The mutants were sorted into three classes: (i and ii) CheW and CheA bound more weakly, or more tightly, to mutants than to wt-CF, and (iii) mutations inhibited kinase activity in a cooperative manner. These data support a model of CF/A/W interaction that is thought to be both cooperative and competitive. These data also are in agreement with studies carried out with intact receptor systems.

Weis and coworkers have also compared and contrasted SUVs (small unilamellar vesicles) and LUVs (large unilamellar vesicles) and how different receptor fragments are formed on these different templates. This work also provided a first look at these complexes via electron microscopic analysis. Perhaps the most intricate work using the TDA system has been provided by Besschetnova et al. Both the excitation and adaptation branches of the chemotaxis system were studied and shown to be interlinked, thus exerting a counterbalancing effect on one another. It was demonstrated that receptor density affected kinase and methyltransferase activity significantly, but in opposite directions. The TDA system allowed the preparation of samples wherein the 2D concentration of the anchored receptor fragments could be varied significantly. It was demonstrated that large concentrations of receptors favored an
increase in kinase activity, while low concentration favored higher CheR activity. This observation can be placed in the larger context of the chemosensory stimulation of ligand binding. It has been shown that ligand binding increases
the distance between receptors, thereby lowering the effective concentration of receptors and lowering kinase activity, while this same event causes an increase in methylation activity.

Furthermore, FRET experiments were carried out using fluorescently labeled C-fragments. These data demonstrated that an increase in the surface concentration of unmodified CF led to an increase in kinase activity from a low 2D concentration (5% mole Ni lipid) to a high 2D concentration (60% mole Ni lipid). Employing the same strategy with fully modified CF showed no increase in activity from the initial low 2D samples and the activity remained at the level over all surface concentrations. These results support the observation that kinase activity depends on both surface concentration and the degree to which the receptor is modified (Ref. 36, see also Ref. 137). These types of experiments can lead to an in-depth understanding of how the various units communicate within the context of the 2D membrane environment while monitoring distance and activity simultaneously.

VII. Tissue Factor

This exemplary work demonstrated that the TDA approach can be utilized for studying extracellular domains as well as cytoplasmic domains, and that the approach can be used for assembling complex signaling components in a simple format that is also amenable to high throughput assays. Most importantly, this work clearly demonstrated that TDA of purified components yields cell-like results that cannot be generated in the absence of complex assembly.138

TF is a noncatalytic transmembrane protein which is responsible for regulating the blood clotting cascade. TF binding to the plasma serine protease, factor VIIa (fVIIa), imparts allosteric activation and the resulting TF-VIIa complex activates downstream factors. Factors IX and X (fIX and fX, respectively) are membrane-bound protease zymogens which are activated by the TF-VIIa complex. Previous studies using a recombinant membrane-localized TF ectodomain (mTF, ectodomain and transmembrane domain) had shown that membrane anchoring is essential for pro-coagulant activity, but the reconstitution processes were quite laborious, time consuming, and difficult to control. In this work, a soluble His-tagged ectodomain fragment (shtTF) that enabled facile expression and purification was used. This soluble fragment lacks the transmembrane segment and can be docked onto liposomes which have the Ni-NTA headgroups (Fig. 6; Ref. 138).

Importantly, the authors note that they had expressed two soluble His-tagged ectodomains with different C-termini. In these experiments, a C-terminal His-tag is appropriate as it positions the ectodomain juxtamembrane residues adjacent to the liposome, thereby, most likely, mimicking their orientation in native assembly. One construct had residues 1–217 with six histidines encoded immediately
adjacent to residue 217, and the other construct (1–219) had five amino acids (a GGAAG flexible spacer) inserted between residue 219 and the hexahistidine affinity tag. Both constructs were active but the latter exhibited more than ten times higher specific activity in clotting assays. The authors propose that the increase in activity exhibited by the latter construct might be due to the linker which allowed better alignment of the ectodomains on the liposome surface. It has been our experience when designing RTK cytoplasmic fragments that we must occasionally employ a shotgun approach, adding flexible linker regions or shortening the juxtamembrane domains to generate active constructs. It is difficult to predict a priori the most functional construct; therefore, it is best to begin with a couple or several recombinant fragments for the initial assessment. Additionally, the liposomes in these studies have phosphoserine headgroups because such negatively charged phospholipids are required for optimal TF-VIIa activity; pointing to a need for assay development.

Purified plasma-derived fVII, fX, and fXa were used to compare the activity of mTF incorporated into liposomes with that of shtTF bound to the Ni-NTA liposomes. Initial assays examined the binding affinity of TF with fVIIa. Previous experiments had shown that wild-type TF, when incorporated into a phospholipid bilayer with the appropriate charge profile, binds to the fVIIa ligand with extremely high affinity ($K_d < 50$ pM), but a recombinant soluble TF fragment (sTF) bound to fVIIa with about 100 times less affinity ($K_d \approx 5$ nM). Upon comparing mTF incorporated into PCPS liposomes with shtTF attached to metal-chelating NiPCPS liposomes, the authors found each bound fVIIa with nearly identical affinities ($K_d \approx 10$ and 10.8 pM, respectively); when the isolated shtTF ectodomain was bound to the liposome surface via an Ni-His interaction, its fVIIa-binding ability was indistinguishable from the membrane spanning mTF construct.
A more relevant test of full function is the ability of the TF-fVIIa complex to activate factor X (fX). When compared with the membrane-associated TF, the soluble ectodomain does not support the same level of fX activation, with or without the addition of PCPS liposomes because the soluble ectodomain is not incorporated or bound by the liposomes. However, when comparing shtTF assembled on NiPCPS liposomes with the membrane-anchored TF, the activation rates of fX were essentially identical, with the catalytic efficiency ($k_{\text{cat}}/K_m$) of the liposome-assembled shtTF being slightly higher.

In the assays described above, autoactivated fVIIa was used. A further test of functionality is to compare the effectiveness of the different TF fragments to enable fVII autoactivation. Earlier work has shown that a membrane-associated, purified, wild-type TF could support fVII autoactivation while a soluble fragment failed to support detectable levels of fVII autoactivation in the presence or absence of liposomes, even after 2 h. By contrast, in this assay, shtTF and mTF both supported fVII autoactivation to a similar extent when tested at identical surface densities, indicating that the TF ectodomain need not be integrated into the membrane, as had been previously suggested. Taken together, these results clearly demonstrate that the soluble TF fragment, when colocalized on a fluid membranous matrix with an appropriate charge profile, can be utilized in biologically relevant assays that use multiple interacting cascade components.

In a final test of relevant TF function, the authors measured the procoagulant activities of sTF, shtTF, and mTF by measuring clotting times in human plasma using a coagulometer. Both the shtTF and mTF showed similar clotting rates after a modification to the nickel-liposomes; this was reasoned to be necessary because of the Ni-NTA interference. Interestingly, the authors also demonstrated that immobilized liposomes could additionally be used for purification of the soluble His-tagged ectodomain from crude mixtures. The captured ectodomain was subsequently assayed in situ for its ability to promote clotting, with excellent results.

In a study to understand the effects of local changes in the phospholipid environment, the Morrissey group utilized the NanoDisc approach discussed earlier. The NanoDisc technology was useful in clearly demonstrating a preference for phosphoserine headgroups in the local lipid during fX activation by the NanoDisc-integrated TF-fVIIa complexes. An identical phosphoserine preference had been observed when generating mTF liposome complexes and shtTF complexes with Ni-NTA liposomes. In this study the authors directly compared the proteolytic activity of TF-fVII with mTF-liposomes and wt TF in NanoDiscs. Interestingly, the NanoDiscs required a higher local concentration of phosphoserine to generate similar activity; this may be attributed to selectivity differences between membrane-associated fX and fX in solution. On the liposomes, fX can accumulate at higher fX to TF ratios,
effectively shuttling the fX to membrane-bound TF-fVIIa complexes. In the case of the NanoDiscs, the number of fX molecules that can bind to TF-NanoDiscs are smaller (ca. 5–6 fX/Nanodisc leaflet) compared to mTF-liposomes. This work also suggests that signaling components requiring larger oligomeric arrays or dynamic exchange of cofactors for full function might be better suited to the TDA approach where a larger number of protein units can be effectively assembled on the lipid scaffold.

VIII. EGFR

The epidermal growth factor family of transmembrane RTKs is an important group of enzymes, largely because of their link to cancers and other diseases. The four members of this family include EGFR/ErbB1/Her1, ErbB2/Her2, ErbB3/Her3, and ErbB4/Her4. EGFR and ErbB4 are both activated in a fairly conventional manner, by ligand-induced dimerization, though the precise mechanism of this activation has eluded researchers for some time. Her2 has no known ligand, yet can be activated by other members of the family, and is the preferred heterodimerization partner for all family members. Her3 is catalytically inactive, but can activate other members as heterodimers or higher order oligomers. Any of these receptors can activate themselves or other family members via heterodimerization, when over expressed.

In a recent structural study by Zhang et al., an allosteric mechanism of activation was proposed for the EGFR kinase domain. Though not the focus of their study, they utilized template-assembled EGFR kinase domains to correlate their structural findings with biochemical activity. Using soluble recombinant fragments of the wild-type or mutant cytoplasmic kinase domains, they challenged their model of allosteric activation via the formation of an asymmetric dimer. The isolated kinase domain of EGFR is autoinhibited and can be activated by mutating the leucine residue at position 834 to an arginine; an activating mutation observed in lung cancer patients. Alternatively, the isolated kinase domain can be activated by increasing its local concentration in a controlled manner or by confining the kinase domain to the 2D surface of liposomes, thus mimicking the membranous matrix in which they normally reside. These membrane-bound kinase domains are free from regulatory control of the extracellular domains. Consequently, this local high concentration ensures transphosphorylation of the activation loop. One might interpret this activation as being the result of an increase in trans/autophosphorylation. In this interpretation, EGFR is its own substrate, and colocalization of the activating enzyme with its enzyme substrate, another EGFR, increases the rate at which the substrate can be phosphorylated. Phosphorylation of Y845 in the activation loop of EGFR, however, is not required for activity, and a nonphosphorylatable
Y845F mutant is activated to a similar degree when colocalized. This result is consistent with the proposal that the assembled constructs are activated by allosteric interactions that result from productive dimer formation rather than a decrease in the kinetic barrier to transphosphorylation. This is analogous to the natural activation that occurs upon release of the ectodomain via a sheddase activity or alternate translation which leaves the cytoplasmic domain anchored to the membrane, and yet unregulated (no longer dependant on ligand-induced dimerization).

In their structural analysis, the authors discuss the relationship of the asymmetric dimer observed in the EGFR structure to the CDK/cyclin complex, where the C-terminal lobe of one EGFR molecule plays a role analogous to cyclin in the activated CDK/cyclin complexes. This C-terminal to N-terminal interaction offers a better explanation for the observation that many activating heterodimer pairs can form between the EGFR family members. ErbB3, despite having an inactive kinase domain, is capable of activating all other EGFR family members, and the sequence of the C-terminal (activating) lobe, in the region described to interact with the N-terminal lobe, is very similar to that of the other family members. In subsequent studies, the authors continue to correlate this template-assembled biochemical analysis to structural analysis and have probed the possibility of identifying inhibitors of the asymmetric dimer interface.

MIG6 (mitogen-induced gene 6; gene symbol ERRFI1) is a 51 kDa cytoplasmic protein which had previously been identified as an inhibitor of EGFR activity.\textsuperscript{147,148} Structural analysis of EGFR-MIG6 complexes revealed that a MIG6 fragment of approximately 25 residues binds to the C-terminal (activating) lobe of the EGFR kinase domain\textsuperscript{149}. In subsequent cell-based assays, the authors showed that while wild-type MIG6 did indeed inhibit EGFR activity, MIG6 constructs with introduced point mutations were unable to inhibit EGFR activity. These point mutations were introduced in the region corresponding to the segment which had been identified as structurally interacting with the C-terminal lobe of EGFR. In biochemical assays, template-assembled EGFR kinase domains can also be inhibited by the addition of peptides which block the formation of the asymmetric dimer. Interestingly, we think that this suggests that template-assembled enzymes are well-suited for identifying inhibitors of protein–protein interactions.

Using a similar approach, Monsey et al. have recently characterized the remaining ErbB family members, demonstrating that the catalytically inactive Her3 readily activates Her2, and that ErbB4 can be activated by TDA in a manner similar to the EGF receptor.\textsuperscript{150} This work paves the way for future analysis of these interactions and demonstrates the plausibility of targeting this family of receptors in a simple, yet meaningful, format that is also readily amenable to the currently used high throughput methods.
IX. Polio Virus Fusion In Vitro Using TDA

The creation of a chemically defined system where a biological membrane acts not only as a surface for organization of proteins but also as an active component in a molecular machine has been technically difficult. The picornaviridae, such as the polio virus, are uncoated viruses which, due to lack of a lipid coat must deliver their charged RNA genome directly through the plasma membrane of the target cell.

Only a single protein from the host cell, specifically the ectodomain of the polio virus receptor (Pvr), is required for viral genome entry.\textsuperscript{151} Thus, the major components of the machine which delivers the viral genome are virally encoded, and enable the viral genome to cross the membrane in the absence of any host transmembrane proteins. Upon binding to the membrane viral proteins, VP1 and VP4 undergo an irreversible, two-step conformational change which results in genome delivery.\textsuperscript{152,153} However, neither the structures derived from VP1 and VP4 conformers in solution in the absence of a membrane nor an electron microscopic analysis of virus entry in cells have been sufficient to elucidate the physical mechanism of this process.

To address these limitations, Dr. Hogel’s group at Harvard employed TDA to create a chemically defined molecular machine which reproduces the process of viral genome entry into cells. NTA-liposomes were decorated with recombinant Pvr with a carboxy-terminal poly-histidine tag. Intact polio virus was shown to bind to the NTA-liposomes in a Pvr-dependent manner.\textsuperscript{154} Cryo-EM and tomographic studies of the virus-Pvr-liposome complex provided compelling results that supported the proposed model that the fivefold symmetry of the virus particle is responsible for contacting five receptor molecules on the surface of the cell membrane. This study also demonstrated that the association of the virus with the ectodomain of the receptor, even in the absence of the transmembrane domains, produces a deformation of the lipid bilayer, an intermediate step required for genome delivery.\textsuperscript{155,156}

This body of work represents a major extension of the utility of TDA; specifically, the creation of a “model membrane system” which facilitates the functional, structural, and mechanistic study of interactions among membrane receptors with their ligands, in a fully defined system.\textsuperscript{155,156}

X. Future Directions

The pharmaceutical industry is in the midst of important new challenges. With a premium on effective, safe, and inexpensive therapeutics, research organizations must focus on finding the best compound for each therapeutic indication, early in the discovery process. This will hopefully eliminate the costs
associated with extensive medicinal chemistry efforts on compounds that fail to achieve the desired efficacy in clinical settings. With no guarantee that a particular therapeutic target will reach that goal, all that development teams can do is ensure that each screening campaign evaluates the target in as natural an environment as possible, using native assay conditions that mirror the in vivo state as accurately as possible. The strategies discussed in the preceding chapter describe a set of tools that can be employed to study membrane-associated targets using high throughput biochemical tools that have significantly advanced in the last 10 years. We hope that this combination of an optimized enzymatic assay system with the high throughput biochemistry and automation will help improve the effectiveness of the industry.

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