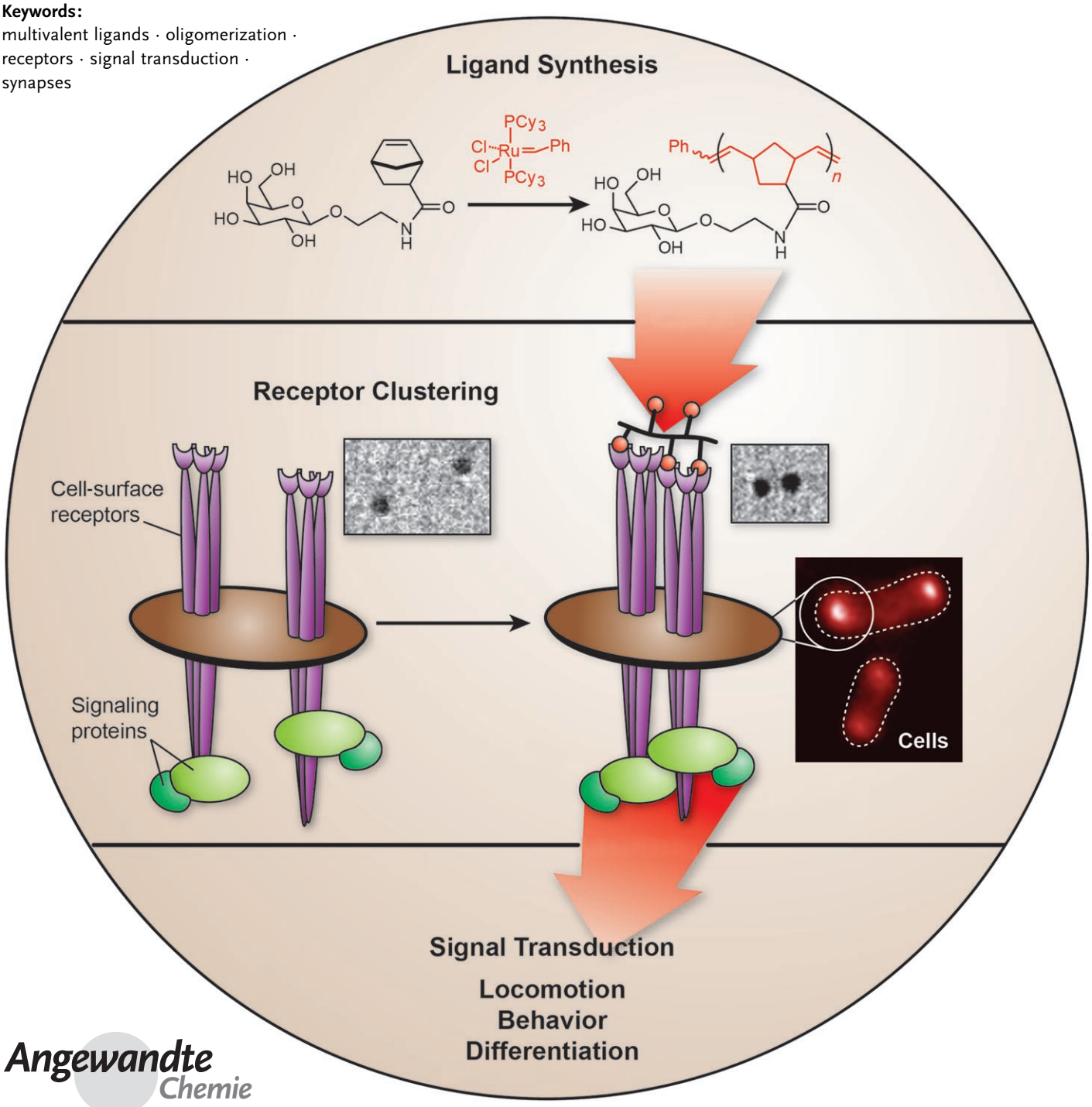
Bioorganic Chemistry

DOI: 10.1002/anie.200502794

Synthetic Multivalent Ligands as Probes of Signal Transduction

Laura L. Kiessling, Jason E. Gestwicki, and Laura E. Strong*

Keywords:
multivalent ligands · oligomerization · receptors · signal transduction · synapses



Cell-surface receptors acquire information from the extracellular environment and coordinate intracellular responses. Many receptors do not operate as individual entities, but rather as part of dimeric or oligomeric complexes. Coupling the functions of multiple receptors may endow signaling pathways with the sensitivity and malleability required to govern cellular responses. Moreover, multireceptor signaling complexes may provide a means of spatially segregating otherwise degenerate signaling cascades. Understanding the mechanisms, extent, and consequences of receptor co-localization and interreceptor communication is critical; chemical synthesis can provide compounds to address the role of receptor assembly in signal transduction. Multivalent ligands can be generated that possess a variety of sizes, shapes, valencies, orientations, and densities of binding elements. This Review focuses on the use of synthetic multivalent ligands to characterize receptor function.

From the Contents

1. Introduction—Receptor Function

To achieve success in the competition for favorable chemical and physical conditions, all organisms (from bacteria to worms to humans) must rapidly and accurately sense changes in their environments. Likewise, the complex orchestrations that govern developmental patterning or immunological responses rely on communication between cells and their environs. The task of monitoring extracellular conditions falls to the cell-surface receptors. These proteins coordinate the cell's internal machinery by collecting, compiling, and translating external information. The pressure for survival demands that responses to stimuli be sensitive and accurate; synergistic and competitive signals must be amplified and integrated. Thus, cells have developed sophisticated and elegant methods for achieving sensitive yet controllable receptor-mediated responses.

One important mechanism for efficient and sensitive signaling is to couple the functions of multiple cell-surface receptors,[1–9] most of which do not typically operate as individual entities, but function in concert. An emerging paradigm is that receptors within multireceptor signaling complexes communicate with each other.[7] Although evidence is mounting for communication between receptors, an understanding of the underlying mechanisms is elusive.

Chemical synthesis can provide a variety of compounds with which to address the role of receptor assembly in signal transduction. Multivalent ligands are well-suited to this task. They are capable of illuminating aspects of interreceptor processes that are not readily probed by using conventional approaches. We suggest that, as the focus shifts from investigations of the function of individual proteins toward the analysis of multireceptor signaling complexes, multivalent ligands will become even more valuable tools to probe sophisticated mechanistic questions. Moreover, multivalent ligands may provide new opportunities for manipulating receptor systems to dissect pathways and, ultimately, treat diseases. This Review focuses on the role of synthetic multivalent ligands as probes for the investigation of receptor collaboration.

2. Direct and Indirect Interactions between Receptors

2.1. Signaling Complexes

Receptors transmit information on extracellular signals to the internal machinery of the cell. Different receptors often share common cytoplasmic components, and therefore information transfer must be constrained to prevent unwanted exchange between disparate pathways.[7,10–12] To understand how signals are transmitted, it is necessary to unravel how cells can respond distinctly to different stimuli by using a set of common components.[7]

The subcellular localization of receptors into signaling complexes is proposed to be one mechanism by which cells achieve spatial and temporal regulation.[13–19] Multiprotein signaling complexes constitute the principle signaling units of neuronal synapses,[20,21] immune synapses,[13,22,23] focal adhesions,[24] and bacterial chemoreceptor arrays (Figure 1).[25,26]

[*] Prof. L. L. Kiessling, Dr. J. E. Gestwicki,[+] Dr. L. E. Strong[$]
Departments of Chemistry and Biochemistry
University of Wisconsin—Madison
1101 University Ave., Madison, WI 53706 (USA)
Fax: (+1) 608-265-0764
E-mail: kiessling@chem.wisc.edu

[+] Present address:
University of Michigan
210 Washtenaw Avenue, Ann Arbor, MI 48109 (USA)

[$] Present address:
Quintessence Biosciences
University Research Park
505 S. Rosa Rd., Madison, WI 53719 (USA)

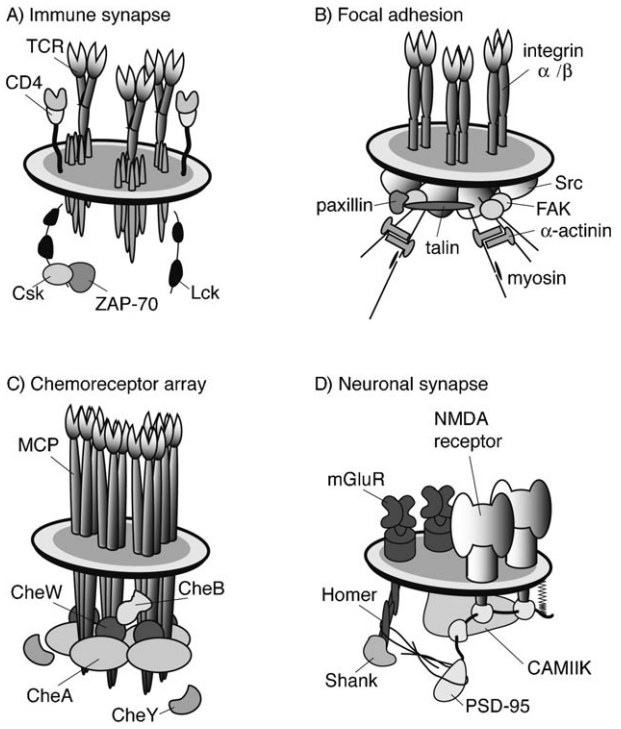


Figure 1. Examples of complexes involved in signal transduction: A) eukaryotic immune recognition; B) eukaryotic cell adhesion; C) prokaryotic chemotaxis; and D) neuronal signaling. These schematic depictions simplify the complexity of biological multiprotein signaling complexes.

These ensembles are composed of receptors, signaling proteins, adapter proteins, and cytoskeletal components.[27–29] Many signaling complexes in eukaryotic cells appear to be associated with detergent-insoluble lipid microdomains that provide unique physical environments for the concentration of signaling components.[30–35] Often it is not known whether cells use microdomains or other mechanisms to organize and assemble signaling components.

The formation of signaling complexes is not restricted to any particular receptor class. Data from microscopy, covalent cross-linking, and X-ray crystallography experiments have revealed that cell-surface receptors from many structural classes assemble into multireceptor complexes; these include some heptahelical G-protein-coupled receptors (GPCRs),[36–38] methyl-accepting chemotaxis proteins (MCPs),[39] gated ion channels,[40] receptor-protein tyrosine kinases (RPTKs),[41,42] and multichain immune recognition receptors (MIRRs).[22,33,35,43–45] The size of these ensembles varies: Some complexes are composed of two receptors while others contain thousands. Some receptors,[46–48] including the ryanodine receptor[40] and MCPs,[39] are so highly concentrated that they dominate certain cellular regions. Unfortunately, little is known about the structures of these assemblies, where they are localized within the cells, and how their localization influences signaling. Understanding these issues, however, could lead to new strategies to precisely control receptor function and therefore cellular responses. Although there remains a need to explore the role of receptor localization and assembly, the data acquired to date suggest some general basic principles for communication between the receptors.

Receptors can exchange information through direct protein–protein contacts or through intermediary proteins. Direct receptor contact typically involves interactions between specific protein regions.[49] For example, helix-mediated interactions are proposed to facilitate the dimerization and oligomerization of GPCRs (Figure 2).[38,50,51] Evidence for the functional importance of these interactions is suggested by engineering disrupting mutations in the proposed contact sites or by adding isolated transmembrane helices to GPCRs.[37,52,53] Although their effects on receptor oligomerization are not yet well-established, these manipulations modulate signaling; presumably, they disrupt receptor–receptor contacts. As mentioned above, an alternative strategy by which receptors exchange information is through intermediary proteins. These scaffolding proteins can organize multireceptor complexes, and thus act as frameworks for protein assembly. Examples of receptors localized by this mechanism include the MCPs and members of the tumor necrosis factor (TNF) receptors (Figure 2B, C).[54–56]

Ligand binding can transduce signals by directly stabilizing or destabilizing protein assemblies.[57–59] For example, data collected from diverse experiments suggest that binding of an attractant to a single MCP can elicit signaling from nearby MCP partners.[46,60–64] Similarly, cross-phosphorylation of some growth-factor receptors is facilitated by ligand-induced contacts.[65] In addition to homodimeric (or oligomeric)

Laura L. Kiessling gained her BS from the Massachusetts Institute of Technology, and completed her PhD in Chemistry at Yale working with Stuart Schreiber. After postdoctoral training at the California Institute of Technology with Peter Dervan, she began her independent career in 1991 at the University of Wisconsin-Madison, where she is currently a professor in both Chemistry and Biochemistry. Besides her long-standing interest in organic synthesis for investigating protein–carbohydrate interactions, she is also interested in multivalent binding events, biomolecular recognition, and signal transduction.

Jason E. Gestwicki gained a BS in Chemistry in 1997 from the State University of New York at Fredonia. In 2002, he completed his PhD in Biochemistry at the University of Wisconsin-Madison with Prof. Laura L. Kiessling. Most recently, he was a Helen Hay Whitney post-doctoral fellow at Stanford University with Prof. Gerald R. Crabtree, where he synthesized potent difunctional inhibitors of protein–protein interactions in neurodegenerative diseases. He joined the University of Michigan Medical School in 2005 as an Assistant Professor of Pathology and Research Assistant Professor in the Life Sciences Institute.

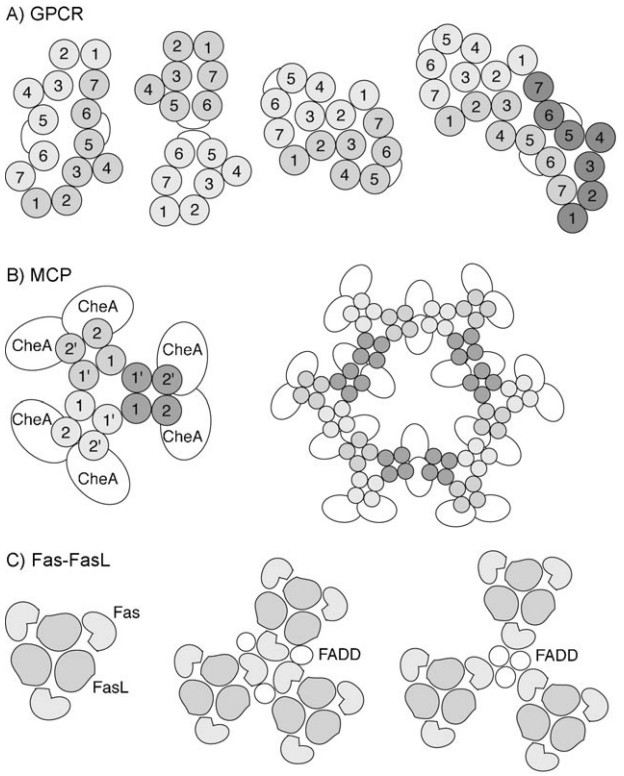


Figure 2. Proposed multireceptor assemblies for some receptors. A) Models of three heptahelical GPCR dimers and a trimer based on mutagenesis results and the structure of bacteriorhodopsin. Each numbered circle represents a transmembrane helix. A variety of 1–7, 5–6, and 2–3 dimers and multimers have been proposed. B) Bacterial chemoreceptors (methyl-accepting chemotaxis proteins, MCPs) are dimeric. These can assemble into a trimer-of-dimers, which can further interact with signaling proteins, such as the kinase CheA. Each MCP passes through the membrane twice (1 and 2) and coiled-coil interactions between these transmembrane domains (1 and 1′) mediate dimerization. A lattice model of MCP organization constructed of six of these trimers-of-dimers and 24 copies of CheA is shown. C) The Fas–FasL interaction has been modeled using protein interfaces suggested by mutagenesis and cross-linking studies. Both the receptor Fas and its corresponding ligand FasL are trimers. The corresponding trimeric complex may be employed as a unit in the lattice stabilized by the adapter protein FADD. Two models are shown with either Fas- or FADD-centered symmetries. See the text for details and literature references.

Laura E. Strong is the Vice President of Product Development at Quintessence Biosciences, Inc. She received her BS in chemistry at the University of Richmond, and earned a PhD in organic chemistry with Prof. Laura L. Kiessling at the University of Wisconsin–Madison (National Institutes of Health predoctoral fellow). While there, she was involved in developing the ring-opening metathesis polymerization to produce biologically active, multivalent ligands. She is co-author of twelve publications and co-inventor on three patent applications.

interactions, interactions between structurally dissimilar receptors have also been demonstrated.[66] The neuronal dopamine and GABA receptors, a gated ion channel, and a GPCR, have been shown to exchange information by direct contact.[67]

An alternative means by which ligands can induce information transfer and co-localization of receptors is by facilitating the recruitment of intermediary proteins to the target receptors; these intermediary proteins are called adapters or scaffolding proteins.[68–73] Specialized modules within intermediary proteins act as information conduits; examples include SH2, SH3, and PDZ domains. These domains participate in a series of protein–protein interactions that transfer information and localize receptors. The simplest adapters directly link one receptor to another. Additionally, many intermediary proteins contain multiple domains that facilitate communication between a receptor and a variety of membrane-associated and soluble signaling proteins.

2.2. Factors that Influence Communication between Receptors

An important first step in investigating and manipulating the function of a multireceptor signaling complex is defining the features. However, the potential diversity created by the presence of multiple receptors and modular adapters raises challenges for those that seek to understand them. The features of the immune synapse illustrate some of the issues relevant for elucidating the function of signaling complexes.

T lymphocytes are critical mediators of effective immune responses to invading pathogens. The contribution of T cells to the immune response is largely controlled by signaling through the T-cell receptor (TCR). Like many other important cell-surface receptors, the TCR can be assembled into a multireceptor complex. When a T cell contacts an antigen-presenting cell (namely, a dendritic or B cell), the cytoskeleton reorganizes, and adhesion receptors and the TCR undergo changes in localization at the interface. This interface has been referred to as the "immune synapse" (Figure 1 A).

The features of the immune synapse can vary. Its structure is influenced by the type of antigen-presenting cell (APC) involved, the type and activation state of the T cell, the duration of the T cell/APC interaction, and the local physiological environment in which it forms.[74] Powerful new imaging methods have been used to visualize immune synapses formed in vitro and in vivo; these studies reveal that dynamic changes in protein localization and organization can occur. At a molecular level, the role of protein organization within the synapse has not yet been established. It is not known whether the observed organization of the receptors on the T cell influences signaling.

It is clear that the TCR[75] and co-receptors, such as CD4,[76,77] are localized at the cell–cell interface. Some of these co-receptors (for example, CD4) augment the signal generated by the TCR; others attenuate it. The balance and orientation of these co-receptors can therefore tune the level of T-cell activation. This tuning is achieved by changes in the phosphorylation of intracellular protein domains and recep-

tors; the immune synapse also contains kinases such as Lck and ZAP-70 that catalyze the phosphorylation of ligated receptors and other proteins.[77,78] Phosphorylation can have either a positive or negative impact on the subsequent recruitment or the enzymatic activity of various components of the signaling complex.

Evidence from microscopy and signaling experiments suggests that the immune synapse can be highly organized.[76] The cytoskeleton plays a critical role in organizing some cell-surface proteins, thus the findings that cytoskeletal components are critical for TCR signaling supports a role for receptor organization.[13,79,80] The positioning of proteins within the immune synapse is also highly dynamic. For example, when a T cell engages with a cell presenting an antigen, the negative regulator CD45 moves to the periphery of the synapse.[76,81–83] Under these circumstances, the TCR is recruited into the inner portion of the synapse. These changes in receptor positioning suggest that synapse organization affects signal transmission: These intricate, orchestrated[76] movements are believed to affect the transfer and propagation of the activating signal. The position of the TCR in relation to the various positive and negative regulators of its function appears to determine the signal intensity.

There are many parameters that can affect signal output, and dissecting the contribution of different factors to T-cell signaling is complicated. Factors that have been proposed to influence signal output[20] include the number of activated receptors, their identity, and stoichiometric ratio (Figure 3).[4,84,85] The stoichiometric composition of a signaling complex (that is, the number of activating versus dampening receptors) can influence the signal strength required to overcome thresholds. In addition to the identity and stoichiometric composition of receptors in the complex, signaling can also be influenced by the proximity and subcellular compartmentalization of receptors. Determining the relative contribution of all of the aforementioned variables remains a major challenge. To isolate and dissect such a complex signaling system a variety of biological and chemical methods are required. Multivalent ligands are valuable tools in the armamentarium.

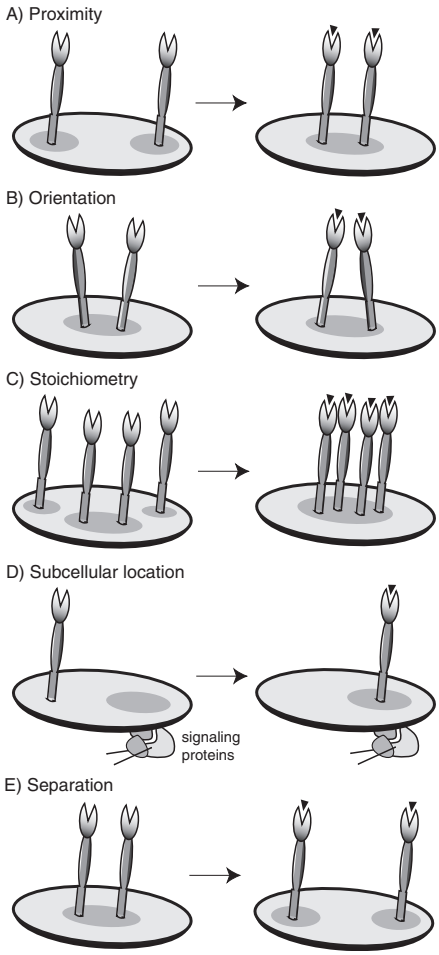


Figure 3. Possible methods for regulating interreceptor communication. A) The distance can influence the transfer of information between receptors or other proteins. B) The relative orientation of two receptors can influence the alignment of enzyme active sites and govern the rates of covalent modifications that result in signal generation. C) The number of receptors in a complex can influence the intensity of a signal. Additionally, the likelihood that receptors will come into contact increases when the numbers of localized receptors is greater. D) The subcellular location of a receptor controls the access of some intracellular signaling proteins to the receptor. Changes in position can govern the flow of information through a receptor or cluster of receptors. E) Ligand binding can lead to activation of receptors by separation when co-receptors act as negative regulators. This receptor mechanism is conceptually related to proximity-induced activation, but the underlying molecular interactions are quite different.

2.3. Challenges in Studying Receptor Function

Historically, cellular functions have been investigated by detailed examination of the structure and activity of a single protein component within a reaction pathway. Signal transduction involves the coordinated interactions of many different proteins. Thus, understanding cellular functions requires uncovering how heterogeneous collections of proteins interact at a supramolecular level. Advances in genomics, transgenic animal technologies, and chemical biology provide the means to identify components of a pathway. Elucidating the functional and structural relationships between these components remains a major challenge.[86–88] Even after evidence for physical association has been obtained, the order in which these interactions occur and their kinetics must be explored. Strategies that provide insight into complex cellular processes are needed.

Understanding multireceptor assemblies requires the use of methods that reveal both molecular and supramolecular detail. For example, immunoprecipitation, confocal microscopy, and Förster resonance energy transfer (FRET) experiments have been influential in exploring changes in subcellular protein distributions.[89,90] Moreover, fluorescent proteins and advanced imaging techniques have revolutionized the ability to follow proteins in live cells in real time.[91–93] Although these approaches provide insight into the operations of multiprotein complexes, the molecular details that underlie interreceptor processes often are obscure. This

limited understanding arises from an old problem: the details revealed by an experiment are limited by the resolution of the investigative methods. For example, the optical resolution of light microscopy is approximately 200 nm. Fluorescence microscopy experiments, therefore, can be used to discern whether receptors are moving from one side of a cell to another, but information concerning the orientation of two adjacent receptors is more difficult to obtain.

Methods with superior resolution are required for investigating receptor–receptor interactions that occur over subnanometer distances. Although FRET can be used to investigate receptor assembly at this level, its application typically requires that cell lines be engineered to produce fluorescent proteins. Electron microscopy, which does not require transfected cell lines, has a limit of resolution of approximately 0.1 nm; thus, it reveals features that are 1000-fold smaller than those observed by optical microscopy. More recently, advances in single-particle methods have started to provide molecular detail for some signaling complexes.[45,92–94] These advances in experimental methodology provide a framework from which to develop reagents for studying the function of multireceptor complexes.

New methods for exploring receptor signaling also are emerging from synthetic organic chemistry and chemical biology. Chemical synthesis provides access to unique compounds that can be used to dissect the role of molecular interactions. These ligands, in concert with the new imaging techniques, can illuminate the roles of protein assemblies. This Review focuses on the application of synthetic multivalent ligands to the analysis of signaling complexes.[9,84]

3. Multivalent Ligands as Probes for Receptor–Receptor Interactions

3.1. Multivalent Ligand Structure and Function

Multivalent ligands present multiple copies of a recognition element (RE) from a central scaffold.[84,95–98] The REs of a multivalent ligand can be a carbohydrate, peptide, protein, or small molecule—any moiety that binds to a receptor. The scaffold determines the structural features of the multivalent ligand; it also dictates how easily they can be varied. These issues are relevant because the architecture of a multivalent ligand—its shape, orientation of the REs, flexibility, size, valency—can influence its biological activity and its mechanism of action.[99–101] For example, in systems that are activated by receptor clustering, the most potent ligands may be those with many, closely spaced REs. Ligands with defined features and tailored biological activities can be attained by chemical synthesis.

An important concept underlies the design of multivalent ligands that activate cellular signaling: many cell-surface receptors are modular. For example, some receptors possess intracellular catalytic domains, which are distinct from their extracellular binding domains; the RE-binding and signal-generating regions of receptors are therefore structurally and spatially distinct. Thus, multivalent binding to sites at the surface of the cell can be used to assemble and thereby influence interactions of distal intracellular (cytoplasmic) components. This situation is distinct from that encountered using typical catalysts for organic reactions, in which the formation of the catalyst–substrate complex results in a chemical transformation. Thus, substrate binding and catalytic activity are inextricably linked. Since binding and catalytic activity are separated for most cell-surface receptors, multivalent ligands can be used to manipulate the localization of intracellular catalytic domains. Ligand binding can promote changes in the receptor and therefore the catalyst environment which can enhance or restrict access of a substrate to the catalytic domain. In this way, a multivalent ligand can influence cellular responses.

As a result of the way in which they function, the nomenclature used to describe the activities of monovalent compounds can lead to confusion.[102] Bioactive and monovalent compounds are often referred to as agonists or antagonists: The former activates a response, while the latter inhibits it. There is often a mechanistic implication in the use of these terms—agonists are believed to induce a conformational change similar to that of the natural ligand, but antagonists induce an inactive conformation. It is these mechanistic implications that complicate the use of this nomenclature to describe multivalent ligands. An individual RE, for example, can be an agonist, partial agonist, or antagonist. Unlike a single RE, however, the ability of a multivalent ligand to activate or inhibit a biological response will be influenced not only by the RE itself but also by the architecture of the scaffold upon which it is displayed. A multivalent display of an RE that serves as a monovalent antagonist, for example, may lead to clustering of receptors; thus, the multivalent ligand could serve as an *agonist*.[103] Likewise, a multivalent ligand composed of REs that bind to a site remote from that of the physiological ligand can induce activation of some systems solely by decreasing the distances between the receptors.[104,105] Conversely, it has been proposed that multiple agonistic REs displayed in an orientation that holds receptors in unproductive arrangements for signaling can result in a multivalent *antagonist*.[106,107] Ligand-induced sequestering of key signaling proteins also can cause antagonist- or agonist-like effects.[108,109] Thus, instead of terming multivalent ligands agonists or antagonists, we refer to them as either multivalent effectors or inhibitors.[84] Inhibitors block receptor function and multivalent effectors activate cellular processes. This terminology does not imply any particular activity for an individual recognition element nor a specific binding mode; it is a function of *both* the RE identity and the architecture of the multivalent ligand.

3.2. Multivalent Ligand Architectures

A variety of natural, synthetic, and semisynthetic scaffolds have been used to probe receptor function (see Figure 4 for examples). These scaffolds vary in size, shape, and physical characteristics. Different scaffolds display REs differently (see Section 3.1); therefore, the structure of a scaffold can have a significant effect on its activity. For example, a globular

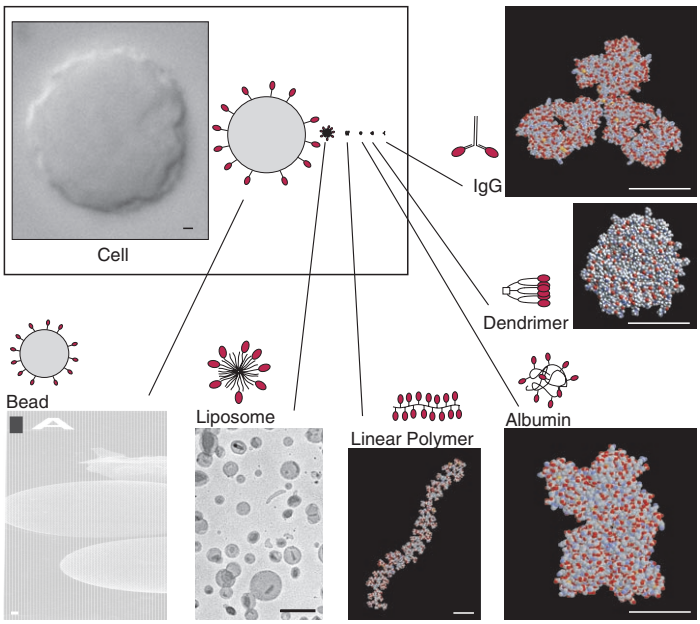


Figure 4. Scaled diagram of the scaffold structures of multivalent ligands. The boxes show a size comparison of the ligands to a mammalian lymphocyte. Size bars are as follows: cell and bead 1 μm; liposome and polymer 0.5 μm; antibody, dendrimer, and albumin 0.05 μm. The immunoglobulin structure is based on PDB 1IGT and the albumin is human serum albumin from PDB 1BM0. The beads are reproduced with permission from reference [262]. The liposomes are courtesy of A. Menon, the cell is a cultured Jurkat cell,[263] the dendrimer model was generated at the Caltech Molecular and Process Simulation Center, and the ROMP-derived polymer was designed by C. W. Cairo (unpublished results).

scaffold, such as a protein or dendrimer, may not be capable of spanning the large distances needed to cluster multiple proteins, but may effectively occupy multiple binding sites on an oligomeric receptor. Thus, the architecture of a ligand influences its ability to form different types of macromolecular receptor complexes.[84,100,110]

It is useful to examine the structural complexity of natural multivalent ligands to understand how to design synthetic multivalent ligands. The architectures of natural ligands are enormously diverse. For example, the TNF family member sTALL was found to be an ensemble of 60 monomer units that interact noncovalently to generate a symmetric "virus-like" complex.[94,111,112] It is hypothesized that the complex architecture of sTALL is required to organize multiple copies of its cell-surface receptor into an active signaling complex. Other natural ligands, such as lipopolysaccharides, mucins, and glycosaminoglycans, present a heterogeneous display of potential receptor binding sites, even within the same molecule.[113,114] A visual comparison between the architecture of sTALL and a typical glycosaminoglycan will reveal very few similarities in either size, shape, or RE density. However, despite their dramatic structural differences, both sTALL and glycosaminoglycans are involved in clustering cell-surface receptors. This simple observation can prompt hypotheses about how differences in the scaffold architecture might have an impact on the underlying signaling process. Often, however, it is difficult to determine which of the many possible ligand features influence activity. For example, naturally occurring polysaccharides vary in length, branching, and monosaccharide composition. Tracing the specific feature of a physiological multivalent ligand that is critical for activity can be arduous.

Synthetic organic chemistry can provide a wide range of defined multivalent ligand architectures. This diversity means that synthetic ligands can be used to dissect the features of physiological ligands that are responsible for their activity. For example, unlike natural ligands, the valency of a synthetic ligand can be systematically altered by varying the length or size of the scaffold. The effect of valency on the activity can be assessed by generating polymers of defined lengths. Similarly, the effect of dendrimer valency on activity can be explored by testing different generations. Examining the impact of these changes on the biological response illuminates the mechanisms underlying the function of natural receptor–ligand complexes to be illuminated. In the following sections we briefly introduce the synthesis and application of some structural classes of multivalent ligands. Finally, we discuss how synthetic multivalent ligands have been used to elucidate and exploit the mechanisms by which key receptors function.

3.3. Synthesis of Multivalent Ligands

The most versatile methods for synthesizing multivalent ligands involve adding REs to a preformed synthetic or naturally occurring scaffold. Scaffolds have been used from many structure classes: proteins, dendrimers, polymers, and solid supports.[115] With an appropriately functionalized scaffold (or RE), multiple copies of an RE can be appended. This approach provides straightforward access to ligands with variable valency and density of REs, since the mole fraction of functionalized RE used in the conjugation reaction can be readily controlled.[115] An alternative to this strategy is the approach typically used in creating polymer-based multivalent ligands: A single step can be used to assemble RE-bearing monomers into a multivalent product.

In most of the examples presented herein, the REs have been added to a preformed scaffold to generate the multivalent ligands. The examples that we discuss can guide the selection of scaffold; the corresponding literature references can be used to design the most efficient synthetic route.

3.3.1. Low-Molecular-Weight Ligands

We use the descriptor "low-molecular-weight ligands" to refer to compounds that present fewer than ten REs and are typically less than 1000 Da. The synthesis of such compounds can be quite challenging; however, some general strategies have been developed.[116–119] Although methods for the direct dimerization of individual REs are known,[119] low-molecular-weight multivalent ligands are typically generated by conjugation of REs to a core scaffold. An advantage of these ligands is that the core scaffold can be rigid to give rise to a specific orientation of the REs. Additionally, low-molecular-weight ligands can be generated by combinatorial approaches,[120,121] therefore allowing their shape, flexibility, and other structural features to be varied. Despite the

opportunities to exert precise control over ligand features, most low-molecular-weight ligands are dimeric. Dimeric ligands can be more readily synthesized but can interact with only two cell-surface receptors; thus their utility for investigating the role of higher order receptor organization in signaling is limited.

3.3.2. Protein Conjugates

Semisynthetic routes to multivalent ligands often involve the incorporation of REs onto well-characterized carrier proteins such as streptavidin, bovine serum albumin (BSA), and keyhole limpet hemocyanin (KLH).[122,123] The size and shape of the scaffolds vary: the tetrameric streptavidin has a rectangular structure with dimensions of approximately 6 nm × 10 nm, while human serum albumin has a globular shape with a diameter of approximately 10 nm. The drawback of protein conjugates and other multivalent compounds generated by semisynthesis is their relative heterogeneity compared to scaffolds generated by chemical synthesis. Protein conjugation reactions typically depend on the presence and accessibility of specific endogenous amino acid side chains. Thus, the opportunities for controlling the orientation of the epitope are limited. Interestingly, this limitation has been partially overcome recently by introducing functionality to viral coat proteins. Viral particles bearing these modified proteins organize the functionality in defined patterns.[124–126] This is a promising strategy that is just beginning to be explored, and the scope of its application is not yet known.

3.3.3. Solid Supports

Receptor binding, activation, and endocytosis have been studied using functionalized beads and surfaces.[127–130] The beads employed in these investigations vary in composition: they can be derived from polystyrene, latex, polysaccharides, or other insoluble materials. The typical reactions used to conjugate binding epitopes to beads are straightforward and general, although rarely chemoselective or regioselective. A variety of small and large synthetic and natural REs have been incorporated. The number of potential sites that can be functionalized on the surface varies with the bead composition. Although beads are widely used, the orientation and availability of their binding sites has not been characterized; generally, the distribution of sites on the bead (and perhaps on the RE) is assumed to be random. Bead size is another variable that can influence bead activity. Indeed, the diameter of the beads can vary widely. For example, sepharose beads, which are used for size-exclusion chromatography, have diameters of 30 to 300 μm; latex beads, with diameters of 0.2 to 2 μm, are much smaller. The number of receptors that a functionalized bead can occupy will depend on many variables: the number of functional REs presented, the density of RE sites, and the size of the beads. The only variable that can be controlled readily is the size of the beads. As with protein scaffolds, a drawback of the beads is the lack of control they offer.

3.3.4. Liposomes

Liposomes are typically noncovalent assemblies that can be used to present multiple REs. It is the lipid–lipid interactions that give rise to the array;[131–133] thus, liposome composition can be controlled by varying the ratio of RE-bearing and unmodified lipids. Liposomes can be generated in a wide range of sizes. For example, liposomes generated by treatment with biobeads affords species with diameters of 0.05 to 0.5 μm.[134] Large liposomes (termed giant vesicles) with diameters of 5–200 μm can also be produced.[135] The size of the liposomes can be nearly homogeneous; however, the arrangement of REs within each liposome is difficult to regulate. Fluctuations in RE presentation within a liposome can be an advantage in generating RE displays that are highly active. The orientation and density of REs can change as the lipid components undergo two-dimensional diffusion; thus allowing the most active arrangement of REs to be found. This dynamic behavior is detrimental for mechanistic studies. Another potential drawback of liposomes is that individual components can partition into biological membranes; therefore, the use of liposomes can be problematic for mechanistic studies involving organisms or even cells.

3.3.5. Dendrimers

Dendrimers are often used as multivalent ligand scaffolds.[96,136–141] These ligands can be fairly homogeneous,[96] which can aid in relating ligand features to biological activity. Beads and carrier–protein conjugates often form heterogeneous populations of conjugates since the sites of RE conjugation are often unknown. In contrast, the architectural features of a dendrimeric ligand are defined by the choice of scaffold and the methods used for its synthesis. For example, starburst (PAMAM) dendrimers of generation 0 have diameters of 1.5 nm and valencies of four.[142] Each increase in generation increases the diameter by 0.7 to 1.6 nm while also doubling the maximum valency. Thus, as the size of the dendrimer increases, so does the RE density. Some of the REs of such highly functionalized dendrimers can be inaccessible to proteins.

3.3.6. Polymers

Modern polymer chemistry is providing new opportunities for the synthesis of tailored, biologically active polymeric (or oligomeric) ligands. The multivalent ligands derived from polymers are typically composed of a central backbone that presents multiple copies of an RE. Ligands of this type can be produced by using a variety of synthetic methods. Polymers are generally assembled in a single step by methods such as radical, ionic, or ring-opening metathesis polymerization of RE-bearing monomers.[101,143–149] Alternatively, REs can be appended to a preformed polymeric scaffold.[101,147] Certain polymerization reactions are more tolerant of biologically active functionality than others; thus, the type of epitopes to be incorporated will determine the most effective synthetic strategy.

Selective polymerization reactions enable the biologically active ligand to be synthesized with controlled valency.[84] Living polymerization reactions, in which the rates of chain termination are low, are powerful methods for generating well-defined multivalent ligands. Polymers of narrow polydispersity can be generated when the polymerizations have fast initiation and slow propagation rates.[150] Such polymerization reactions can be used to synthesize multivalent ligands with a variety of sizes. For example, linear polymers with molecular weights of approximately 3000 generated by ring-opening metathesis polymerization (ROMP) can span 100 nm.[151] Polymers displaying dendrimeric appendages can reach lengths of 10 to 100 nm.[152] In addition to allowing control over the degree of polymerization, living polymerization reactions can be used to synthesize block copolymers that display different REs (or simply different functionality) within each block.[153]

Polymers generated by ROMP have found increasing use as biologically active multivalent ligands.[101,149,154–158] Ruthenium–carbene catalysts for ROMP can be used to generate materials of distinct valency.[150,159–161] These ligands have been generated as inhibitors of saccharide–protein interactions,[151,162–164] as ligands for combating vancomycin-resistant bacteria,[156] and as effectors of biological responses.[46,165] Importantly, this polymerization method allows control over the display and density of REs.[166,167] The differences in activity between these compounds can, therefore, be attributed to specific aspects of ligand structure.

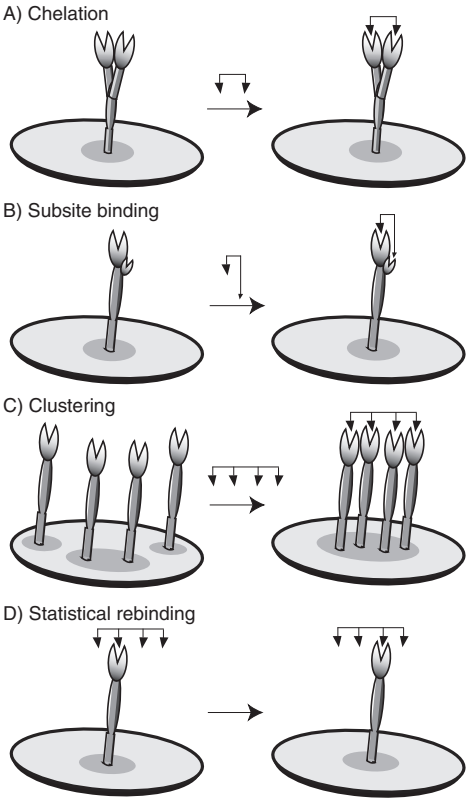


Figure 5. Receptor binding mechanisms that are unique to multivalent ligands.

3.3.7. Combinatorial Synthesis of Multivalent Ligands

Combinatorial methods offer a new approach to the synthesis of multivalent ligands. Combinatorial and diversity-oriented synthesis of monovalent molecules has facilitated the discovery of low-molecular-weight ligands that are optimally designed to bind to a target receptor.[168–173] Similarly, libraries of multivalent ligands might be expected to contain compounds that possess diverse activities.[119–121,166,167,174]

The technical hurdles to overcome in the synthesis of small-molecule libraries are different from those encountered with multivalent ligand libraries. For example, multivalent ligands bearing different REs can have different physical properties; therefore, they will require different purification methods. Combinatorial approaches to multivalent ligand synthesis are in their infancy, and only a few synthetic strategies for generating diverse multivalent ligands have been described.[119–121,153,166,167,174] Future advances in synthetic methods and purification technologies will be instrumental in providing multivalent materials with a broad range of architectural diversity.

3.4. Mechanisms of Multivalent Ligand Binding

Multivalent ligands can bind receptors in ways that are inaccessible to monovalent compounds (Figure 5).[100,115,175] For example, a ligand with multiple copies of an RE can bind to multiple binding sites on a single oligomeric receptor (examples of such receptors are immunoglobulins and some lectins). The cost for translational entropy is paid with the first receptor–ligand contact, and subsequent binding interactions proceed without additional penalties in translational entropy.[176] Excellent examples of this chelation binding mode are presented in recent studies directed at devising multivalent inhibitors of pentavalent toxins.[177–179] Similarly, certain receptors possess binding subsites in addition to their primary site of interaction. Unlike monovalent ligands, which can only access subsites adjacent to their primary binding pocket, multivalent ligands may gain binding energy from contacting more remote secondary sites.[180] Either an RE or another component of the scaffold can contact these subsites. In situations where the multivalent ligand occupies more than one binding site—whether it is a subsite or a primary site within an oligomeric receptor—the ligand typically will bind with a high functional affinity.[181] Another mechanism by which a multivalent ligand can act is through steric stabilization: The steric bulk of the multivalent ligand may preclude the engagement of a bound receptor with an opposing viral particle or cell.[182] This aspect of multivalent ligand activity is particularly relevant for inhibitors of binding interactions at the cellular or viral surface. All these binding modes together are unique to multivalent ligands and have led to numerous applications of these reagents as inhibitors of macromolecular interactions.

Multivalent ligands do not have to inhibit a process, they can act as *activators* of signal transduction. Multivalent ligands can bind avidly to multiple receptors on the cell surface, a process that is facilitated in the fluid lipid bilayer by

the two-dimensional diffusion of receptors. The multivalent ligands can activate signaling pathways if they can cluster signaling receptors (Figure 5c). It is this mechanism, which is uniquely available to multivalent ligands, that is the focus of the remainder of this Review.

We have outlined the three major concepts that are critical for the application of multivalent ligands as probes of signal transduction: 1) signal transduction cascades are mediated by receptor–receptor interactions, and promoting receptor assembly is critical for signaling (Figure 3). 2) Multivalent ligands can interact with the target receptors through multiple binding modes (Figure 5). 3) The structure of a multivalent ligand will determine the favored binding modes (Figure 4). Thus, the structure of the ligand can be optimized to elicit the desired biological response.[84] In the next sections, we provide some examples that illustrate the utility of multivalent ligands as mechanistic probes.

4. Using Multivalent Ligands to Gain Insight into Receptor Function

The binding of a multivalent ligand to a cell surface can assemble a multiprotein complex with distinct features. The valency of the ligand, the orientation of REs, and the stability of the interaction can be used to control features of the complex: its stoichiometric composition, its size, the orientation of the receptors within it, and the lifetime of the complex. Thus, a multivalent ligand can have a marked influence on the output response of a signaling cascade. The following examples describe how specific multivalent ligands can be used to analyze signaling.

4.1. Receptor Proximity

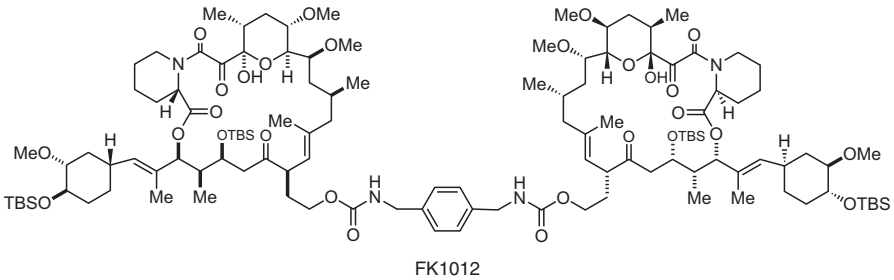
The binding of multivalent ligands can result in clustering of the receptors in the membrane.[84] Here, we discuss some of the many examples in which the ability of multivalent ligands to promote clustering has been used to reveal aspects of receptor function.

4.1.1. Integrins

Integrins are cell-surface receptors that mediate cell adhesion; they also play a central role in the focal adhesion signaling complex.[24,183–185] Integrin function is important in multicellular organisms, and targeted deletion of integrin subunits can strongly impair tissue formation.[186] Given their fundamental physiological roles, it is not surpising that integrin-mediated adhesion and signaling events are carefully regulated.

A key feature of integrins is their ability to switch between low- and high-affinity binding modes.[187] These two modes are thought to allow rapid and reversible adjustments of the strength of the cell adhesion. This switch likely involves changes in the integrin conformation, proximity, or both.[188] Integrins are proposed to adopt at least two conformational states: a bent inactive state and an extended active state.[187] In addition, they can undergo changes in receptor proximity, which can be induced either by interaction with a ligand (an "outside-in" change) or by cellular activation (an "inside-out" change). Although there is evidence that both proximity and conformation influence signaling and adhesive strength, more specific and useful therapeutics may result from a deeper understanding of the relative contributions of these mechanisms. Insight into the influence of the receptor proximity in focal adhesion function is emerging from experiments with synthetic multivalent ligands.

One group of synthetic ligands used to probe integrin clustering are chemical inducers of dimerization (CIDs). CIDs are low-molecular-weight, cell-permeable, divalent ligands that can mediate the clustering of two receptors.[4,116,189–191] A key feature of CIDs is that they can oligomerize receptors fused to a specific ligand-binding protein (Figure 6). This strategy first utilized fusions to FKBP, a protein that binds to the immunosuppressant FK506.[189] FK506 is a low-molecular-weight drug that can be functionalized and converted into the dimer FK1012, which is capable of dimerizing FKBP. FKBP fusion proteins can be transfected or otherwise introduced into cells, thus permitting examination of the functional consequences of FK1012-mediated dimerization of the target fusion proteins. Ligand addition can induce formation of higher order assemblies if multiple copies of FKBP are fused to the target protein of interest.[192] Such strategies have been expanded to include heterodimerization and dimerization



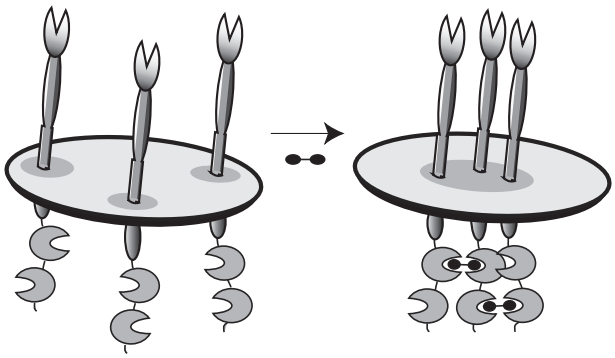


Figure 6. Depiction of the intracellular-mediated alteration of receptor proximity by CID. A receptor fused to a binding protein is expressed in a target cell. Addition of a small-molecule dimerization agent (FK1012) induces the clustering of the receptor. TBS = *tert*-butyldimethylsilyl.

agents that avoid endogenous proteins (for example, FKBP) and specifically target engineered fusion proteins.[192–194]

Both the proximity and conformation of the integrin contribute to the change in the binding affinity. A CID strategy was employed to dissect the role of conformational change from that of receptor proximity.[195] A gene encoding two copies of FKBP in tandem was fused to that encoding αIIb; the latter is a part of the integrin complex αIIb-β3. The addition of the dimerization agent AP1510 elicits integrin clustering in cells producing the fusion protein. If proximity alone is important for the switch between low and high affinity, one would expect that AP1510 would fully replace the need for a natural binding partner. As a measure of the switch to the high-affinity binding mode, an antibody (PAC1) was used that binds specifically to the high-affinity form of the integrin. Addition of the FKBP dimerization agent AP1510 to the cells resulted in a dose-dependent increase in the binding of PAC1. To investigate the role of the conformational change of the receptor, these authors used the monovalent antibody fragment LIBS that binds and potently activates integrins. The increase in binding observed in the presence of AP1510 was modest; the effect from LIBS treatment was more dramatic. These studies with CIDs indicate that, although there is a contribution from receptor proximity, other factors, such as adopting the high-affinity conformational state, contribute significantly to integrin-mediated cell adhesion.

Integrins are cell-surface proteins that are not only involved in adhesion but also in intracellular signaling. Signaling from integrins involves the activation of multiple pathways. As is the case for most complex signaling systems, efforts to deconvolute the contributions of each pathway are ongoing. The two kinases Syk and FAK are implicated in transducing signals through different pathways in integrin signaling. The formation of integrin clusters may favor information transfer through one of these pathways (Syk or FAK) over others. To test this hypothesis, the role of receptor proximity in signaling by Syk and FAK was explored with the dimeric ligand AP1510. When cells were treated with AP1510 alone to cluster the integrin αIIb-β3, an increase in tyrosine phosphorylation by Syk was observed. The requirements for activation of FAK, however, include both the receptor in the high-affinity state (treatment with LIBS) and oligomerization (treatment with AP1510). These results demonstrate the complex interplay between signaling, adhesion, and cellular response that can be achieved by changes in integrin occupancy, proximity, or both. These investigations illustrate the utility of the CID strategy for deconvoluting these contributions. One minor disadvantage of the CID approach, however, is that the cells need to be genetically engineered to express the fusion proteins.

Multivalent surfaces can be used to probe integrin function directly. Integrins can facilitate cell adhesion to extracellular matrix components as well as other cells.[185,196] The extracellular matrix, a naturally occurring multivalent display, may therefore also influence integrin activity.[197] In one study, surfaces were modified with monovalent fibrinogen or monovalent fibrin for use in cell-adhesion studies.[198] Lymphoblastoid B cells were allowed to adhere to the coated microtiter wells (Figure 7 A). The multivalent fibrin was able

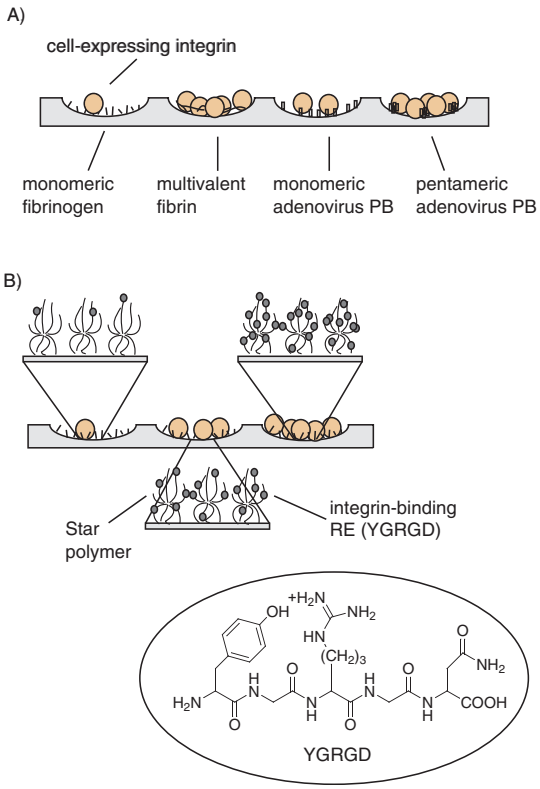


Figure 7. The density of REs presented influences the adhesion of cells to surfaces bearing integrin ligands. A) Surfaces coated with multivalent ligands have a greater functional affinity for B cells. B) Star polymers are depicted that display variable copies of the pentapeptide YGRGD (an integrin-binding RE). The relative potency of each surface is indicated by the number of cells bound.

to support cell adhesion but unactivated cells did not adhere to immobilized monovalent fibrinogen.

In a series of related experiments adenovirus PB, a ligand specific for the integrin $\alpha_v\beta_3$, was coated in wells in either its monomeric or pentameric form (Figure 7b). As in the fibrin experiment, the B cells attached selectively to the wells coated with multivalent ligand. To understand the mechanisms responsible for the differences in binding, the number of possible integrin binding sites in the wells displaying monomeric ($800\,\text{sites}\,\text{mm}^{-2}$) or pentameric ligand ($125\,\text{sites}\,\text{mm}^{-2}$) was determined by radioimmunoassay. Although it had sixfold fewer sites per mm^2, the surface displaying the pentameric integrin ligand was more adhesive. These results suggest that the local arrangement of binding sites, and not the overall number of sites, is critical for integrin-mediated adhesion.

These results are supported by additional data that indicate local RE density is important for the adhesion strength of integrin.[127] Maheshwari et al. used star polymers that display variable numbers of copies of the minimal integrin ligand YGRGD to investigate the impact of binding-site density on integrin-mediated cell migration and adhesion of $\alpha_v\beta_3$-bearing cells. The integrin $\alpha_v\beta_3$ binds RGD sequences, and surfaces with more YGRGD were more effective at interacting with cells (Figure 7B). Interestingly, however, when surfaces with equal numbers of REs but different local

densities were compared, those with closely packed REs were more efficient at mediating cell migration and adhesion. These results suggest that clustering of the integrins improves their adhesive strength, probably through both enhanced functional affinity for the ligand and by activation of the signaling pathways.

These experiments with synthetic multivalent surfaces have highlighted a role for receptor proximity in integrin function. These studies also provide information about the most effective types of natural integrin ligands. Unfortunately, these multivalent surfaces cannot be used to explore integrin function in living organisms. Experiments utilizing soluble multivalent ligands could be used to investigate the consequences of integrin clustering in physiological settings.

4.1.2. G-Protein-Coupled Receptors

GPCRs are one of the largest families of mammalian cell-surface receptors. They participate in many important physiological processes. Their significance is underscored by their importance to medicine: over 50% of therapeutics on the market act through GPCRs.[199] Mounting evidence implicates GPCR homo- and heterodimerization in the regulation of signaling from these receptors.[117,200] Therefore, it would be valuable to elucidate the contribution of GPCR oligomerization to understand GPCR signaling and to exert control over it. The role of the ligand in modulating the clustering of GPCRs is unknown, but experiments using synthetic multivalent ligands are beginning to address this issue.

Low-molecular-weight divalent ligands have been used to assess the consequences of receptor dimerization.[103,117,186,201–207] An illustration of this approach is the use of the dimer of a GPCR ligand to activate neutrophil chemotaxis.[208] Neutrophils are attracted to sites of bacterial infection by by-products of bacterial protein synthesis, such as *N*-formylmethionine-containing peptide fragments. Perhaps the most widely studied neutrophil chemoeffector is *N*-formylmethionine-leucine-phenylalanine (fMLF), which is recognized by the formyl peptide receptor (FPR)—a GPCR. Dimers presenting two copies of fMLF were, therefore, employed to explore the role of receptor proximity in the regulation of FPR signaling.

Mono-, di-, and triethylene glycol-linked dimers of fMLF were generated by coupling a linker to the C terminus of the peptide. The monoethylene glycol dimer would not be expected to be capable of dimerizing the FPR; consequently, any enhanced chemotactic activity relative to fMLF is likely due to an increase in avidity. In contrast, the authors suggest that the triethylene glycol linked dimer may facilitate dimerization of the receptor, although the linker length required to simultaneously occupy two copies of the FPR is not known. The authors generated a series of divalent ligands, hoping that those with longer linkers might dimerize the FPR.

Two assays were used to explore FPR function: chemotaxis and superoxide production.[208] Neutrophils are recruited in vivo to the location of the invading pathogen through chemotaxis; the release of superoxide serves a killing role. These two cellular responses must occur with strict spatial and temporal control to minimize damage to healthy tissue. Specifically, chemotaxis towards a site of bacterial infection must precede superoxide formation or the neutrophils could prematurely release toxic oxygen species. Both of these responses can be elicited in response to ligand binding to FPR. It is important, therefore, to understand what factors influence the triggering of these distinct cellular responses.

The synthetic dimers were added to cells expressing FPR, and the chemotaxis and superoxide levels were monitored. The triethylene glycol linked dimer was the most active chemoattractant, while the monoethylene glycol dimer elicited the highest level of superoxide production. Although FPR dimerization was not tested directly, these results suggest that dimerization of the receptor may influence chemotactic responses. The increased ability of the monoethylene glycol dimer to elicit superoxide release was attributed to it having higher affinity for the FPR. Although the specific mechanisms of action of these divalent agents were not probed further, these initial results suggest that synthetic multivalent ligands will be useful in illuminating the role of receptor clustering in GPCR signaling in general, and chemotactic signaling in neutrophils in particular.

Receptor heterooligomerization can provide a means of integrating information from multiple pathways into a coherent cellular response. The consequences of GPCR heterodimer formation have been probed by creating heterodimeric ligands.[103] This approach was taken to study the effects of coclustering a GPCR that responds to enkephalin with one that is activated by neurotensin. Both natural ligands are important regulatory neuropeptides that function as synergistic activators of GPCR signaling.[209] The goal of the studies was to determine whether there might be a direct potentiation of signal through assembly of a heteroligomeric GPCR complex.

Cells expressing both receptors were treated with neurotensin, a mixture of neurotensin and enkephalin, or a difunctional neurotensin–enkephalin conjugate[209] The cytosolic cyclic GMP (cGMP) concentration was measured at a variety of ligand doses. Only a subtle increase in cGMP production was observed when both hormones were added separately. In contrast, the covalent heterodimer was a potent inducer. These data suggest that the forced proximity of the two receptors is the primary cause for the increased level of cGMP. As in the previous example, however, direct data in support of heterodimerization are lacking.

Further experimentation with ligands bearing higher valencies or more diverse architectures may afford more definitive insights into the effects of receptor proximity on GPCR-mediated signaling. It will be especially useful to couple signaling studies with those that address the influence of ligands on GPCR assembly. Since so many drugs target GPCRs, these investigations may facilitate the design of a new generation of therapeutics.[210]

4.1.3. T-Cell Receptors

T lymphocytes are key mediators of mammalian immunity.[13,22,78,211] These cells recognize foreign antigens through their T-cell receptors (TCRs). Binding at T-cell receptors can result in cellular activation, which can precipitate the killing

of tumor cells, pathogens, and virally infected cells. As discussed in Section 1, when T cells encounter antigen-presenting cells, an immune synapse can form at the cell–cell interface. This immune synapse, which contains the TCR as well as other co-receptors and signaling components, possesses exquisite organization.[76]

There is evidence that changes in the organization of the TCR and other signaling components are important for T-cell activation; however, the contribution of various factors to TCR signaling remains unclear. Despite the large body of literature on TCR organization and signaling, questions remain concerning the molecular details of TCR function. For example, it is uncertain as to whether the dimerization of two TCRs is sufficient stimulus for T-cell activation or whether TCR oligomerization is required. The role of simple monovalent ligation of individual TCRs is also unknown.[212] The purpose of the synapse is unclear if individual or small groups of TCRs can activate signaling. Multivalent ligands will undoubtedly play prominent roles in addressing these fundamental issues.

One strategy for studying TCR function uses multivalent protein conjugates which are formed from the high-affinity interaction between biotin and the tetravalent protein streptavidin.[213] The four identical biotin-binding sites of streptavidin each bind with a dissociation constant of 10^{-15} M; therefore, biotinylated recognition elements can be readily displayed from a streptavidin scaffold. Since streptavidin is tetravalent, multivalent complexes can be generated that present 1, 2, 3, or 4 recognition epitopes per scaffold (Figure 8). Davis, McConnell, and co-workers developed streptavidin as a scaffold for the presentation of peptide-loaded major histocompatibility complexes (MHCs).[213] Since peptide-loaded MHCs can serve as ligands for the TCR, streptavidin-bound biotinylated MHC complexes can bind (in principle) up to four TCRs.

To generate these multivalent presentations, a peptide derived from moth cytochrome *c* (MCC) was added to the MHC. The resulting MCC–MHC complex was singly biotinylated and mixed with streptavidin; this procedure should yield a tetravalent complex that can bind the TCR (Figure 8). The amount of active MHC complex in the assembly reaction was varied to favor formation of mono-, di-, tri-, or tetrameric structures. Biotinylated MHC that lacks MCC was also produced, and these proteins, which cannot bind the TCR, were added to occupy the remaining streptavidin sites. A complex displaying four unloaded MHCs, which does not display foreign antigen, served as a control.

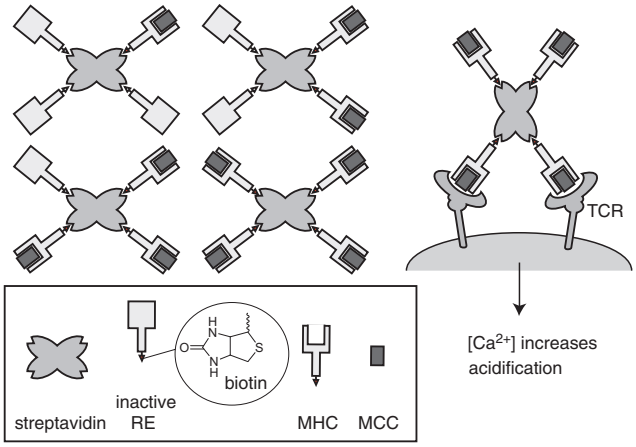


Figure 8. Schematic representation of streptavidin-MHC-MCC complexes. Left: Four complexes with one, two, three, or four biotinylated MHC-MCC moieties; right: a model for the activation of TCRs by multivalent engagement by the highest valency ligand.

To test the effects of the assemblies on T-cell activation, decreases in extracellular pH values and increases in the concentration of intracellular Ca^{2+} were measured. In both cases, the tetramer was the only ligand to elicit a significant increase in activity. Although effects arising from changes in binding kinetics cannot be ruled out, these experimental results suggest that changes in TCR proximity influence T-cell activation. Similar results were obtained using chemically defined peptide-based multivalent ligands, although a dimeric ligand was sufficient to activate TCR signaling.[214,215] These data taken together suggest that valency is an important feature of natural TCR ligands and that receptor proximity is important for the amplification of TCR-mediated signals.

Factors other than TCR ligation also contribute to activation and signal modulation. Indeed, numerous co-receptors serve to positively or negatively modulate the activation signal. One limitation of using streptavidin-based scaffolds is that it is difficult to incorporate multiple REs that can engage co-receptors and TCRs simultaneously. Thus, scaffolds that can be functionalized to present multiple types of REs have been employed.

A variety of beads and surfaces have been used as multivalent displays to explore T-cell signaling.[9,129,216–218] In this way, a study addressing the mechanism by which T-cell responses are enhanced by the co-receptor CD28 was undertaken.[87] Ligand binding to the TCR is necessary and sufficient for activation of T cells; however, co-stimulation of CD28 yields a more potent response. To determine the mechanism for enhanced signaling, beads were used to mimic the physical size of the surface of an antigen-presenting cell (APC), which presents ligands on its cell surface for the TCR and CD28.

Latex beads were modified to assess the consequences of simultaneous binding to receptors. Specifically, they were designed to cocluster the TCR and CD28: an antibody that recognizes the TCR and another that binds the co-receptor CD28 was attached. The beads were tested for their ability to induce cell proliferation, which occurs upon lymphocyte activation. If T-cell activation requires simultaneous binding of both the TCR and CD28, only the beads displaying REs for both receptors should activate the cells. Beads displaying only the TCR clustering element (anti-TCR) did not promote T-cell proliferation. In contrast, beads displaying both anti-TCR and anti-CD28 were potent activators of proliferation.

One explanation for the data is that simultaneous binding of CD28 and TCR promotes the co-localization of these receptors to a region of the membrane termed a lipid microdomain. It has been suggested that lipid microdomains concentrate signaling components and facilitate formation of mature signaling complexes. Support for a role for microdomains in enhancing signaling came from the concentration

of a fluorescent marker of lipid rafts (fluorescein-labeled cholera toxin B subunit) into a dense fluorescent patch at the point of contact between the bead and the T cell. These results demonstrate that co-stimulation of the TCR and co-receptors can modify their distribution on the cell surface, which may modulate immune responses. An important caveat to these experiments, however, is that the REs on a bead-based multivalent ligand are immobilized. Thus, unlike cell-surface REs, the REs attached to the bead cannot undergo rearrangement. However, similar results were obtained when an antigen-presenting cell was used as a natural "ligand". This comparison suggests that the RE-decorated bead effectively mimics some aspects of an antigen-presenting cell.[219] Thus, a role for the CD28 co-receptor in regulating receptor proximity and immune function was implicated from studies using functionalized beads as multivalent ligands.

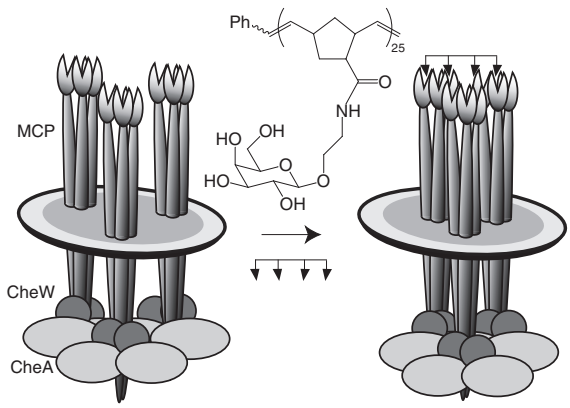


Figure 9. Investigation of receptor proximity effects in bacterial chemotaxis by using synthetic multivalent polymers. Addition of a multivalent ligand with sufficient valency can induce the reorganization of MCPs which potentiates signaling and activates bacterial locomotion.

4.1.4. ***Bacterial Chemoreceptors***

Bacteria must migrate towards nutrients and away from toxins to survive. Bacterial chemotaxis is driven by a well-characterized signaling pathway that is responsible for the detection of these nutrients and toxins.[220–222] Five types of membrane-bound chemoreceptors (MCPs) mediate chemotaxis in *Escherichia coli*. Each MCP type is responsible for detecting and mediating chemotaxis towards a subset of small molecules or other stimuli. Bacteria require integration of signals from multiple stimuli into a coherent response to initiate appropriate locomotion. Moreover, responses are mounted against stimuli at very low concentrations, which requires a significant level of signal amplification.[223–225]

The mechanism used by bacteria to amplify and integrate chemotactic signals has long been debated. One recent hypothesis is that signal amplification is achieved through interreceptor communication.[26,54,60,226,227] Interestingly, the MCPs are organized within the cell: They are concentrated at the poles of *Escherichia coli* and other bacterial species.[39,228] This organization of chemoreceptors into an array has been proposed to be important in signal transduction. Recent experiments using synthetic multivalent ligands and other approaches support such a view; they suggest that communication between homologous and heterologous MCPs within the chemoreceptor array is responsible for chemotactic responses.[46,54,60,62,229–231]

The first goal was to generate multivalent attractants that could cluster the chemoreceptors. ROMP was used to synthesize multivalent ligands that display chemotactic carbohydrate residues in sufficient valency (ca. 25 monomer units) to mediate MCP clustering, as shown by microscopy. This change in receptor proximity influenced signaling through the MCPs: ligands capable of clustering MCPs were also potent activators of chemotaxis (Figure 9). It was shown by using a fluorescent probe that galactose-bearing multivalent ligands cluster the galactose-sensing receptor Trg. Surprisingly, they also cluster the serine-sensing receptor Tsr. Tsr does not bind galactose; its presence in the cluster suggests that chemoreceptor–chemoreceptor interactions bring it into the cluster. These data indicate that there are interactions between different types of chemoreceptors.

Evidence in support of the functional significance of chemoreceptor–chemoreceptor interactions was obtained by examining the effects of receptor clustering on signal output. Specifically, a multivalent galactose derivative was introduced to cluster the chemoreceptors. When the bacteria had adapted to the multivalent attractant, the monovalent attractant serine was added. Under these conditions the chemotactic response to serine was potentiated by 100- to 1000-fold. This result indicates that heterologous MCPs communicate so as to amplify and integrate chemotactic signals. The proximity of multiple types of receptors is critical for signal amplification. Thus, a single receptor is not all that is needed to sense a particular compound—all chemoreceptor types contribute to proper sensing and signal amplification.

The application of multivalent chemoattractants to examine chemoreceptor proximity in chemotaxis illustrates their power: They can be used to explore signal transduction even when receptor dimerization is known to be required. Changes in receptor organization had not been implicated in chemotactic signal transduction: the role of receptor organization might have been overlooked because the known chemoattractants are monomeric, and the MCPs are dimeric in the absence of ligands. Nevertheless, studies indicate that changes in MCP proximity influence signal amplification, and evidence supporting such a model was obtained using multivalent ligands. Moreover, the use of these ligands did not require genetic manipulation of the bacteria; thus, the behavioral response elicited by the multivalent ligand could be directly analyzed under physiological conditions and in wild-type genetic backgrounds. These results underscore that soluble, structurally defined multivalent ligands are valuable probes.

4.2. Receptor Orientation

Many cell-surface receptors possess domains with intrinsic enzymatic activity, while some interact with proteins with catalytic domains. The substrates of these enzymatic activities are often other signaling proteins. The relative orientations of

the active site of the receptor-associated enzyme as well as the substrate may influence the amount of product generated; this enzymatic efficiency, in turn, will influence signal transduction.[225,229,230,232] It is often unclear whether the activity of an assembled signaling complex is the result of a specific orientation of receptors within the complex or simply the localization of signaling components.[233] Since multivalent ligands can organize receptors into specific orientations, they have the potential to serve as tools for investigating the role of protein orientation in signaling.

4.2.1. G-Protein-Coupled Receptors (GPCRs)

One key feature of multivalent ligands is that they can occupy multiple binding sites on a single receptor and thereby exploit the chelate effect (Figure 5a).[176] Multivalent ligands that use this binding mode can interact with high functional affinities, and is an important determinant of agonist or antagonist potency. In a recent example, a low-molecular-weight dimer was constructed that can bind simultaneously to two sites on the corticotrophin-releasing factor receptor (CRFR-1). CRFR-1 is a GPCR that is a key regulator of adrenocorticotropic hormone (for example, testosterone) release during the stress response.[234]

A series of dimers was generated, in which different orientations of the REs were controlled by using helical and rigid linkers. The addition of these dimers to cells expressing CRFR-1 resulted in the release of testosterone. Potent activity was elicited by the dimers that maintained a *trans* orientation between the terminal REs. Ligands that did not maintain this orientation were tenfold less effective at causing testosterone release. The experiments conducted do not eliminate the possibility that the observed differences arise from ligand-induced changes in receptor oligomerization, but the results suggest that the orientation of REs can influence receptor activation.[117,235]

4.2.2. ZAP-70

Chemical synthesis can be used to create ligands to probe the role of protein orientation in assembled signaling complexes. In the previous example, the relative orientation of the binding moieties within a multivalent ligand influence its ability to elicit signaling through a target receptor. When a multivalent ligand acts by clustering receptors, however, the geometric constraints on the binding epitopes might be relaxed. In the few examples studied to date, the localization of receptors to a signaling complex is often more important than their specific orientation within the complex.[233,236] The ability of dimeric ligands to activate signaling by ZAP-70 is consistent with this view and highlights that different RE orientations often give rise to only subtle effects.[107]

ZAP-70 is a kinase whose function is required for the varied signaling functions of the T cell. If the orientation of ZAP-70 is important for its function, multivalent ligands that control its orientation should possess varying abilities to elicit T-cell activation. To this end, a series of small-molecule dimeric ligands was generated; each possessed conformationally restricted linkers between the two identical REs. The activity of this series was assessed by adding CIDs to cells encoding a membrane-docking protein (three copies of FKBP fused to a myristoylation domain of v-Src) and a ZAP-70–FKBP fusion protein (Figure 10). Presumably, these CIDs can

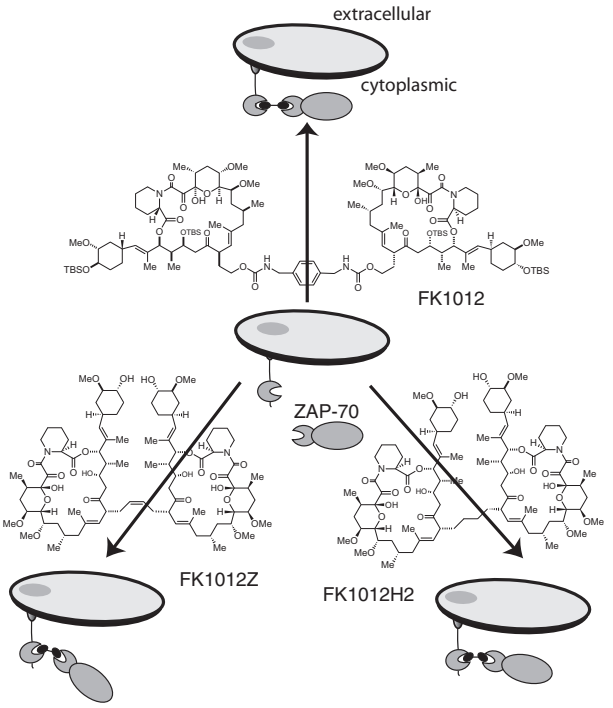


Figure 10. Investigation of the influence of the orientation of ZAP-70 on its kinase function by a CID strategy. The chemical structures of three dimerization agents FK1012, FK1012H2, and FK1012Z are shown. These dimers present REs in three distinct relative orientations, but their abilities to induce ZAP-70 activity were similar.

bind and cluster the fusion proteins, thereby recruiting ZAP-70 to the membrane. All of the synthetic CIDs mediated ZAP-70 recruitment and all activated signaling. While large changes in orientation may influence ZAP-70 signaling, the presumed changes in the orientation afforded by the CIDs had little effect.

The difference in the results of the CRFR-1 versus the ZAP-70 studies suggests a plasticity in the assembly of signaling complexes: For signal activation, the REs of dimeric ligands that occupy subsites within a receptor must be more carefully aligned than those that act by inducing changes in receptor proximity. Interestingly, these findings are consistent with studies in which fusion proteins have been used to test whether different assemblies of signaling domains influence output.[233,236] As with the ZAP-70 investigations, the orientation of different signaling domains was much less important than their recruitment to a signaling complex. It will be interesting to explore the generality of these findings further.

4.3. Composition of Receptor Clusters

The number of receptors in a complex can influence its biological activity. Cell adhesion is expected to be especially

sensitive to the number of receptors in a complex; the functional affinity of the array is expected to be directly related to the number of receptor–ligand complexes formed.[100] Signal transduction processes also may be sensitive to the number of receptors in the complex; however, it can be difficult to determine the quantitative role of receptor composition in signaling. Multivalent ligands can be generated that vary in the density and valency of REs so as to investigate either cell adhesion or cell signaling. These ligands, therefore, may have the ability to generate specific clusters of receptors that vary in the number of proteins included. This strategy has been utilized to investigate the role of composition for receptor function.

4.3.1. *B-Cell-Antigen Receptor*

The B-cell-antigen receptor (BCR) is a complex comprised of proteins involved in the recognition of antigens and the generation of antibodies during acquired immune responses.[23,85,237] The activation of BCR-mediated functions must be strictly regulated, since inappropriate activation can cause autoimmune disease. Understanding the minimal requirements for BCR-mediated activity is therefore critical. Synthetic multivalent ligands have played key roles in investigating the importance of antigen stoichiometry in the activation of this system.

Pioneering work by Dintzis et al. and others in the field of BCR signaling in the 1970s and 1980s utilized synthetic multivalent ligands to explore the role of valency in B-cell responses.[238–242] The binding of a multivalent antigen to the B-cell receptors (BCRs) can activate B-cell signaling. A series of multivalent ligands were generated using polyacrylamide, dextran, carboxymethylcellulose, and polyvinyl alcohol as scaffolds to test how the valency of T-cell-independent antigens influences antibody production.[243] These ligands were injected into mice, and the efficiency of antibody production was measured. In every case, irrespective of the scaffold structure or the amount of polymer branching, the immunogenicity of the ligands strictly depended on the RE valency. From these data, the authors hypothesize that the number of BCRs included in a cluster is a principle determinant of function. Moreover, they propose that occupation of approximately 12 BCRs is required for B-cell activation. There are many steps in this complex system between B-cell activation and antibody production. These investigations highlight, however, the power of synthetic multivalent ligands for answering questions of fundamental biological importance.

4.3.2. *L-Selectin*

Leukocyte migration from the blood to lymphatic tissues is dependent on the function of L-selectin; L-selectin also mediates leukocyte recruitment to sites of inflammation.[244] L-selectin is displayed on the surface of leukocytes, and it typically localizes to patches at the tips of the cellular microvilli (Figure 11).[237,241–246] The natural ligands for L-selectin are glycoproteins displayed on the endothelium of the blood vessel.[247] The binding of L-selectin to these ligands slows the progress of cells through the blood vessels and allows tight adherence of the leukocyte to the endothelium. Physiological L-selectin ligands typically present multiple copies of derivatives of the sulfated sialyl Lewis x antigen (sLe^x); synthetic multivalent ligands that display these and related carbohydrate epitopes have been shown to be effective selectin ligands.[248–253] Multivalency, therefore, may be an important determinant of L-selectin function in vivo. Experiments using synthetic multivalent ligands have started to reveal the importance of multivalency and stoichiometry of selectin–ligand clusters for L-selectin recognition.

The role of the receptor composition in regulating the adhesion function of L-selectin was investigated using synthetic multivalent ligands derived from ROMP.[254] The polymers were specifically end-labeled with a single fluoro-

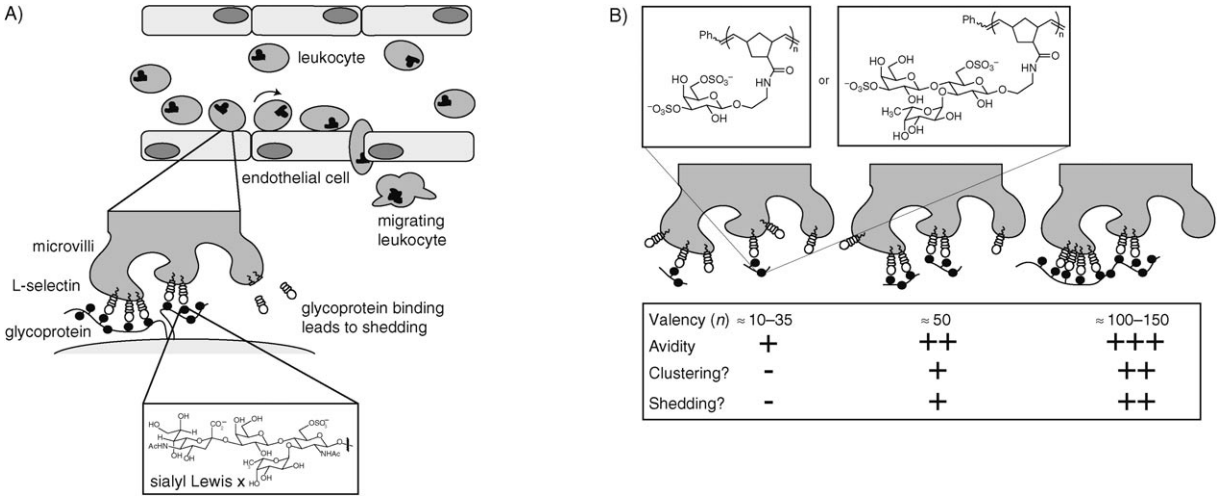


Figure 11. Multivalent ligands for L-selectin mimic cell-surface glycoproteins. A) L-selectin expressed on lymphocytes binds to glycoproteins on the endothelium. This interaction slows lymphocyte progression through the vessel and triggers proteolytic release of L-selectin. B) Multivalent polymers displaying sulfated carbohydrates also bind multiple copies of L-selectin, which leads to receptor clustering and proteolytic shedding. There is a direct relationship between the valency of the polymer, the number of L-selectin proteins bound, and the avidity of the interaction.

phore to visualize binding and to determine how many copies of a multivalent ligand bind L-selectin-positive cells.[255] It was found that the number of copies of L-selectin that bind to a multivalent sulfated carbohydrate derivative depends on its valency (Figure 11).[254] A related observation is that the functional affinity of a ligand for cells displaying L-selectin is directly related to its valency. Increasing the valency from 35 monomer units to 150 resulted in an increase in the relative ratio from 1.1 to nearly 5 copies of L-selectin. The functional affinity of the interaction improved 10-fold. These results suggest that the adhesive strength of L-selectin is related to the stoichiometry of the selectin–ligand complex.

Intriguingly, these polymers not only bind L-selectin but also promote its proteolytic release, or shedding, from the cell surface.[256,257] It appears that cell signaling is important for triggering the release of L-selectin:[165,258] it is possible that clustering L-selectin activates the signaling cascade that leads to its cleavage; or perhaps physiological L-selectin ligands also influence L-selectin levels in vivo. These results taken together suggest that the stoichiometry of L-selectin–ligand interactions is an important determinant of both the adhesive and signaling functions of this protein.

5. Summary and Outlook

Cell-surface receptors mediate the essential tasks of cell adhesion and signaling. In these roles receptors are rarely left to function as isolated entities. Rather, they collaborate as constituents of higher order macromolecular assemblies. Understanding the mechanisms underlying the function of these assemblies is critical. Multivalent ligands can serve as powerful tools for the deconvolution of complex multireceptor networks. The use of synthetic multivalent ligands is complementary to other approaches; therefore, they provide the means to address important new questions.

In addition to utility as agents for examining receptor function, synthetic multivalent ligands have potential applications in the treatment of disease. Multivalent ligands can participate in binding mechanisms that are not available to small molecules. For example, Whitesides, Collier, and coworkers took advantage of this unique aspect of multivalent ligands to generate potent polyacrylamide-based multivalent ligands that function as inhibitors of the anthrax toxin.[259] The success of these and other[260,261] in vivo applications of multivalent ligands indicates that they may function not only as probes of biological processes but even as therapeutic agents. In addition to their potential uses as inhibitors, multivalent ligands can mediate the clustering of cell-surface proteins and thereby function as effectors. While many small-molecule inhibitors are known, it is typically more difficult to find small molecules that serve as activators of signal transduction. The ability of multivalent ligands to activate signal transduction pathways suggest that they may have complementary therapeutic applications.

Multireceptor complexes are the requisite entities that convert extracellular stimuli into appropriate cellular responses. Communication and coordination between receptors provides a means to amplify, integrate, and process signals. Understanding how these processes occur at a molecular level is crucial to illuminating the function of biological *systems*. While monovalent ligands are powerful tools for disrupting such complexes, they cannot be used to examine the importance of the formation of complexes. Multivalent ligands have the necessary attributes to direct the formation of multiprotein complexes and/or control the localization of multiple proteins within the cell. We envision that such ligands, which can be used in a similar manner as small molecules for temporal control, will illuminate how protein assembly and/or protein localization direct cellular responses.

In this Review, we have provided an overview of some of the uses of synthetic multivalent ligands as probes. Additional advances in target-oriented and diversity-oriented syntheses of multivalent ligands will afford novel compounds that address increasingly complex biological questions. Such investigations demand a multidisciplinary approach that combines chemical and biological concepts to yield, ultimately, a coherent understanding of the molecular mechanisms governing cellular systems.

Received: August 7, 2005

[1] J.-P. Chaneux, J. Thiéry, Y. Tung, C. Kittel, *Proc. Natl. Acad. Sci. USA* **1966**, *57*, 335.
[2] C.-H. Heldin, *Cell* **1995**, *80*, 213.
[3] J. Schlessinger, *Trends Biochem. Sci.* **1988**, *13*, 443.
[4] J. D. Klemm, S. L. Schreiber, G. R. Crabtree, *Annu. Rev. Immunol.* **1998**, *16*, 569.
[5] H. Metzger, *J. Immunol.* **1992**, *149*, 1477.
[6] J. C. Sacchettini, L. G. Baum, C. F. Brewer, *Biochemistry* **2001**, *40*, 3009.
[7] F. D. Smith, J. D. Scott, *Curr. Biol.* **2002**, *12*, R32.
[8] A. Weiss, J. Schlessinger, *Cell* **1998**, *94*, 277.
[9] J. T. Groves, *Angew. Chem.* **2005**, *117*, 3590; *Angew. Chem. Int. Ed.* **2005**, *44*, 3524.
[10] M. Mellado, J. Rodríguez-Frade, A. J. Vila-Coro, S. Fernández, A. Martín de Ana, D. R. Jones, J. L. Toran, C. Martínez-A, *EMBO J.* **2001**, *20*, 2497.
[11] H. D. Madhani, *Cell* **2001**, *106*, 9.
[12] D. J. Riese II, D. F. Stern, *BioEssays* **1998**, *20*, 41.
[13] M. L. Dustin, A. C. Chan, *Cell* **2000**, *103*, 283.
[14] D. Bray, *Annu. Rev. Biophys. Biomol. Struct.* **1998**, *27*, 59.
[15] S. Tsunoda, J. Sierralta, Y. Sun, R. Bodner, E. Suzuki, A. Becker, M. Socolich, C. S. Zuker, *Nature* **1997**, *388*, 243.
[16] E. Stein, A. A. Lane, D. P. Cerretti, H. O. Schoecklmann, A. D. Schroff, R. L. Van Etten, T. O. Daniel, *Genes Dev.* **1998**, *12*, 667.
[17] M. N. Levit, Y. Liu, J. B. Stock, *Biochemistry* **1999**, *38*, 6651.
[18] M. N. Teruel, T. Meyer, *Cell* **2000**, *103*, 181.
[19] D. A. Lauffenburger, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 5031.
[20] M. B. Kennedy, *Science* **2000**, *290*, 750.
[21] J. H. Kim, R. L. Huganir, *Curr. Opin. Cell Biol.* **1999**, *11*, 248.
[22] R. N. Germain, *Curr. Biol.* **1997**, *7*, R640.
[23] L. Matsuuchi, M. R. Gold, *Curr. Opin. Immunol.* **2001**, *13*, 270.
[24] A. van der Flier, A. Sonnenberg, *Cell Tissue Res.* **2001**, *305*, 285.
[25] T. S. Shimizu, N. Le Novére, M. D. Levin, A. J. Beavil, B. J. Sutton, D. Bray, *Nat. Cell Biol.* **2000**, *2*, 792.
[26] D. Bray, M. D. Levin, C. J. Morton-Firth, *Nature* **1998**, *393*, 85.
[27] P. T. Martin, *Glycobiology* **2002**, *12*, 1R.
[28] S. Damjanovich, R. Gaspar, Jr., C. Pieri, *Q. Rev. Biophys.* **1997**, *30*, 67.

[29] D. Holowka, B. Baird, *Annu. Rev. Biophys. Biomol. Struct.* **1996**, *25*, 79.
[30] P. W. Janes, S. C. Ley, A. I. Magee, P. S. Kabouridis, *Semin. Immunol.* **2000**, *12*, 23.
[31] A. Cherukuri, M. Dykstra, S. K. Pierce, *Immunity* **2001**, *14*, 657.
[32] D. A. Brown, E. London, *Annu. Rev. Cell Dev. Biol.* **1998**, *14*, 111.
[33] C. Langlet, A.-M. Bernard, P. Drevot, H.-T. He, *Curr. Opin. Immunol.* **2000**, *12*, 250.
[34] M. Edidin, *Curr. Opin. Struct. Biol.* **1997**, *7*, 528.
[35] E. D. Sheets, D. Holowka, B. Baird, *Curr. Opin. Chem. Biol.* **1999**, *3*, 95.
[36] T. E. Hebert, M. Bouvier, *Biochem. Cell Biol.* **1998**, *76*, 1.
[37] T. Bartfai, J. L. Benovic, J. Bockaert, R. A. Bond, M. Bouvier, A. Christopoulos, O. Civelli, L. A. Devi, S. R. George, A. Inui, B. Kobilka, R. Leurs, R. Neubig, J. P. Pin, R. Quirion, B. P. Roques, T. P. Sakmar, R. Seifert, R. E. Stenkamp, P. G. Strange, *Nat. Rev. Drug Discovery* **2004**, *3*, 574.
[38] P. R. Gouldson, C. R. Snell, R. P. Bywater, C. Higgs, C. A. Reynolds, *Protein Eng.* **1998**, *11*, 1181.
[39] J. R. Maddock, L. Shapiro, *Science* **1993**, *259*, 1717.
[40] C. C. Yin, F. A. Lai, *Nat. Cell Biol.* **2000**, *2*, 669.
[41] L. Pellegrini, D. F. Burke, F. von Delft, B. Mulloy, T. L. Blundell, *Nature* **2000**, *407*, 1029.
[42] J. Schlessinger, *Cell* **2000**, *103*, 211.
[43] T. Brümmendorf, V. Lemmon, *Curr. Opin. Cell Biol.* **2001**, *13*, 611.
[44] C. Chan, A. J. T. George, J. Stark, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 5758.
[45] A. D. Douglass, R. D. Vale, *Cell* **2005**, *121*, 937.
[46] J. E. Gestwicki, L. L. Kiessling, *Nature* **2002**, *415*, 81.
[47] D. J. Irvine, A. M. Mayes, L. G. Griffith, *Biomacromolecules* **2001**, *2*, 85.
[48] I. Markovic, E. Leikina, M. Zhukovsky, J. Zimmerberg, L. V. Chernomordik, *J. Cell Biol.* **2001**, *155*, 833.
[49] A. H. Elcock, J. A. McCammon, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 2990.
[50] T. E. Hébert, M. Bouvier, *Biochem. Cell Biol.* **1998**, *76*, 1.
[51] M. K. Dean, C. Higgs, R. E. Smith, R. P. Bywater, C. R. Snell, P. D. Scott, G. J. G. Upton, T. J. Howe, C. A. Reynolds, *J. Med. Chem.* **2001**, *44*, 4595.
[52] D. L. Milligan, D. E. Koshland, Jr., *Science* **1991**, *254*, 1651.
[53] S. Terrillon, M. Bouvier, *EMBO Rep.* **2004**, *5*, 30.
[54] V. Sourjik, H. C. Berg, *Nature* **2004**, *428*, 437.
[55] S. W. Fesik, *Cell* **2000**, *103*, 273.
[56] H. Wu, *Cell Surf. Recept.* **2004**, *68*, 225.
[57] J. J. Skehel, D. C. Wiley, *Annu. Rev. Biochem.* **2000**, *69*, 531.
[58] E. W. Yu, D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 9517.
[59] T. L. Blundell, D. F. Burke, D. Chirgadze, V. Dhanaraj, M. Hyvonen, C. A. Innis, E. Parisini, L. Pellegrini, B. L. Sayed, *Biol. Chem.* **2000**, *381*, 955.
[60] P. J. Gardina, M. D. Manson, *Science* **1996**, *274*, 425.
[61] I. Tatsuno, M. Homma, K. Oosawa, I. Kawagishi, *Science* **1996**, *274*, 423.
[62] P. Ames, C. A. Studdert, R. H. Resier, J. S. Parkinson, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 7060.
[63] S.-H. Kim, W. Wang, K. K. Kim, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11611.
[64] P. A. Thomason, P. M. Wolanin, J. B. Stock, *Curr. Biol.* **2002**, *12*, R399.
[65] A. Ullrich, J. Schlessinger, *Cell* **1990**, *61*, 203.
[66] F. Alderton, S. Rakhit, K. C. Kong, T. Plamer, B. Sambi, S. Pyne, N. J. Pyne, *J. Biol. Chem.* **2001**, *276*, 28578.
[67] F. Liu, Q. Wan, Z. B. Pristupa, Y. T. Yu, H. B. Niznik, *Nature* **2000**, *403*, 274.
[68] C. C. Garner, J. Nash, R. L. Huganir, *Trends Cell Biol.* **2000**, *10*, 274.
[69] T. Pawson, P. Nash, *Science* **2003**, *300*, 445.
[70] M. Sheng, C. Sala, *Annu. Rev. Neurosci.* **2001**, *24*, 1.
[71] S. N. Gomperts, *Cell* **1996**, *84*, 659.
[72] K. E. Prehoda, W. A. Lim, *Curr. Opin. Cell Biol.* **2002**, *14*, 149.
[73] W. A. Lim, *Curr. Opin. Struct. Biol.* **2002**, *12*, 61.
[74] P. Friedl, A. T. den Boer, M. Gunzer, *Nat. Rev. Immunol.* **2005**, *5*, 532.
[75] M. F. Krummel, M. M. Davis, *Curr. Opin. Immunol.* **2002**, *14*, 66.
[76] R. N. Germain, I. Stefanova, *Annu. Rev. Immunol.* **1999**, *17*, 467.
[77] E. O. Long, *Annu. Rev. Immunol.* **1999**, *17*, 875.
[78] M. L. Hermiston, Z. Xu, R. Majeti, A. Weiss, *J. Clin. Invest.* **2002**, *109*, 9.
[79] L. Tuosto, I. Parolini, S. Schröder, M. Sargiacomo, A. Lanzavecchia, A. Viola, *Eur. J. Immunol.* **2001**, *32*, 345.
[80] P. A. van der Merwe, S. J. Davis, A. S. Shaw, M. L. Dustin, *Semin. Immunol.* **2000**, *12*, 5.
[81] A. Viola, S. Schroeder, Y. Sakakibara, A. Lanzavecchia, *Science* **1999**, *283*, 680.
[82] S. K. Bromley, W. R. Burack, K. G. Johnson, K. Somersalo, T. N. Sims, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, M. L. Dustin, *Annu. Rev. Immunol.* **2001**, *19*, 375.
[83] Y. M. Vyas, K. M. Mehta, M. Morgan, H. Maniar, L. Butros, S. Jung, J. K. Burkhardt, B. Dupont, *J. Immunol.* **2001**, *167*, 4358.
[84] L. L. Kiessling, J. E. Gestwicki, L. E. Strong, *Curr. Opin. Chem. Biol.* **2000**, *4*, 696.
[85] J. I. Healy, C. C. Goodnow, *Annu. Rev. Immunol.* **1998**, *16*, 645.
[86] A. R. Asthagiri, D. A. Lauffenburger, *Annu. Rev. Biomed. Eng.* **2000**, *2*, 31.
[87] T. Ideker, T. Galitski, L. Hood, *Annu. Rev. Genomics Hum. Genet.* **2001**, *2*, 343.
[88] S. Krauss, M. D. Brand, *FASEB J.* **2000**, *14*, 2581.
[89] J. Matko, M. Edidin, *Methods Enzymol.* **1997**, *278*, 444.
[90] C. Philipona, Y. Chevolot, D. Léonard, H. J. Mathieu, H. Sigrist, F. Marquis-Weible, *Bioconjugate Chem.* **2001**, *12*, 332.
[91] J. Zhang, R. E. Campbell, A. Y. Ting, R. Y. Tsien, *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906.
[92] J. Lippincott-Schwartz, E. Snapp, A. Kenworthy, *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 444.
[93] F. S. Wouters, P. J. Verveer, P. I. H. Bastiaens, *Trends Cell Biol.* **2001**, *11*, 203.
[94] Y. Liu, L. Xu, N. Opalka, J. Kappler, H.-B. Shu, G. Zhang, *Cell* **2002**, *108*, 383.
[95] D. C. Tully, J. M. J. Fréchet, *Chem. Commun.* **2001**, 1229.
[96] R. Roy, *Curr. Opin. Struct. Biol.* **1996**, *6*, 692.
[97] N. V. Bovin, *Glycoconjugate J.* **1998**, *15*, 431.
[98] J. J. Lundquist, E. J. Toone, *Chem. Rev.* **2002**, *102*, 555.
[99] R. Roy, D. Page, S. F. Perez, V. V. Bencomo, *Glycoconjugate J.* **1998**, *15*, 251.
[100] M. Mammen, S.-K. Choi, G. M. Whitesides, *Angew. Chem.* **1998**, *110*, 2908; *Angew. Chem. Int. Ed.* **1998**, *37*, 2754.
[101] L. L. Kiessling, L. E. Strong, *Top. Organomet. Chem.* **1998**, *1*, 199.
[102] T. Kenakin, *FASEB J.* **2001**, *15*, 598.
[103] M. D. Carrithers, M. R. Lerner, *Chem. Biol.* **1996**, *3*, 537.
[104] M. B. Macek, L. C. Lopez, B. D. Shur, *Dev. Biol.* **1991**, *147*, 440.
[105] H. Schneider, W. Chaovapong, D. J. Matthews, C. Karkaria, R. T. Cass, H. J. Zhan, M. Boyle, T. Lorenzini, S. G. Elliot, L. B. Giebel, *Blood* **1997**, *89*, 473.
[106] D. Somjen, Y. Amirzaltsman, B. Gayer, G. Mor, N. Jaccard, Y. Weisman, G. Barnard, F. Kohen, *J. Endocrinol.* **1995**, *145*, 409.
[107] I. A. Graef, L. J. Holsinger, S. Diver, S. L. Schreiber, G. R. Crabtree, *EMBO J.* **1997**, *16*, 5618.
[108] C. Torigoe, J. K. Inman, H. Metzger, *Science* **1998**, *281*, 568.

[109] H. Nishizumi, K. Horikawa, I. Mlinaric-Rascan, T. Yamamoto, *J. Exp. Med.* **1998**, *187*, 1343.
[110] D. Wright, L. Usher, *Curr. Org. Chem.* **2001**, *5*, 1107.
[111] Y. Liu, X. Hong, J. Kappler, L. Jiang, R. Zhang, L. Xu, C.-H. Pan, W. Martin, R. C. Murphy, H.-B. Shu, S. Dai, G. Zhang, *Nature* **2003**, *423*, 49.
[112] H. M. Kim, K. S. Yu, M. E. Lee, D. R. Shin, Y. S. Kim, S.-G. Paik, O. J. Yoo, H. Lee, J.-O. Lee, *Nat. Struct. Biol.* **2003**, *10*, 342.
[113] R. Sasisekharan, G. Venkataraman, *Curr. Opin. Chem. Biol.* **2000**, *4*, 626.
[114] J. D. Esko, U. Lindahl, *J. Clin. Invest.* **2001**, *108*, 169.
[115] L. L. Kiessling, L. E. Strong, J. E. Gestwicki, *Annu. Rep. Med. Chem.* **2000**, *35*, 321.
[116] P. A. Clemons, *Curr. Opin. Chem. Biol.* **1999**, *3*, 112.
[117] P. S. Portoghese, *J. Med. Chem.* **2001**, *44*, 2259.
[118] D. L. Boger, J. Goldberg, *Bioorg. Med. Chem.* **2001**, *9*, 557.
[119] H. E. Blackwell, P. A. Clemons, S. L. Schreiber, *Org. Lett.* **2001**, *3*, 1185.
[120] M. Pattarawarapan, K. Burgess, *Angew. Chem.* **2000**, *112*, 4469; *Angew. Chem. Int. Ed.* **2000**, *39*, 4299.
[121] J. Goldberg, Q. Jin, S. Satoh, J. Desharnais, K. Capps, D. L. Boger, *J. Am. Chem. Soc.* **2002**, *124*, 544.
[122] M. J. Krantz, N. A. Holtzman, C. P. Stowell, Y. C. Lee, *Biochemistry* **1976**, *15*, 3963.
[123] Y. C. Lee, C. P. Stowell, M. J. Krantz, *Biochemistry* **1976**, *15*, 3956.
[124] E. Gillitzer, D. Willits, M. Young, T. Douglas, *Chem. Commun.* **2002**, 2390.
[125] Q. Wang, T. Lin, L. Tang, E. Johnson, M. G. Finn, *Angew. Chem.* **2002**, *114*, 477; *Angew. Chem. Int. Ed.* **2002**, *41*, 459.
[126] T. L. Schlick, Z. Ding, E. W. Kovacs, M. B. Francis, *J. Am. Chem. Soc.* **2005**, *127*, 3718.
[127] G. Maheshwari, G. Brown, D. A. Lauffenburger, A. Wells, L. G. Griffith, *J. Cell Sci.* **2000**, *113*, 1677.
[128] N. Paran, B. Geiger, Y. Shaul, *EMBO J.* **2001**, *20*, 4443.
[129] N. Horan, L. Yan, H. Isobe, G. M. Whitesides, D. Kahne, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11782.
[130] C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides, D. E. Ingber, *Biotechnol. Prog.* **1998**, *14*, 356.
[131] B. T. Houseman, M. Mrksich, *Angew. Chem.* **1999**, *111*, 876; *Angew. Chem. Int. Ed.* **1999**, *38*, 782.
[132] M. Mrksich, *Chem. Soc. Rev.* **2000**, *29*, 267.
[133] E. A. Smith, W. D. Thomas, L. L. Kiessling, R. M. Corn, *J. Am. Chem. Soc.* **2003**, *125*, 6140.
[134] D. Lévy, A. Bluzat, M. Seigneuret, J.-L. Rigaud, *Biochim. Biophys. Acta* **1990**, *1025*, 179.
[135] F. M. Menger, J. S. Keiper, *Curr. Opin. Chem. Biol.* **1998**, *2*, 726.
[136] E. K. Woller, M. J. Cloninger, *Org. Lett.* **2002**, *4*, 7.
[137] Y. Kim, S. C. Zimmerman, *Curr. Opin. Chem. Biol.* **1998**, *2*, 733.
[138] Y. Aoyama, *Chem. Eur. J.* **2004**, *10*, 588.
[139] M. J. Cloninger, *Curr. Opin. Chem. Biol.* **2002**, *6*, 742.
[140] W. B. Turnbull, S. A. Kalovidouris, J. F. Stoddart, *Chem. Eur. J.* **2002**, *8*, 2988.
[141] T. K. Lindhorst, *Host-Guest Chem.* **2002**, *218*, 201.
[142] C.-H. Lin, G.-J. Shen, E. Garcia-Junceda, C.-H. Wong, *J. Am. Chem. Soc.* **1995**, *117*, 8031.
[143] N. V. Bovin, H.-J. Gabius, *Chem. Soc. Rev.* **1995**, *24*, 413.
[144] J. D. Reuter, A. Myc, M. M. Hayes, Z. Gan, R. Roy, D. Qin, R. Yin, L. T. Piehler, R. Esfand, D. A. Tomalia, J. R. Baker, Jr., *Bioconjugate Chem.* **1999**, *10*, 271.
[145] K. Matyjaszewski, J. Xia, *Chem. Rev.* **2001**, *101*, 2921.
[146] D. J. Irvine, A.-V. G. Ruzette, A. M. Mayes, L. G. Griffith, *Biomacromolecules* **2001**, *2*, 545.
[147] B. R. Griffith, B. L. Allen, A. C. Rapraeger, L. L. Kiessling, *J. Am. Chem. Soc.* **2004**, *126*, 1608.
[148] V. Ladmiral, E. Melia, D. M. Haddleton, *Eur. Polym. J.* **2004**, *40*, 431.
[149] H. D. Maynard, S. Y. Okada, R. H. Grubbs, *J. Am. Chem. Soc.* **2001**, *123*, 1275.
[150] T. M. Trnka, R. H. Grubbs, *Acc. Chem. Res.* **2001**, *34*, 18.
[151] M. Kanai, K. H. Mortell, L. L. Kiessling, *J. Am. Chem. Soc.* **1997**, *119*, 9931.
[152] A. D. Schlüter, J. P. Rabe, *Angew. Chem.* **2000**, *112*, 860; *Angew. Chem. Int. Ed.* **2000**, *39*, 864.
[153] J. K. Pontrello, M. J. Allen, E. S. Underbakke, L. L. Kiessling, *J. Am. Chem. Soc.* **2005**, *127*, 14536.
[154] L. E. Strong, L. L. Kiessling, *J. Am. Chem. Soc.* **1999**, *121*, 6193.
[155] D. D. Manning, L. E. Strong, X. Hu, P. J. Beck, L. L. Kiessling, *Tetrahedron* **1997**, *53*, 11937.
[156] H. Arimoto, K. Nishimura, T. Kinumi, I. Hayakawa, D. Uemura, *Chem. Commun.* **1999**, 1361.
[157] V. C. Gibson, E. L. Marshall, M. North, D. A. Robson, P. J. Williams, *Chem. Commun.* **1997**, 1095.
[158] K. H. Mortell, R. V. Weatherman, L. L. Kiessling, *J. Am. Chem. Soc.* **1996**, *118*, 2297.
[159] D. M. Lynn, B. Mohr, R. H. Grubbs, *J. Am. Chem. Soc.* **1998**, *120*, 1627.
[160] E. L. Dias, S. T. Nguyen, R. H. Grubbs, *J. Am. Chem. Soc.* **1997**, *119*, 3887.
[161] C. Fraser, R. H. Grubbs, *Macromolecules* **1995**, *28*, 7248.
[162] K. H. Mortell, M. Gingras, L. L. Kiessling, *J. Am. Chem. Soc.* **1994**, *116*, 10253.
[163] S. Iyer, S. Rele, G. Grasaa, S. Nolan, E. L. Chaikof, *Chem. Commun.* **2003**, 1518.
[164] D. A. Mann, M. Kanai, D. J. Maly, L. L. Kiessling, *J. Am. Chem. Soc.* **1998**, *120*, 10575.
[165] E. J. Gordon, W. J. Sanders, L. L. Kiessling, *Nature* **1998**, *392*, 30.
[166] C. W. Cairo, J. E. Gestwicki, M. Kanai, L. L. Kiessling, *J. Am. Chem. Soc.* **2002**, *124*, 1615.
[167] J. E. Gestwicki, C. W. Cairo, L. E. Strong, K. A. Oetjen, L. L. Kiessling, *J. Am. Chem. Soc.* **2002**, *124*, 14922.
[168] S. L. Schreiber, *Science* **2000**, *287*, 1964.
[169] W. J. Hoekstra, B. L. Poulter, *Curr. Med. Chem.* **1998**, *5*, 195.
[170] S. R. Wilson, A. W. Czarnik, *Combinatorial Chemistry: Synthesis and Applications*, Wiley, New York, **1997**.
[171] K. C. Nicolaou, J. A. Pfefferkorn, *Biopolymers* **2001**, *60*, 171.
[172] J. A. Ellman, M. A. Gallop, *Curr. Opin. Chem. Biol.* **1998**, *2*, 317.
[173] M. A. Shogren-Knaak, P. J. Alaimo, K. M. Shokat, *Annu. Rev. Cell Dev. Biol.* **2001**, *17*, 405.
[174] V. Wittmann, S. Seeberger, *Angew. Chem.* **2000**, *112*, 4508; *Angew. Chem. Int. Ed.* **2000**, *39*, 4348.
[175] L. L. Kiessling, N. L. Pohl, *Chem. Biol.* **1996**, *3*, 71.
[176] W. P. Jencks, *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 4046.
[177] P. I. Kitov, J. M. Sadowska, G. Mulvey, G. D. Armstrong, H. Ling, N. S. Pannu, R. J. Read, D. R. Bundle, *Nature* **2000**, *403*, 669.
[178] Z. Zhang, E. A. Merritt, M. Ahn, C. Roach, Z. Hu, C. L. M. J. Verlinde, W. G. J. Hol, E. Fan, *J. Am. Chem. Soc.* **2002**, *124*, 12991.
[179] E. Fan, Z. Zhang, W. E. Minke, Z. Hou, C. L. M. J. Verlinde, W. G. J. Hol, *J. Am. Chem. Soc.* **2000**, *122*, 2663.
[180] A. L. Banerjee, D. Eiler, B. C. Roy, X. Jia, M. K. Haldar, S. Mallik, D. K. Srivastava, *Biochemistry* **2005**, *44*, 3211.
[181] S. Howorka, J. Nam, H. Bayley, D. Kahne, *Angew. Chem.* **2004**, *116*, 860; *Angew. Chem. Int. Ed.* **2004**, *43*, 842.
[182] M. Mammen, G. Dahmann, G. M. Whitesides, *J. Med. Chem.* **1995**, *38*, 4179.
[183] M. P. Sheetz, D. P. Felsenfeld, C. G. Galbraith, *Trends Cell Biol.* **1998**, *8*, 51.
[184] M. A. Schwartz, *Trends Cell Biol.* **2001**, *11*, 466.

[185] S. M. Schoenwaelder, K. Burridge, *Curr. Opin. Cell Biol.* **1999**, *11*, 274.
[186] D. Bouvard, C. Brakebusch, E. Gustafsson, A. Aszódi, T. Bengtsson, A. Berna, R. Fässler, *Circ. Res.* **2001**, *89*, 211.
[187] R. O. Hynes, *Cell* **2002**, *110*, 673.
[188] R. H. Li, N. Mitra, H. Gratkowski, G. Vilaire, R. Litvinov, C. Nagasami, J. W. Weisel, J. D. Lear, W. F. DeGrado, J. S. Bennett, *Science* **2003**, *300*, 795.
[189] D. M. Spencer, T. J. Wandless, S. L. Schreiber, G. R. Crabtree, *Science* **1993**, *262*, 1019.
[190] I. A. Graef, F. Chen, A. Kuo, G. R. Crabtree, *Cell* **2001**, *105*, 863.
[191] A. S. Yap, W. M. Brieher, M. Pruschy, B. M. Gumbiner, *Curr. Biol.* **1997**, *7*, 308.
[192] S. K. Muthuswamy, M. Gilman, J. S. Brugge, *Mol. Cell. Biol.* **1999**, *19*, 6845.
[193] J. F. Amara, T. Clackson, V. M. Rivera, T. Guo, T. Keenan, S. Natesan, R. Pollock, W. Yang, N. L. Courage, D. A. Holt, M. Gilman, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 10618.
[194] P. A. Clemons, B. G. Gladstone, A. Seth, E. D. Chao, M. A. Foley, S. L. Schreiber, *Chem. Biol.* **2002**, *9*, 49.
[195] T. Hato, N. Pampori, S. J. Shattil, *J. Cell Biol.* **1998**, *141*, 1685.
[196] N. J. Boudreau, P. L. Jones, *Biochem. J.* **1999**, *339*, 481.
[197] E. R. Welsh, D. A. Tirrell, *Biomacromolecules* **2000**, *1*, 23.
[198] D. G. Stupack, E. Li, S. A. Silletti, J. A. Kehler, R. L. Geahlen, K. Hahn, G. R. Nemerow, D. A. Cheresh, *J. Cell Biol.* **1999**, *144*, 777.
[199] S. R. George, B. F. O'Dowd, S. P. Lee, *Nat. Rev. Drug Discovery* **2002**, *1*, 808.
[200] M. Mellado, J. Rodríguez-Frade, S. Mañes, C. Martínez-A, *Annu. Rev. Immunol.* **2001**, *19*, 397.
[201] R. H. Kramer, J. W. Karpen, *Nature* **1998**, *395*, 710.
[202] G. D. Glick, P. L. Toogood, D. C. Wiley, J. J. Skehel, J. R. Knowles, *J. Biol. Chem.* **1991**, *266*, 23660.
[203] B. P. England, P. Balasubramanian, I. Uings, S. Bethell, M.-J. Chen, P. J. Schatz, Q. Yin, Y.-F. Chen, E. A. Whitehorn, A. Tsavaler, C. L. Martens, R. W. Barrett, M. McKinnon, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 6862.
[204] Y. He, J. W. Karpen, *Biochemistry* **2001**, *40*, 286.
[205] J. M. Paar, N. T. Harris, D. Holowka, B. Baird, *J. Immunol.* **2003**, *169*, 856.
[206] D. Chan, L. Gera, J. Stewart, B. Helfrich, M. Verella-Garcia, G. Johnson, A. Baron, J. Yang, J. P. Bunn, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4608.
[207] R. G. Bhushan, S. K. Sharma, Z. Xie, D. J. Daniels, P. S. Portoghese, *J. Med. Chem.* **2004**, *47*, 2969.
[208] M. Miyazaki, H. Kodama, I. Fujita, Y. Hamasaki, S. Miyazaki, M. Kondo, *J. Biochem.* **1995**, *117*, 489.
[209] K. Yano, S. Kimura, Y. Imanishi, *Eur. J. Pharm. Sci.* **1998**, *7*, 41.
[210] L. He, J. Fong, M. von Zastrow, J. L. Whistler, *Cell* **2002**, *108*, 271.
[211] M. L. Dustin, *J. Clin. Invest.* **2002**, *109*, 155.
[212] W. W. A. Schamel, I. Arechaga, R. M. Risueno, H. M. van Santen, P. Cabezas, C. Risco, J. M. Valpuesta, B. Alarcon, *J. Exp. Med.* **2005**, *202*, 493.
[213] J. J. Boniface, J. D. Rabinowitz, C. Wülfing, J. Hampl, Z. Reich, J. D. Altman, R. M. Kantor, C. Beeson, H. M. McConnell, M. M. Davis, *Immunity* **1998**, *9*, 459.
[214] J. R. Cochran, D. Aivazian, T. O. Cameron, L. J. Stern, *Trends Biochem. Sci.* **2001**, *26*, 304.
[215] J. R. Cochran, L. J. Stern, *Chem. Biol.* **2000**, *7*, 683.
[216] S. Miyamoto, S. K. Akiyama, K. M. Yamada, *Science* **1995**, *267*, 883.
[217] J. A. Hubbell, *Curr. Opin. Biotechnol.* **1999**, *10*, 123.
[218] B. Lowin-Kropf, V. S. Shapiro, A. Weiss, *J. Cell Biol.* **1998**, *140*, 861.
[219] C. R. F. Monks, B. A. Freiberg, H. Kupfer, N. Sciaky, A. Kupfer, *Nature* **1998**, *395*, 82.
[220] J. Stock, M. Levit, *Curr. Biol.* **2000**, *10*, R11.
[221] G. L. Hazelbauer, H. C. Berg, P. Matsumura, *Cell* **1993**, *73*, 15.
[222] J. J. Falke, R. B. Bass, S. L. Butler, S. A. Chervitz, M. A. Danielson, *Annu. Rev. Cell Dev. Biol.* **1997**, *13*, 457.
[223] R. Jasuja, Y. Lin, D. R. Trentham, S. Khan, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11346.
[224] A. M. Stock, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 10945.
[225] J. P. Armitage, C. J. Dorman, K. Hellingwerf, R. Schmitt, D. Summers, B. Holland, *Mol. Microbiol.* **2003**, *47*, 583.
[226] X. Feng, J. W. Baumgartner, G. L. Hazelbauer, *J. Bacteriol.* **1997**, *179*, 6714.
[227] B. A. Mello, Y. Tu, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 8223.
[228] J. E. Gestwicki, A. C. Lamanna, R. M. Harshey, L. L. McCarter, L. L. Kiessling, J. Adler, *J. Bacteriol.* **2000**, *182*, 6499.
[229] K. K. Kim, H. Yokota, S. H. Kim, *Nature* **1999**, *400*, 787.
[230] J. E. Gestwicki, C. W. Cairo, D. A. Mann, R. M. Owen, L. L. Kiessling, *Anal. Biochem.* **2002**, *305*, 149.
[231] V. Sourjik, *Trends Microbiol.* **2004**, *12*, 569.
[232] R. S. Syed, S. W. Reid, C. Li, J. C. Cheetham, K. H. Aoki, B. Liu, H. Zhan, T. D. Osslund, A. J. Chirino, J. Zhang, J. Finer-Moore, S. Elliott, K. Sitney, B. A. Katz, D. J. Matthews, J. J. Wendoloski, J. Egrie, R. M. Stroud, *Nature* **1998**, *395*, 511.
[233] J. E. Dueber, B. J. Yeh, R. P. Bhattacharyya, W. A. Lim, *Curr. Opin. Struct. Biol.* **2004**, *14*, 690.
[234] M. Beyermann, S. Rothemund, N. Heinrich, K. Fecher, J. Furkert, M. Dathe, R. Winter, E. Krause, M. Bienert, *J. Biol. Chem.* **2000**, *275*, 5702.
[235] D. Qin, R. Sullivan, W. F. Berkowitz, R. Bittman, S. A. Rotenberg, *J. Med. Chem.* **2000**, *43*, 1413.
[236] S. H. Park, A. Zarrinpar, W. A. Lim, *Science* **2003**, *299*, 1061.
[237] P. Hasler, M. Zouali, *FASEB J.* **2001**, *15*, 2085.
[238] H. M. Dintzis, R. Z. Dintzis, B. Vogelstein, *Proc. Natl. Acad. Sci. USA* **1976**, *73*, 3671.
[239] R. Z. Dintzis, B. Vogelstein, H. M. Dintzis, *Proc. Natl. Acad. Sci. USA* **1982**, *79*, 884.
[240] B. Sulzer, A. S. Perelson, *Mol. Immunol.* **1997**, *34*, 63.
[241] P. K. A. Mongini, C. A. Blessinger, P. F. Highlet, J. K. Inman, *J. Immunol.* **1992**, *148*, 3892.
[242] G. Ragupathi, L. Howard, S. Capello, R. R. Koganty, D. Qiu, B. M. Longnecker, M. A. Reddish, K. O. Lloyd, P. O. Livingston, *Cancer Immunol. Immunother.* **1999**, *48*, 1.
[243] R. Z. Dintzis, M. Okajima, M. H. Middleton, G. Greene, H. M. Dintzis, *J. Immunol.* **1989**, *143*, 1239.
[244] R. P. McEver, K. L. Moore, R. D. Cummings, *J. Biol. Chem.* **1995**, *270*, 11025.
[245] S. R. Hasslen, U. H. von Andrian, E. C. Butcher, R. D. Nelson, S. L. Erlandsen, *Histochem. J.* **1995**, *27*, 547.
[246] S. R. Hasslen, A. R. Burns, S. I. Simon, W. Smith, K. Starr, A. N. Barclay, S. A. Michie, R. D. Nelson, S. L. Erlandsen, *J. Histochem. Cytochem.* **1996**, *44*, 1115.
[247] S. D. Rosen, *Annu. Rev. Immunol.* **2004**, *22*, 129.
[248] E. E. Simanek, G. J. McGarvey, J. A. Jablonowski, C.-H. Wong, *Chem. Rev.* **1998**, *98*, 833.
[249] G. Thoma, R. O. Duthaler, J. L. Magnani, J. T. Patton, *J. Am. Chem. Soc.* **2001**, *123*, 10113.
[250] G. Thoma, J. T. Patton, J. L. Magnani, B. Ernst, R. Ohrlein, R. O. Duthaler, *J. Am. Chem. Soc.* **1999**, *121*, 5919.
[251] D. D. Manning, X. Hu, P. Beck, L. L. Kiessling, *J. Am. Chem. Soc.* **1997**, *119*, 3161.
[252] W. J. Sanders, E. J. Gordon, O. Dwir, P. J. Beck, R. Alon, L. L. Kiessling, *J. Biol. Chem.* **1999**, *274*, 5271.
[253] R. Stahn, H. Schäfer, F. Kernchen, J. Schreiber, *Glycobiology* **1998**, *8*, 311.
[254] R. M. Owen, J. E. Gestwicki, T. Young, L. L. Kiessling, *Org. Lett.* **2002**, *4*, 2295.

[255] E. J. Gordon, J. E. Gestwicki, L. E. Strong, L. L. Kiessling, *Chem. Biol.* **2000**, *7*, 9.
[256] E. J. Gordon, L. E. Strong, L. L. Kiessling, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1293.
[257] P. Mowery, Z. Q. Yang, E. J. Gordon, O. Dwir, A. G. Spencer, R. Alon, L. L. Kiessling, *Chem. Biol.* **2004**, *11*, 725.
[258] E. J. Gordon, L. E. Strong, L. L. Kiessling, *Bioorg. Med. Chem.* **1998**, *6*, 1293.
[259] M. Mourez, R. S. Kane, J. Mogridge, S. Metallo, P. Deschatelets, B. R. Sellman, G. M. Whitesides, R. J. Collier, *Nat. Biotechnol.* **2001**, *19*, 958.
[260] L. Bracci, L. Lozzi, A. Pini, B. Lelli, C. Falciani, N. Niccolai, A. Bernini, A. Spreafico, P. Soldani, P. Neri, *Biochemistry* **2002**, *41*, 10194.
[261] G. W. Byrne, A. Schwarz, J. R. Fesi, P. Birch, A. Nepomich, I. Bakaj, M. A. Velardo, C. Jiang, A. Manzi, H. Dintzis, L. E. Diamond, J. S. Logan, *Bioconjugate Chem.* **2002**, *13*, 571.
[262] Y. Minamoto, T. Hato, S. Nakatani, S. Fujita, *Thromb. Haemost.* **1996**, *76*, 1072-1079.
[263] J. E. Gestwicki, L. L. Kiessling, unpublished results.

