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Published on Web 03/21/2003

Cooperative Binding at Lipid Bilayer Membrane Surfaces

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Abstract: The binding of copper(II) ions to membrane-bound synthetic receptors has been investigated. Complexation fitted a 4:1 receptor:copper(II) model, and the observed binding constants are significantly enhanced at the membrane relative to solution; these effects can be explained by the lower polarity of the membrane−water interface and the concentrating effect of the membrane, with no observed contribution from receptor preorganization. The stoichiometry of the complex formed is very sensitive to the concentration of the receptor in the membrane, and at low concentrations, binding is reduced relative to solution controls. This implies that by increasing or decreasing the number of receptors in their membranes, cells can finely tune biological responses such as chemotaxis that depend on the size of the receptor−ligand clusters formed.

Cooperative binding to receptors constrained to a membrane surface is a fundamentally important process in biology. For example, in chemotaxis bacteria can detect very small changes in the concentration of ligands, such as sugars, over many orders of magnitude.[1] A recent mathematical model proposed to explain this remarkable behavior suggested that signal transduction is regulated by changes in lateral clustering of the chemoreceptors.[2] The importance of lateral clustering was confirmed when multivalent ligands with pendant sugars were shown to induce aggregation and give a chemotactic response.[3] Furthermore, the size of the cluster formed is also important; for example, tetramers of the chemotaxic receptor Tar are significantly more active during in vitro chemotaxic signaling than individual or dimeric receptors.[4]

Another important biological process initiated by receptor aggregation is the allergic response. This is mediated by membrane-bound receptors that are formed when IgE immunoglobulins bind to membrane-embedded FCϵRI stems on the cell surface. During an allergic reaction a multivalent antigen binds to the IgE-FCϵRI receptors, inducing receptor aggregation. The formation of these aggregates initiates a cascade of signaling events, resulting in the release of chemical messengers that trigger the immune response. The size of the aggregates is unknown, but model studies with IgE oligomers have shown that dimers do not trigger the response as effectively as larger aggregates. Again, both antigen affinity and the size of the aggregate is important.[5]

Receptors that are anchored to a membrane surface can only move in two-dimensions, and so binding interactions between them are expected to be more thermodynamically favorable than the corresponding interactions in solution, where the molecules are free to move in three dimensions. Thus preorganization of receptors on a membrane surface could account for the cooperative intra-membrane binding interactions described above. However, there have been few quantitative experimental studies that shed light on the relative importance of the various factors that might contribute to cooperative receptor aggregation in membranes. There are two problems with such studies. If we consider the aggregation of membrane-bound receptors around a multivalent ligand, we cannot simply compare the association constant for binding the ligand from solution (K_1), with the apparent association constants for subsequent interactions within the membrane (K_2), because the values of the latter will change as a function of the concentration of the receptor in the membrane.[6] Second, the membrane interface is a quite different environment from bulk solution, and this is likely to have a dramatic effect on the intrinsic binding constant for any interaction.

Here we report our results concerning the aggregation of membrane-anchored receptors, using a model system that allows us to quantify the membrane environment and therefore investigate the relationship between receptor concentration and the cooperativity of multicomponent assembly processes on the membrane surface.

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Table 1. Calculated Binding Constants to Copper(II) for Receptor **1** in Aqueous Buffer (pH 6, 50 mM MES), Receptor **1** in 4% Aqueous Buffer in Methanol, and Receptor **2** in Vesicles[a]

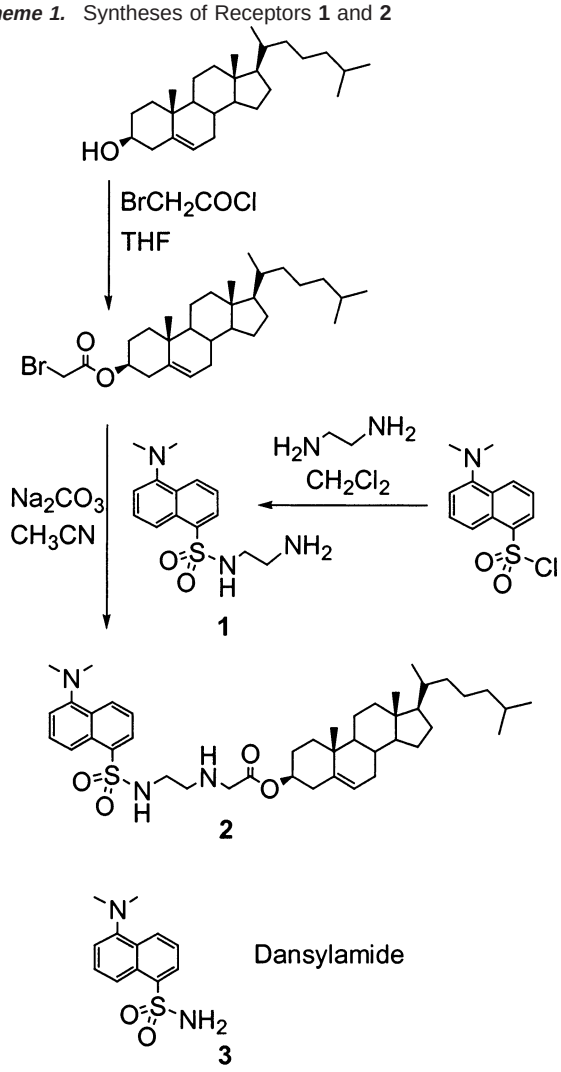
	solution binding constants (M^{-1})				membrane binding constants (M^{-1})		
receptor (medium)	K_1	K_2	K_3	K_4	K_2^{memb}	K_3^{memb}	K_4^{memb}
1 (aq buffer)	$1.2 \pm 0.2 \times 10^2$	< 0.1	nd	nd			
1 (4% aq buffer in MeOH)	$8 \pm 1 \times 10^3$	$7 \pm 1 \times 10^3$	nd	nd			
2 (vesicles)	$1.4 \pm 0.2 \times 10^4$				$1.0 \pm 0.2 \times 10^2$	8 ± 4	$1.5 \pm 0.7 \times 10^3$

[a] nd: too low to be determined accurately.

Results and Discussion

We chose a dansyl-ethylenediamine conjugate as our headgroup and cholesterol as the membrane anchor.[7] The dansyl group has a strongly environmentally sensitive fluorophore, which provides information about the microenvironment surrounding the probe,[8] and the coordination of copper(II) to related ligands quenches the fluorescence.[9,10] Analysis of the degree of quenching caused by titrating copper(II) into vesicular suspensions of the membrane-anchored dansyl ethylenediamine conjugate therefore allows direct monitoring of the binding processes. Membrane receptor **2** was synthesized in two steps from cholesterol (Scheme 1). Heating cholesterol with bromoacetyl chloride in THF under reflux afforded the bromoacetyl ester. Subsequent displacement of the bromide with **1** in acetonitrile, available through the condensation of dansyl chloride with ethylenediamine, gave the receptor **2**. Compound **1** serves as a useful control system for quantifying the copper(II)−receptor interactions in solution.

Scheme 1. Syntheses of Receptors **1** and **2**



Initial Binding Studies. The binding constants for complexation of **1** by copper(II) in aqueous solution were measured to provide reference binding constants for calibrating the analogous interactions at vesicle surfaces. The affinity of **1** (0.2 mM) for copper(II) was low in aqueous solution at pH 6: only the first binding constant could be determined (K_1, Table 1), and there was no observable 2:1 (Cu$\mathbf{1}_2$) binding ($K_2 < 0.1$ M^{-1}). This behavior strongly suggests a monodentate interaction rather than a bidentate metal−ligand interaction; for glycine ethyl ester binding to copper(II) $K_1 \approx 200$ M^{-1} at pH 6, but for ethylenediamine binding to copper(II) $K_1 \approx 3 \times 10^5$ M^{-1} at pH 6.[11]

We then prepared unilamellar vesicles (800 nm diameter) containing 1 mol % **2**, keeping the bulk concentration of receptor at 0.2 mM as before**.** Aqueous copper(II) chloride was titrated into this vesicular solution, and the decrease in the fluorescence of the dansyl group monitored. Initial analysis of the titration curve indicated that a 2:1 complex (Cu$\mathbf{2}_2$) formed with observed binding constants K_1^{obs} and K_2^{obs} both approximately 10^4 M^{-1}.

Thus the membrane-bound ligands have a much higher affinity for copper(II) than the corresponding ligands in solution. The effect is particularly pronounced for K_2^{obs} which shows a $>10^5$ fold enhancement, apparently confirming that restricting the ligands to a two-dimensional environment leads to strong cooperative intra-membrane binding interactions. However, the observation of a 100-fold enhancement of K_1^{obs} is important, because anchoring the receptor in a membrane should have no entropic advantage for this process. The increase suggests that the environment at the membrane interface plays a significant role in modulating binding interactions involving membrane-anchored receptors.

The Polarity of the Membrane-Water Interface. To quantify the effect of the membrane-water interface on binding, we used the fluorescent headgroup to probe the polarity of the environment experienced by the receptor. The fluorescence

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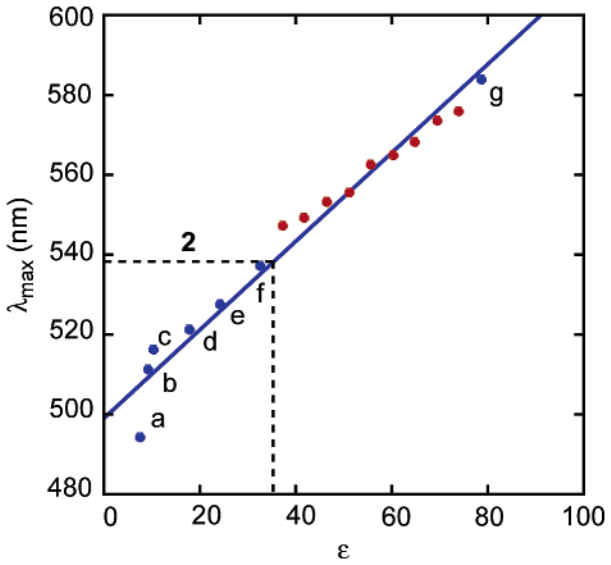

Figure 1. Calibration curve showing the change in λ_{max} of dansylamide fluorescence with changing solvent polarity. The dansylamide fluorescence was measured both in pure solvents (Blue dots: a, THF; b, CH_2Cl_2; c, 1-octanol; d, 1-butanol; e, ethanol; f, methanol; g, water) and water/methanol mixtures. (Red dots, 10% increments. There is a linear relationship between the volume fraction of a water-methanol mixture and its dielectric constant.[12]) The dotted line shows the interpolation of λ_{max} (538.4 nm) found for receptor **2** in vesicles.

emission spectra of both **1** and **3** were measured in a series of solvents. As solvent polarity increased, the dansyl fluorescence intensity I_{max} decreased and wavelength maximum λ_{max} increased. The position of λ_{max} and solvent polarity ϵ were used to construct a calibration curve (Figure 1). A good linear fit was obtained for both **1** and dansylamide **3** with little difference between the two compounds, showing that altering the polarity of the surroundings is the major contributor to the changes in λ_{max}, and that changing the structure of the headgroup has a negligible effect. The fluorescence emission spectrum of a vesicular solution of **2** (1 mol % loading in 800 nm unilamellar vesicles) had $\lambda_{max} = 538.4$ nm, and comparison with the calibration curve indicates that the bilayer environment surrounding the headgroups of the receptor **2** is akin to $4 \pm 3\%$ water in methanol ($\epsilon \sim 35$, Figure 1). This value is in good agreement with other estimates of the polarity of the interface between lipid bilayers and bulk aqueous solution,[13] and shows that the receptor is positioned in a polar environment, although clearly not in a fully aqueous environment. This clearly has to be taken into account when interpreting the observed binding constants. Theoretical studies by Sakurai et al.[14] predict that binding interactions dependent either on hydrogen bonding or between charged species are strongly affected by the presence of the bilayer interface even when the local binding interaction is positioned in the aqueous subphase.

To obtain more appropriate solution control association constants for **1**, we measured the binding constants in 4% aqueous buffer in methanol. A 2:1 Cu**1**$_2$ complex was formed, with both K_1 and K_2 significantly greater than in purely aqueous buffer (Table 1). Both values are very close to those obtained in the vesicular system for **2**. Thus the improvement in the binding affinity due to constraining the receptors in vesicles appears to be primarily due to solvation effects in the local environment. Previous studies of interactions with membrane-anchored receptors have not taken the effect of the membrane environment into account, and the large cooperative effects observed in these experiments could be caused by similar effects.[15]

The Effect on Binding of Vesicle Receptor Concentration. The membrane concentration of the receptor should also be an important factor in determining the magnitude of the observed binding constants, and so far we have ignored this parameter.[7a,16a] With an appropriate solution control established, i.e. K_1 for binding **1** in solution is the same as K_1^{obs} for the membrane-anchored receptor **2**, we investigated how the membrane concentration of **2** affects the value of K_2^{obs} relative to that measured for **1** in solution.

We maintained the bulk concentration of **2** at 0.2 mM and the vesicle size at 800 nm, but varied the concentration of phospholipid. This has the effect of varying the number of vesicles and hence the number of receptors per vesicle. Keeping the overall concentration of the receptor constant ensures that any differences observed in the binding behavior are purely due to changes in the observed association constants. Five different vesicular solutions were prepared containing 0.2, 1, 2.5, 5, and 7.5 mol % **2** in the lipid bilayer. Titration of copper(II) into these solutions revealed two effects as the membrane concentration of receptor increased: the overall binding affinity increased dramatically and the stoichiometry changed from Cu**2**$_2$ to Cu**2**$_4$ (Figure 2). There are clearly huge cooperative effects in this system to the extent that the 4:1 complex, which is never observed in solution, becomes the most stable species when the receptors are anchored to the membrane.

We used an ML_4 binding model and an iterative curve fitting program to determine apparent bulk association constants at each phospholipid concentration. The first binding constant K_1^{obs} is independent of the number of receptors per vesicle as expected, but the observed values of K_2^{obs}, K_3^{obs}, and K_4^{obs} increase with increasing receptor to phospholipid ratio. The increase in K_2^{obs} with increasing mole percent of receptor in the membrane is shown in Figure 3. There is a clear linear correlation, and the slope is related to the association constant in the membrane K_n^{memb}, which is the true measure of the strength of the intramembrane binding interaction (Figure 4).

To understand this relationship, we define a binding constant K_n^{memb} in terms of the membrane concentration of the receptor:

$$K_n^{memb} = \frac{[MR_n]_{memb}}{[MR_{n-1}]_{memb}[R]_{memb}} \quad (1)$$

The membrane concentration of each species X is related to its bulk solution concentration by the fraction of the overall solvent volume occupied by phospholipid:

$$[X]_{solution} = [X]_{memb}(V_m[PL]) \quad (2)$$

where V_m is the molar volume of phospholipid[17] and [PL] is

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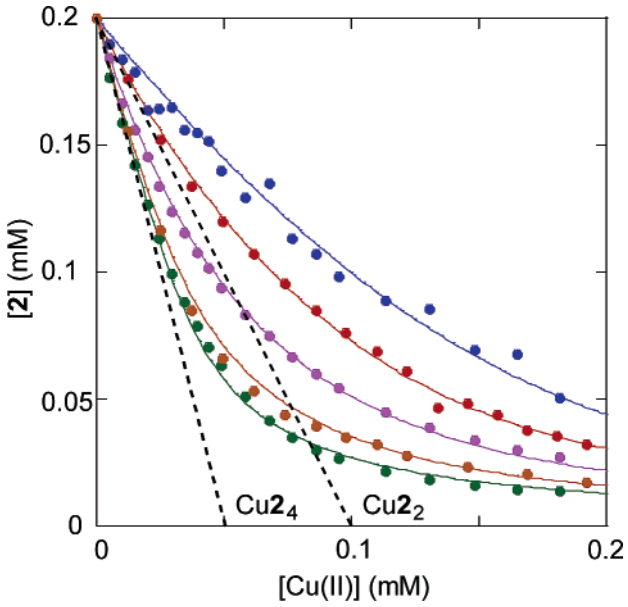

Figure 2. Titration curves showing the decrease in the fluorescence of vesicle-bound receptor **2** upon the addition of copper(II). Receptor **2** (0.2 mM) was dissolved in vesicles with a receptor loading of 0.2 mol % (blue dots), 1 mol % (red dots), 2.5 mol % (purple dots), 5 mol % (orange dots), and 7.5 mol % (green dots). The solid lines represent curve fits calculated using the binding constants in Table 1. The dotted lines represent curves calculated at the tight binding limit for the Cu**2**$_2$ and Cu**2**$_4$ complexes.

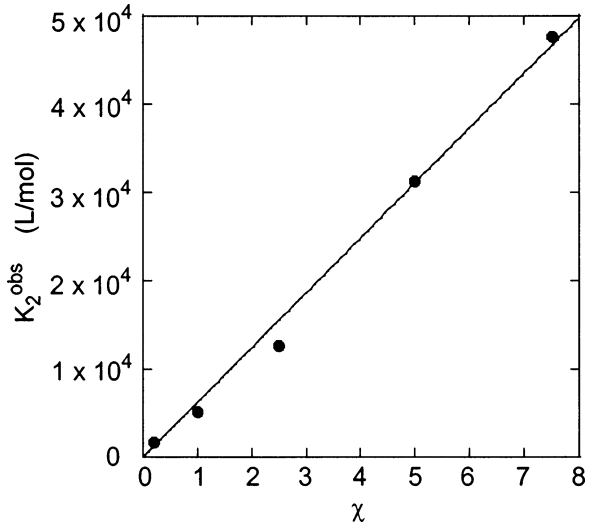

Figure 3. Change in the observed binding constant K_2^{obs} as a function of the mole percentage of receptor in the vesicle membranes χ.

the concentration of phospholipid. Combining eqs 1 and 2 gives

$$K_n^{obs} = \frac{[MR_n]_{solution}}{[MR_{n-1}]_{solution}[R]_{solution}} = \frac{K_n^{memb}}{[PL]V_m} \qquad (3)$$

which can be expressed as

$$K_n^{obs} = \left(\frac{K_n^{memb}}{100[R]_T V_m}\right)\chi \qquad (4)$$

where $[R]_T$ is the total concentration of the receptor in the solution (0.2 mM in our case) and χ is the mole percentage of the receptor in the membrane. Using eq 4 and the slope of the plot in Figure 3 (6200 M^{-1}) we find that K_2^{memb} is 100 M^{-1},

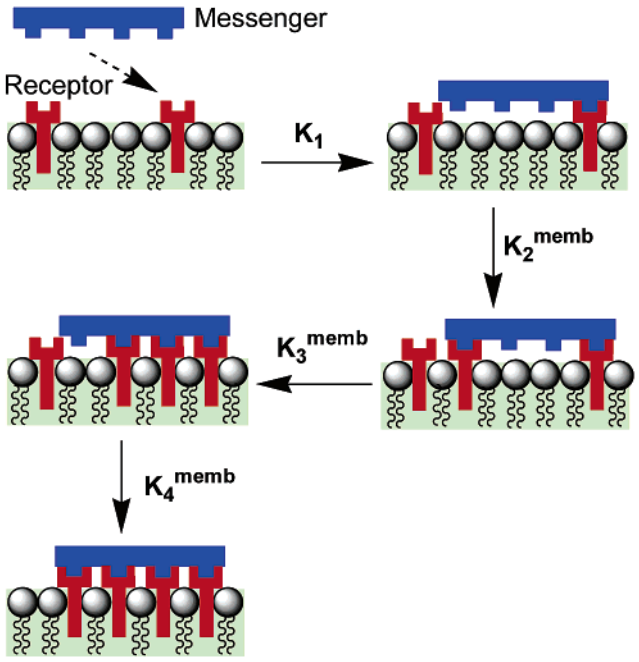

Figure 4. Sequential binding events during the complexation of the messenger (copper(II)) to membrane embedded receptor **2**.

significantly less than K_2 in solution. In other words, the intrinsic binding strength for this interaction is lower in the membrane than in solution. This presumably reflects difficulties in attaining an appropriate geometric arrangement for binding. The potential cooperativity associated with reducing the number of degrees of freedom, i.e. constraining the receptors to move in two-dimensions, does not appear to be significant in this system.

In this approach, we have assumed that the membrane-anchored receptor is constrained to the volume of the lipid membrane in order to define the membrane concentration, and so K_n^{memb} are three-dimensional binding constants. In reality, it is unlikely that the polar headgroups can sample the full volume of the membrane. They are constrained to the interfacial regions, and so the association constants determined by this method overestimate the true membrane binding affinities. However, the advantage of this approach is that we can directly compare membrane association constants with solution association constants, even though membrane binding events are more properly expressed as two-dimensional binding constants.[18]

Using the data obtained at different phospholipid concentrations, we used eq 4 to obtain three self-consistent membrane binding constants (Table 1)[19] that fit all of the titration data

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(17) We calculate V_m (0.84 dm^3 mol^{-1}) from the dimensions of unilamellar EYPC vesicles (thickness of the phospholipid leaflet ~20 Å, cross sectional area of the headgroup ~70 $Å^2$. Balgavy, P.; Dubnickova, M.; Uhrikova, D.; Yaradaikin, S.; Kiselev, M.; Gordeliy, V. *Acta Phys. Slov.* **1998**, *48*, 509−533) and so this factor relates to the relatively disordered bilayer phase, rather than the crystalline value.

(18) It is useful to also express the binding events at the surface of the membrane as two-dimensional binding constants. These two-dimensional binding constants can be related to the membrane binding constants K_n^{obs} and K_n^{memb} by $K_n^{obs} = K_n^{2D}/A_m[PL]$ and $K_n^{memb} = d_{memb}K_n^{2D}$ where d_{memb} is the thickness of a phospholipid leaflet (~20 Å) and A_m is the molar cross-sectional area of the phospholipid headgroup (4.2×10^7 dm^2 mol^{-1}). Data from Balgavy, P.; Dubnickova, M.; Uhrikova, D.; Yaradaikin, S.; Kiselev, M.; Gordeliy, V. *Acta Phys. Slov.* **1998**, *48*, 509−533.

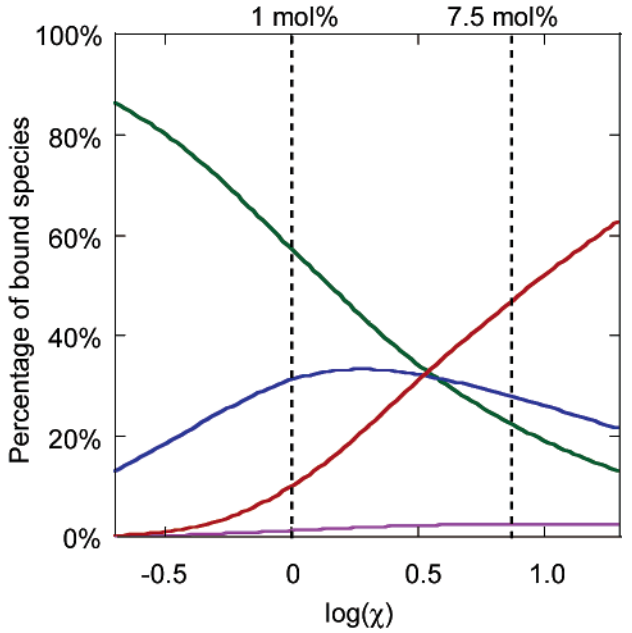

Figure 5. Speciation plot showing the amounts of the Cu**2** (green), Cu$\mathbf{2}_2$ (blue), Cu$\mathbf{2}_3$ (purple), Cu$\mathbf{2}_4$ (red) complexes as fractions of all bound species, as a function of the logarithm of the mole percent of receptor in the membrane χ. The copper concentration is 0.1 mM and the concentration of receptor **2** is 0.2 mM.

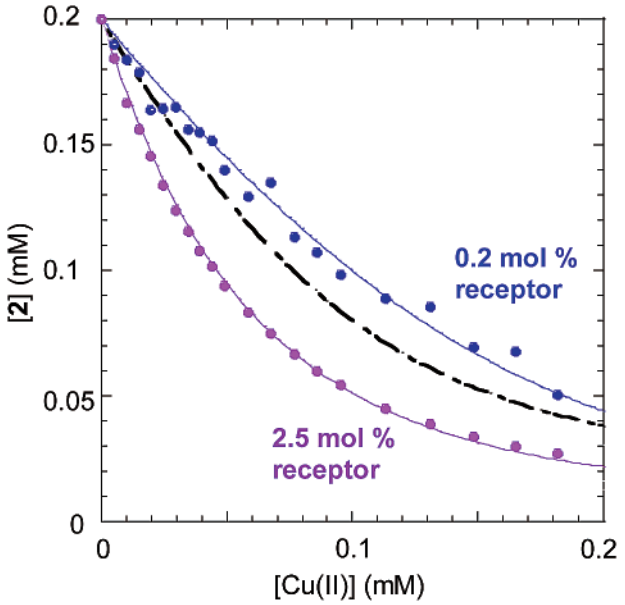

Figure 6. Titration curves showing the decrease in the fluorescence of receptor **2** in vesicles with a receptor-to-phospholipid ratio of (blue) 0.2 mol % and (purple) 2.5 mol % upon the addition of copper(II). The dashed black line (- −) represents the titration curve constructed from the binding constants of copper(II) to the headgroup **1** in 4% MES buffer pH 6 in methanol.

(Figure 2). The stoichiometry of the complex is strongly dependent on the membrane concentration of the receptor. The speciation plot in Figure 5 shows that at a copper(II) concentration of 0.1 mM, a membrane concentration of 1 mol % gives largely the Cu$\mathbf{2}_2$ species, whereas 7.5 mol % gives the Cu$\mathbf{2}_4$ species preferentially. The Cu$\mathbf{2}_3$ species is never formed to any significant extent, and in the absence of any structural information, we prefer not to speculate as to the reasons.

At high mole percentages, species are formed that cannot be observed in solution; this is simply because much higher local concentrations of receptor are achieved in the membrane. Conversely, at low mole percentages of receptor, aggregation of the receptor is inhibited by localization in the membrane. This is a straightforward implication of eq 4 and is demonstrated by the experimental data in Figure 3. At membrane receptor loadings above 1.1 mol %, positive cooperativity is observed since K_2^{obs} is greater than 7000 M^{-1}, the corresponding solution value for **1** in 4% buffer in methanol. However, at loadings less than 1.1 mol %, K_2^{obs} is less than K_2 and the system shows negative cooperativity. This negative cooperativity is highlighted in Figure 6 which shows the titration data for **2** at membrane loadings of 0.2 and 2.5 mol % compared with **1** in solution of similar polarity.

Thus at high receptor loadings, binding at the membrane is more favorable than in solution, resulting in intra-vesicle aggregation of the receptors. Conversely, at low receptor loadings interactions within the membrane are less favorable than interactions with species in solution, and so we might expect inter-vesicle interactions to dominate. However, if inter-vesicle interactions dominated at low loadings, then as the receptor loading decreased, K_2^{obs} would decrease until it reached K_2 (7000 M^{-1}), at which point inter-vesicular binding should maintain $K_2^{obs} = K_2$. The fact that we observe inhibition of binding ($K_2^{obs} < K_2$) at low receptor loadings implies that there are steric effects that prevent vesicle−vesicle interactions in this system.

Therefore, both the binding strength and the type of complex formed are highly dependent on the membrane concentration of the receptor (Figure 7). This shows that by controlling the concentration of receptor in its membrane a cell could control both the strength of binding to a messenger, the type of cluster formed, and interactions with other cells. Since both chemotaxis and the immune response are sensitive to clusters of a particular size, this effect gives the cell an extra degree of control over the response to an external messenger molecule. For example, overexpression of a receptor in a membrane could result in a decrease in the cellular response by decreasing the number of smaller active clusters in favor of larger inactive clusters.

Conclusions

Cooperativity during membrane binding events has only been studied quantitatively in a few other systems. The effect of changing the surface concentration of ligand on the formation of the 1:2 complex between chloroeremomycin dimer and its vesicle-bound ligand, *N*-docosanoyl-Gly-Ala-D-γ-Glu-Lys(N-ϵ-acetyl)-D-Ala-D-lactate, has been studied. The values of K_2^{obs} were much larger than the equivalent process in solution, the binding of chloroeremomycin to the *N*-acetyl-Gly-Ala-D-γ-Glu-Lys(*N*-ϵ-acetyl)-D-Ala-D-lactate, suggesting a large degree of cooperativity in this system. [7(a)] Although several values of K_2^{obs} were obtained at different concentrations of phospholipid, the membrane binding constant K_2^{memb} was not determined. A two-dimensional binding constant was directly determined for the bivalent binding of IgM to its hapten immobilized in EYPC monolayers, but K_2 in solution was not determined, so no

(19) Although this treatment ignores factors such as changes in surface charge, this has been approximately calculated for 1 mol/mol % receptor and is a minor effect. Data from Gennis, R. B. *Biomembranes: Molecular Structure and Function*; Springer-Verlag: London, 1989.

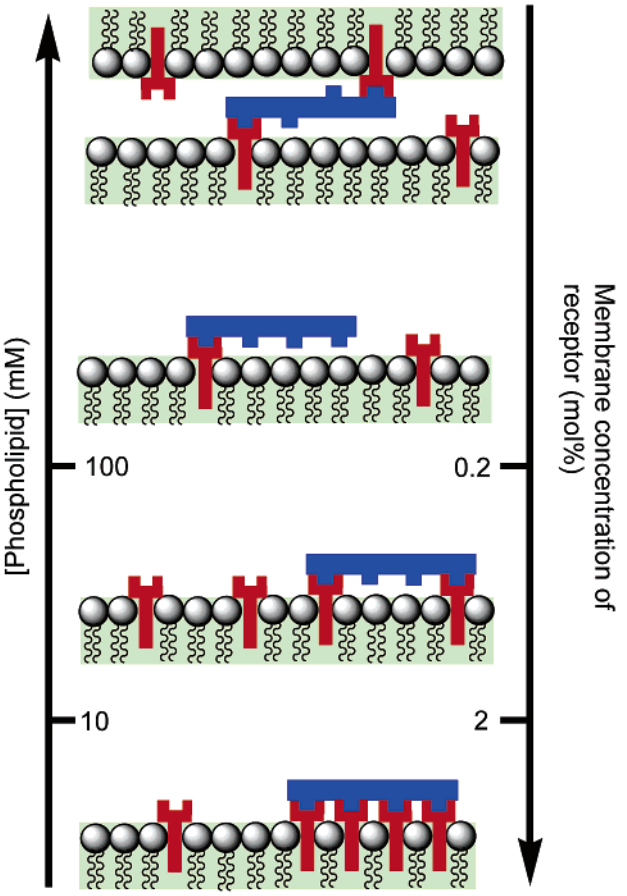

Figure 7. Tuning the concentration of receptor in the membrane can be used to control the identity of the major complex formed between copper(II) and receptor **2**. As the membrane concentration of receptor increases, the size of the complex increases. At low membrane concentrations (top), intra-vesicular binding is inhibited and inter-vesicular binding should predominate, though this was precluded in our system due to steric constraints.

comparison could be drawn between solution and membrane binding events.[7b]

These and other such studies of the binding of multivalent ligands at vesicular and cellular interfaces do not take into account the difference in environment between the membrane interface and bulk aqueous solution. Often K_1 for binding to a membrane-bound receptor is assumed to be the same as K_1 for binding to the receptor in aqueous solution.[7a,16] In fact, changes in polarity and the hydration sphere around species embedded in membranes are known to be important for many binding processes, such as the binding of antibodies to surface-bound haptens or the binding of antibiotics to bacterial cell wall analogues.[20] A large effect has been observed for the antibiotic teichoplanin A_3-1. This antibiotic, which does not insert into vesicles, binds tightly to the vesicle-bound ligand (*N*-docosanoyl-Lys(*N*-ϵ-acetyl)-D-Ala-D-lactate) to form a 1:1 complex with a binding constant of 6×10^4 M^{-1}, whereas binding to the water soluble derivative (*N*-acetyl-Lys(*N*-ϵ-acetyl)-D-Ala-D-lactate) was too weak to be observed (<10 M^{-1}).[7c] Therefore large increases in binding strength can result exclusively due to the effect of the membrane environment on binding, which can lead to overestimation of the degree of cooperativity.

The use of the dansyl fluorophore in our system has allowed us to quantify the effect of the membrane environment on binding, revealing that this is the dominant factor in determining the strength of the receptor−ligand binding. Hence, we were able to use a suitable control system to accurately quantify the effect of membrane concentration on intra-membrane interactions.

Receptor **2** bound in the membrane of phosphatidylcholine vesicles formed Cu**2**, Cu$\mathbf{2}_2$, and Cu$\mathbf{2}_4$ complexes with copper(II). The affinity of **2** for copper(II) increased dramatically with an increase in the membrane concentration of **2**. In addition, the nature of the complex formed is highly dependent on the membrane concentration of **2**. At membrane loadings below 2 mol % the Cu$\mathbf{2}_2$ complex was the major species, as in the solution binding experiments. However, at very low loadings of receptor (<0.75 mol %), the intra-membrane binding interactions were inhibited relative to interactions in solution, and Cu**2** was the dominant complex. In effect, under these conditions the receptors are kept apart despite localization in the membrane. The behavior of this system illustrates how cells might control the production of a particular type of ligand−receptor cluster by either increasing or decreasing the amount of receptor expressed in the membrane. Receptor aggregation gives a cell a more versatile mechanism to control its response to external messengers compared to other signaling mechanisms.

Experimental Section

NMR spectra were recorded on Bruker AC 250 or AMX 400 spectrometers, UV−vis spectra on a Varian Cary 1 Bio spectrophotometer and fluorescence spectra on a Hitachi F-4500 fluorescence spectrophotometer. ES^+, CI^+, and FAB^+ (using an *m*-nitrobenzyl alcohol matrix) mass spectra were obtained on Micromass Prospec and Micromass Platform spectrometers, and MALDI-TOF mass spectra were recorded on a Bruker Reflex III MALDI-TOF mass spectrometer.

Column chromatography was carried out on 60 mesh silica gel. Water was doubly distilled before use. Dansylamide **3** was used as purchased from Aldrich.

Preparation of Dansyl Ethylenediamine, 1.[10] A solution of dansyl chloride (200 mg, 0.74 mmol) in dichloromethane (6 mL) was dropped into 1,2-ethylenediamine (6.5 mL, 445 mg, and 7.42 mmol) while stirring and cooling over ice. The mixture was stirred while warming to room temperature for 1 h. The reaction mixture was acidified with dilute HCl and then extracted with dichloromethane (2 × 20 mL). The aqueous layer was made basic (pH 9) using 10 M NaOH and again extracted with dichloromethane (2 × 20 mL). The organic layer was dried over $MgSO_4$, filtered through a sinter and the solvent removed under reduced pressure to give (2-aminoethyl)-dansylamide as a yellow solid (200 mg, 92%). ^{1}H NMR: ($CDCl_3$, 250 MHz); δ 2.63 (t, $J = 5.8$ Hz, 2H; $NHCH_2CH_2NH_2$), 2.80 (s, 6H; $N(CH_3)_2$ and m, 2H; $NHCH_2$-CH_2NH_2), 7.19 (d, $J = 7.6$ Hz, 1H; 6-CH-dansyl), 7.52 (m, 2H; 7-CH-dansyl and 3-CH-dansyl), 8.24 (m, 2H; 8-CH-dansyl and 4-CH-dansyl), 8.47 (d, $J = 8.2$ Hz, 1H; 2-CH-dansyl). MS (ES^+): *m*/*z* 294 (MH^+); Anal. Calcd. for $C_{14}H_{19}N_3O_2S_1 \cdot {}^1/_2H_2O$: C, 55.61; H, 6.67; N, 13.90; S, 10.60. Found: C, 55.72; H, 6.40; N, 13.77; S, 10.36.

Preparation of Cholesteryl Bromoacetate.[21] Cholesterol (383 mg, 1 mmol) was dissolved in dry THF (5 mL). Bromoacetyl chloride (250 μL, 3 mmol) was added dropwise and the reaction mixture was stirred at reflux for 1.5 h. The solution was concentrated to an oil under reduced pressure, and the residue recrystallized from ethyl acetate to give the product as a white powder (337 mg, 0.67 mmol, 67%). mp 160−162 °C; ^{1}H NMR ($CDCl_3$, 250 MHz): δ 0.6−2.25 (m, 43H, cholesterol

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protons), 3.8 (s, 2H, CH_2Br), 4.6 (m, 1H, 3-CH-cholesterol), 5.4 (d, 1H, 3.9 Hz 6-CH-cholesterol). MS (CI^+) *m*/*z* 524 ($M+NH_4^+$).

Preparation of 3-*O*-(2-(2-Aminoethyl)dansylamide)acetyl)cholesteryl Ester, 2. Dansyl ethylenediamine **1** (21.9 mg, 74.6 μmol), cholesteryl bromoacetate (34 mg, 67.8 μmol), and sodium carbonate (18 mg, 169.5 μmol) were suspended in ca. 5 mL of acetonitrile. The reaction was stirred at reflux overnight. The resulting solution was concentrated, dissolved in chloroform, filtered, and concentrated again. The crude product was purified by column chromatography on silica (chloroform/ ethyl acetate, 4:1). The solvent was removed from the product containing fractions under reduced pressure and the residue recrystallized by slow evaporation of the solvent from dichloromethane/ hexanes to give the product as a pale green solid (42 mg, 58 μmol, 86%). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 0.6−2.25 (m, 43H, cholesterol protons), 2.5 (t, 2H, ethylamine CH_2), 2.6 (s +t, 8H, $N(CH_3)_2$, ethylamine CH_2), 3.0 (s, 2H, $2H_c$), 4.5 (m, 1H, 3-CH-cholesterol), 5.6 (d, 1H, 6-CH-cholesterol), 7.1 (d, 1H, J = 7.3 Hz, 1H, 6-CH-dansyl), 7.5 (m, 2H, 3- and 7-CH-dansyl), 8.2 (d, 1H, J = 7.3 Hz, 8-CH-dansyl), 8.3 (d, 1H, J = 8.6 Hz, 1H, 4-CH-dansyl) 8.5 (d, 1H, J = 8.2 Hz, 1H, 2-CH-dansyl). MS (FAB^+) *m*/*z:* found 720.475077; calcd for MH^+, 720.477405. Anal. Calcd. for $C_{43}H_{64}N_3O_4S.C_6H_{14}\cdot 0.5H_2O$: C, 72.20; H;9.90; N; 5.15; S, 3.95. Found: C, 72.0; H, 9.60; N, 4.95; S, 3.85. Analysis by HPLC (silica, eluant: 9:1 ^{i}PrOH:petrol with 0.1% triethylamine, retention time 16.6 min) showed >98% purity.

Synthesis of Vesicles. We used egg yolk phosphatidylcholine (EYPC, used as purchased from Sigma (type XVI from fresh egg yolk)) to make our vesicles as bilayers constructed from EYPC are in a fluid state at temperatures above −7°.[22] Using phosphatidylcholines rather than ethanolamines avoided complications arising from competitive complexation to copper(II) by the phospholipid. The EYPC phospholipid bilayers, though impermeable to fluorescein on the time scale of the binding experiments, were permeable to copper(II) ions.[23] This eliminated the need to differentiate between binding to receptors on the interior of the vesicle and receptors on the exterior of the vesicle.

Unilamellar vesicles were prepared by dissolving egg yolk phosphatidylcholine (64 mg, 320 mg for vesicles 0.2 mol % in the receptor) and the required amount of **2** (0.2, 0.5, 1, 2.5, 5, and 7.5 mol %) in spectroscopic grade ethanol-free chloroform (20 mL), followed by removal of the solvent to give a thin film of phospholipid on the interior of the flask. The buffer was added (MES 50 mM pH 6.0) to the flask, and the thin film detached by vortex mixing to give a suspension of multilamellar vesicles. These were extruded through a single 800 nm polycarbonate membrane using an Avestin Liposofast extrusion apparatus to give unilamellar vesicles. Vesicle size was characterized by static light scattering and membrane integrity confirmed through carboxyfluorescein encapsulation.

Calculation of Membrane Environment. (a) Calibration Curve. Monomer probes **1** or **3** (2×10^{-8} mol) were transferred from stock solution (l00 μL from 1×10^{-4} M solution in THF) into each of eight 10 mL volumetric flasks. Solutions were made up to the 10 mL mark using dichloromethane, THF, *n*-octanol, *n*-butanol, ethanol, methanol, water, and MES buffer (pH 6) to give 2×10^{-6} M solutions. The fluorescence emission spectrum of each solution was measured at 25 °C. A calibration plot was constructed using λ_{max} of emission versus the dielectric constant of each of the solvents.

(b) Measurements in Vesicles. Aliquots of the 1×10^{-4} M vesicular stock solutions (200 μL) were diluted 100 fold using MES buffer (pH 6). The fluorescence emission spectrum of each of these solutions was then recorded at 25 °C. The results were interpolated into the calibration plot.

Titrations with Copper(II) Chloride. Aliquots of a 5 mM copper-(II) chloride solution dissolved in MES buffer (20 mM pH 6.0) were added to the appropriate solutions. The fluorescence spectrum was recorded after each addition. The stoichiometry and binding affinity was determined by using an iterative curve-fitting program to fit the decrease in emission intensity with increasing copper(II) concentration.

Acknowledgment. This work was supported by the BBSRC (Grant Ref. B50/08373), EPSRC, and the Royal Society.

Supporting Information Available: Details of experiments to demonstrate membrane permeability to copper(II) and UV−vis titration of recptors with copper(II). Tables detailing observed binding constants for receptor **2** to copper(II) in different concentrations of phospholipid, fluorescence emission λ_{max} for dansylamide and headgroup **1** in different solvents, fluorescence emission λ_{max} for dansylamide in mixtures of water and methanol, fluorescence data for the titration of **1** in 4% MES buffer in methanol with copper(II), fluorescence data for the titration of vesicles containing **2** with copper(II), and values used to calculate the curve fits. This material is available free of charge via the Internet at http://pubs.acs.org.

JA021048A

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(23) Copper(II) ions rapidly diffused into vesicles containing the ion-sensitive dye xylenol orange; see supplementary information.