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Synthetic Mimics of Small Mammalian Cell Surface Receptors

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Abstract: Receptors on the surface of mammalian cells promote the uptake of cell-impermeable ligands by receptor-mediated endocytosis. To mimic this process, we synthesized small molecules designed to project anti-dinitrophenyl antibody-binding motifs from the surface of living Jurkat lymphocytes. These synthetic receptors comprise *N*-alkyl derivatives of 3β-cholesterylamine as the plasma membrane anchor linked to 2,4-dinitrophenyl (DNP) and structurally similar fluorescent 7-nitrobenz-2-oxa-1,3-diazole (NBD) headgroups. Insertion of two β-alanine subunits between a DNP derivative and 3β-cholesterylamine yielded a receptor that avidly associates with cell surfaces (cellular $t_{1/2} \sim 20$ h). When added to Jurkat cells at 10 μM, this receptor enhanced uptake of an anti-DNP IgG ligand by ~200-fold in magnitude and ~400-fold in rate within 4 h (ligand internalization $t_{1/2} \sim 95$ min at 37 °C). This non-natural receptor mimics many natural receptors by dynamically cycling between plasma membranes and intracellular endosomes (recycling $t_{1/2} \sim 3$ min), targeting of protein ligands to proposed cholesterol and sphingolipid-enriched lipid raft membrane microdomains, and delivery of protein ligands to late endosomes/lysosomes. Quantitative dithionite quenching of fluorescent extracellular NBD headgroups demonstrated that other 3β-cholesterylamine derivatives bearing fewer β-alanines in the linker region or *N*-acyl derivatives of 3β-cholesterylamine were less effective receptors due to more extensive trafficking to internal membranes. Synthetic cell surface receptors have potential applications as cellular probes, tools for drug delivery, and methods to deplete therapeutically important extracellular ligands.

Introduction

Receptors on the surface of mammalian cells function as sensors and mediators of uptake of specific ligands in the extracellular environment. These biomolecules reside on the cellular plasma membrane, the protective lipid bilayer that envelops the cell to guard the inner cellular machinery from potentially toxic or opportunistic extracellular materials. Only small hydrophobic compounds passively diffuse across this hydrophobic barrier to rapidly penetrate into cells. More hydrophilic macromolecules, such as proteins and DNA, generally require specific active transport mechanisms to access the cell interior. Cell surface receptors are components of this active transport machinery and deliver cargo, such as nutrients and growth factors, into cells via the uptake process of receptor-mediated endocytosis.[1]

Mammalian cell surface receptors comprise at least three basic components: a ligand recognition motif projecting from the cell surface, a linker region, and a membrane-binding element that anchors the receptor to the plasma membrane. As an example, the small receptor ganglioside GM1 (**1**, Figure 1) projects a pentasaccharide headgroup from the cell surface that binds the bacterial protein cholera toxin as a ligand. This molecular recognition event enables cholera toxin to penetrate into cells by receptor-mediated endocytosis.[2,3] Binding of ligands to internalizing cell surface receptors generally results in clustering of receptor/ligand complexes in plasma membrane subdomains that invaginate and pinch off as intracellular endocytic vesicles. These internalized vesicles become increasingly acidified by the activation of proton pumps and fuse in the cytoplasm to form larger acidic (pH ~ 6) endosomes.[4] The altered chemical environment within endosomes typically dissociates internalized receptor/ligand complexes. The free receptor can traffic to other endosomes, can cycle back to the cell surface via exocytosis, and can often be reused for uptake of ligand up to several hundred times.[4] The free ligand either exits endosomes and proceeds to other intracellular locations or more often becomes trapped in lysosomes, more acidic (pH ~ 5.0) organelles containing degratory enzymes that are highly active at this low pH.[4]

Cell surface receptors involved in receptor-mediated endocytosis have been extensively targeted to enable cellular uptake of poorly permeable molecules. Ligands of these receptors have been used to deliver linked drugs, molecular probes, and macromolecules into cells.[5] Receptors targeted in this way include the glycosylphosphatidylinositol (GPI) lipid-anchored

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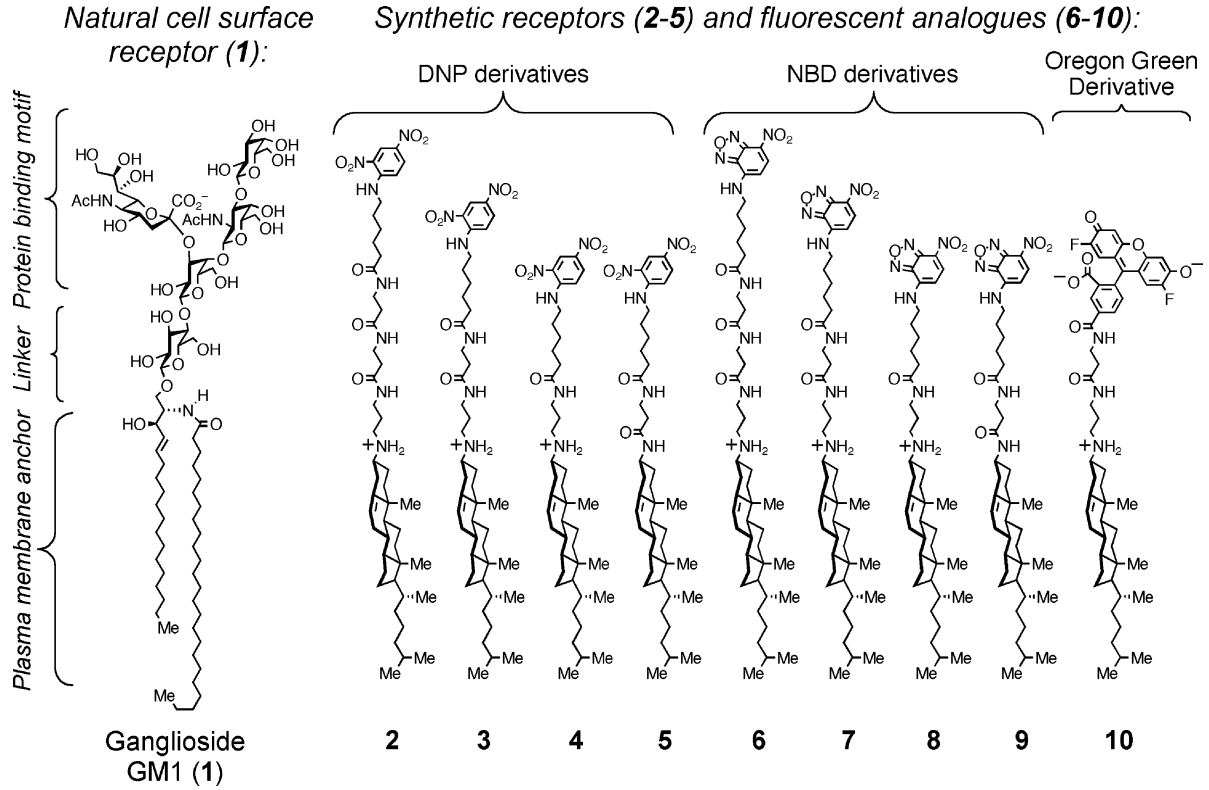

Figure 1. Structures of the small natural receptor ganglioside GM1, novel synthetic receptors, and related control compounds.

folate receptors,[6−8] transferrin receptors,[9] and low-density lipoprotein (LDL) receptors[10] that are often overexpressed on rapidly proliferating cells.

As an alternative to natural receptors targeted for the delivery of impermeable molecules, our laboratory is investigating synthetic mimics of cell surface receptors. Synthetic receptors derived from 3β-cholesterylamine linked to fluorescein, biotin, and peptides as protein-binding motifs have been directly loaded into plasma membranes of living mammalian cells to promote the endocytosis of impermeable protein ligands.[11−13] This approach has been termed "synthetic receptor targeting" (Figure 2).[12] Other elegant approaches for cell surface engineering have also been reported[14−18] and reviewed.[19] A chemical approach for rewiring related cellular adhesion processes has also been described.[20] The cellular uptake of ligands mediated by 3β-cholesterylamine-derived receptors has been proposed[12] to mimic endocytic cellular penetration mechanisms employed by

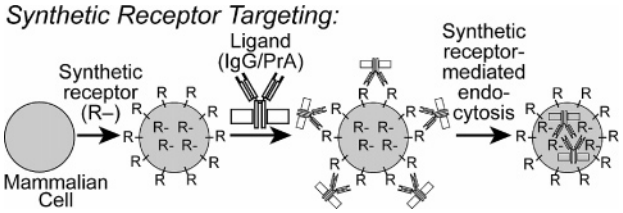

Figure 2. Synthetic receptor targeting approach for enhancing the cellular uptake of impermeable ligands. Synthetic mimics of cell surface receptors are added to living mammalian cells. These cells internalize cognate ligands, such as macromolecular antibodies (IgG), bound to bacterial protein A (PrA) by synthetic receptor-mediated endocytosis.

certain protein toxins and viruses.[21] These mechanisms are highly complex at the molecular level and are thought to involve plasma membrane subdomains termed lipid rafts[22,23] and/or endocytosis involving the protein clathrin.[1,24,25]

Lipid rafts are proposed to comprise liquid-ordered phases of the cellular plasma membrane enriched in cholesterol and sphingolipids, such as ganglioside GM1 (**1**).[23,26,27] These domains have recently been visualized by two-photon microscopy studies of living cells,[28] and aspects of lipid rafts have been reconstituted in model systems.[29] Many proteins covalently or noncovalently associated with cholesterol, sphingolipids, or saturated lipids of cellular membranes are thought to associate

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with lipid rafts[30] that segregate and concentrate membrane proteins, regulate the activation of specific signal transduction pathways,[31] and control the endocytosis of specific receptors.[32] Binding of cholera toxin to the lipid raft-associated receptor ganglioside GM1 (**1**) promotes uptake of the toxin by endocytosis. In cell lines, such as lymphocytes, that lack the raft-associated protein caveolin, this endocytosis is thought to be mediated by the protein clathrin.[33] However, the molecular mechanisms linking **1** to clathrin are not yet well understood, and small receptors, such as **1**, are also thought to internalize ligands via distinct lipid raft-dependent endocytic pathways.[33,34]

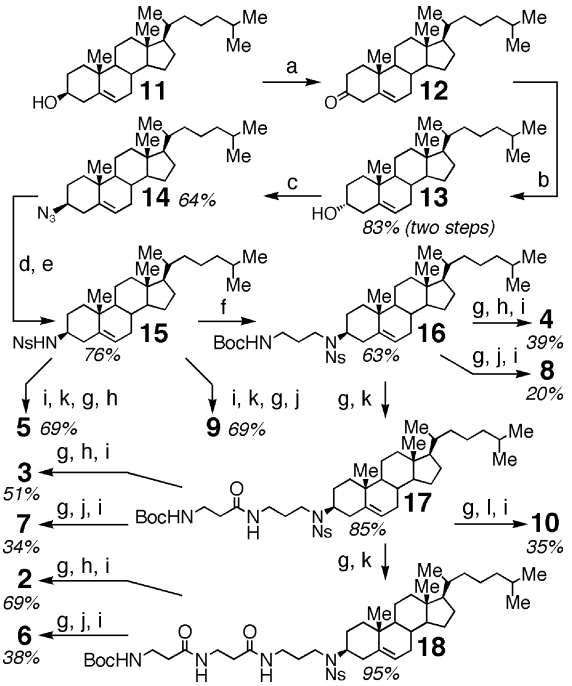
We report here the construction of novel synthetic mimics of small cell surface receptors (**2**−**5**) and related green fluorescent analogues (**6**−**10**) as mechanistic probes (Figure 1). When added to living mammalian cells, these compounds were designed to insert in the cellular plasma membrane, project protein-binding headgroups from the cell surface, capture cognate soluble antibodies (IgG), and internalize these macromolecular ligands. We found that the most efficient synthetic receptor (**2**) mimics many natural receptors by avidly associating with the cell surface, rapidly cycling between plasma membranes and intracellular endosomes, targeting of protein ligands to cholesterol and sphingolipid-enriched lipid rafts, and delivery of protein ligands to late endosomes/lysosomes. Remarkably, subtle molecular modifications substantially altered the trafficking of these compounds to internal membranes, resulting in modulation of the efficiency of synthetic receptors as delivery systems for impermeable ligands.

Results

Design and Synthesis of Receptors for Anti-DNP IgG Ligands. To enable mammalian cells to capture and internalize anti-dinitrophenyl IgG ligands, synthetic receptors were designed that incorporate 2,4-dinitrophenyl (DNP) and structurally similar green fluorescent 7-nitrobenz-2-oxa-1,3-diazole (NBD) headgroups. These headgroups were linked to *N*-alkyl and *N*-acyl derivatives of 3β-cholesterylamine via tethers containing 6-aminohexanoic acid and β-alanine subunits (Figure 1). To evaluate the affinity of these headgroups for rabbit polyclonal anti-DNP IgG, related fluorescent derivatives were evaluated with fluorescence polarization assays. These assays indicated that both DNP and fluorescent NBD derivatives bind tightly to this IgG with apparent K_d values of 23 ± 1.5 nM (DNP) and 820 ± 144 nM (NBD) (data shown in Figure S1, Supporting Information). The weaker binding of NBD compared with that of DNP is consistent with previous biophysical studies of similar receptor/ligand systems.[35] Detailed binding studies of anti-DNP IgG to DNP-linked lipids embedded in model membranes on a solid support were recently reported.[36]

To construct synthetic receptors **2**−**10**, a previously reported[37] synthesis of 3β-cholesterylamine was modified, as shown in Scheme 1. This approach, incorporating a Swern oxidation[38] of cholesterol (**11**) to ketone **12**, provided the nosyl-protected 3β-cholesterylamine **15** in 40% yield over five steps. This compound (**15**) was elaborated to receptors **2**−**10** by Fukuyama's amine synthesis methodology[39] and/or deprotection and sequential amide bond formation reactions. Synthetic procedures and compound characterization data are provided in the Supporting Information.

Scheme 1 [a]



[a] Reagents and conditions: (a) oxalyl chloride, DMSO, CH_2Cl_2, TEA, −78 °C; (b) L-selectride, THF, −78 °C; (c) PPh_3, HN_3, DEAD, benzene; (d) $LiAlH_4$, Et_2O, 0 °C; (e) 2-nitrobenzenesulfonyl chloride, DIEA, THF; (f) boc-3-chloropropylamine, K_2CO_3, DMA, 120 °C; (g) TFA, CH_2Cl_2 (2:25); (h) 6-(2,4-dinitrophenyl)aminohexanoic acid succinimidyl ester, DIEA, CH_2Cl_2; (i) PhSH, K_2CO_3, THF/DMF (1:4); (j) 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid succinimidyl ester, DIEA, CH_2Cl_2; (k) boc-β-alanine NHS ester, DIEA, CH_2Cl_2; (l) 5-carboxyoregongreen NHS ester, DIEA, DMF.

The Linker Region of Synthetic Receptors Controls the Magnitude and Kinetics of Ligand Uptake. Synthetic receptors (**2**−**9**) were evaluated as mediators of cellular uptake of protein ligands in living Jurkat lymphocytes (a human T-cell line). Confocal laser scanning microscopy and flow cytometry were employed to examine cells that were treated with synthetic receptors, washed to remove unincorporated compounds, and subsequently treated with a polyclonal rabbit anti-DNP antibody (IgG) complexed with fluorescent conjugates of the IgG-binding protein A (PrA) from *Staphylococcus aureus*.[40,41] Confocal microscopy of cells treated with the green fluorescent NBD derivative **6** (10 μM, 1 h) and anti-DNP IgG-labeled red fluorescent with PrA-Alexa Fluor-594 (PrA-AF594, 4 h) revealed receptor **6** localized both on the cell surface (embedded in the plasma membrane) and in intracellular compartments (Figure 3, panel A). The red fluorescent IgG ligand was taken

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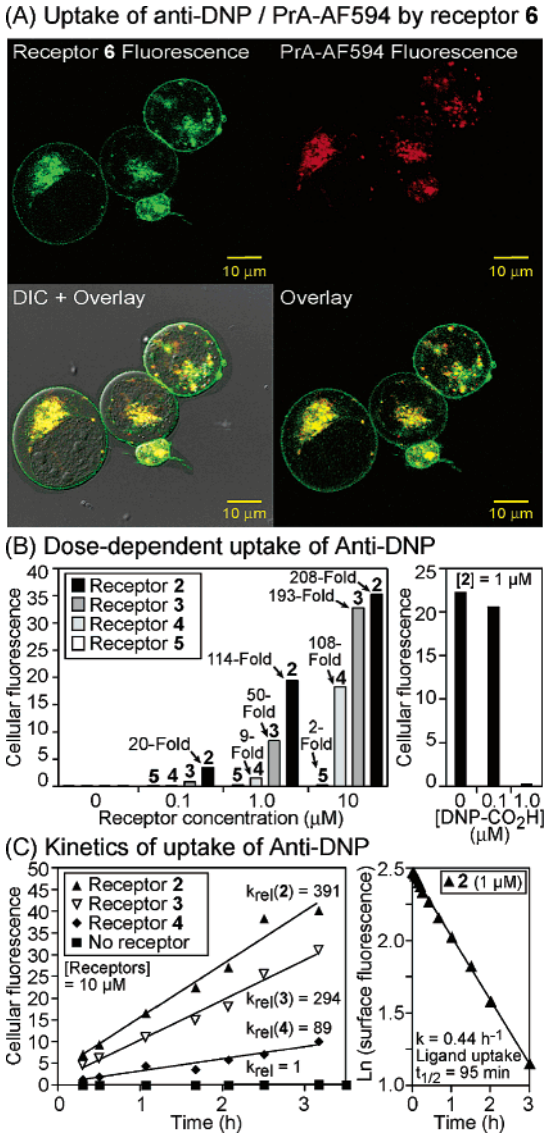

Figure 3. Synthetic receptor-mediated uptake of protein ligands by Jurkat lymphocytes. Cells were pretreated with receptors for 1 h at 37 °C prior to addition of an anti-DNP IgG/PrA complex. Panel A: confocal laser scanning and differential interference contrast (DIC) microscopy of cells treated with the green fluorescent receptor **6** (10 μM) followed by addition of red fluorescent anti-DNP/PrA-AF594 (for 4 h). Yellow pixels indicate co-localization of green and red fluorophores. Panel B: dose-dependent magnitude of uptake of anti-DNP/PrA-AF488 (after 4 h) quantified by flow cytometry. Right: competitive inhibition of this uptake (mediated by receptor **2**, 1 μM) by addition of 6-(2,4-dinitrophenyl)aminohexanoic acid (DNP-CO_2H). Panel C: kinetics of uptake of anti-DNP/PrA-AF488 quantified by flow cytometry. Left: relative differences in receptor uptake rates quantified by accumulation of cellular fluorescence. Right: determination of the rate and half-life of ligand internalization mediated by receptor **2** at 1 μM.

up by cells under these conditions, but it only partially co-localized with intracellular **6**, suggesting dissociation of the receptor/ligand complex within cells (Figure 3, panel A). This intracellular red fluorescence was 59-fold lower in the absence of receptor **6** (Figure S2, Supporting Information), consistent with a synthetic receptor-mediated uptake mechanism. The IgG-loaded intracellular compartments were identified as late endosomes and lysosomes by co-localization studies in cells transfected with EGFP-lgp120, a fluorescent protein marker of these compartments (Figure S3, Supporting Information).[42] Comparison of cells treated with receptor **6** alone with cells treated with receptor **6** followed by excess anti-DNP IgG/PrA-AF594 for 4 h revealed that the protein ligand did not deplete the receptor from the cell surface, despite extensive uptake of this protein complex. These and other results presented here are consistent with a delivery mechanism involving binding of the IgG/PrA ligand to the synthetic receptor **6** at the cell surface, internalization of this complex by endocytosis, dissociation of IgG from **6** in acidic endosomes, and return of receptor **6** to the cell surface by plasma membrane recycling.

To investigate the efficiency of uptake mediated by the higher affinity DNP-based receptors **2**−**5**, the magnitude and kinetics of delivery of a green fluorescent anti-DNP/PrA-AF488 complex were quantified by flow cytometry (Figure 3, panels B and C). Since this technique does not distinguish cell surface-associated fluorescence from intracellular fluorescence, cells were washed with the hapten 6-(2,4-dinitrophenyl)aminohexanoic acid (DNP-CO_2H, 100 μM) prior to analysis to competitively displace any noninternalized antibody. The longest of the synthetic receptors (**2**) proved to be the most efficient in both magnitude and relative rate of delivery of the IgG ligand into cells. After treatment of cells with receptor **2** for 1 h at a final concentration of 10 μM, this receptor enhanced the cellular uptake of added anti-DNP IgG by 208-fold in magnitude and 391-fold in rate compared with basal levels of endocytosis. Dose-dependent competition experiments with DNP-CO_2H added with the antibody further confirmed the specificity of this uptake (Figure 3, panel B, right). Remarkably, sequential removal of β-alanine subunits from the linker of **2**, affording receptors **3** and **4**, substantially reduced receptor efficiency. Additionally, conversion of the secondary amine of receptor **4** to the secondary amide of **5** essentially abolished internalization of IgG, reducing delivery compared with that of **4** by over 50-fold at 10 μM (Figure 3, panel B). The most efficient receptor (**2**, 1 μM) promoted internalization of the fluorescent protein ligand with first-order kinetics (k = 0.44 h^{-1}) corresponding to a ligand half-life on the cell surface of 95 min (Figure 3, panel C, right). This value was quantified by cooling receptor-treated cells to 4 °C to block endocytosis, allowing the protein ligand to bind the receptor (**2**) on the cell surface, warming cells to 37 °C to trigger uptake, and subsequent competitive removal of cell surface protein prior to analysis. Under the ligand uptake conditions shown in Figure 3, no significant cellular cytotoxicity was observed after 24 h (see the Supporting Information for details).

Linker Structure Controls the Subcellular Localization of Synthetic Receptors. To investigate the substantial differences in biological activities of receptors **2**−**5**, cells were treated with structurally similar green fluorescent receptors (**6**−**9**). Epifluorescence microscopy and fluorescence quenching assays were employed to examine differences in cellular fluorescence and receptor subcellular localization. As shown in Figure 4 (panel A), the overall cellular fluorescence resulting from treatment with 10 μM of these compounds was within 2-fold (Figure 4 and Figure S2 of the Supporting Information). However, the subcellular localization of these compounds differed significantly. Receptor **6**, bearing two β-alanine linker subunits, was nearly equally distributed between the cell surface and internal membranes. However, the single β-alanine-containing receptor **7** exhibited lower cell surface localization, and the absence of β-alanine in the linker of **8** rendered this compound

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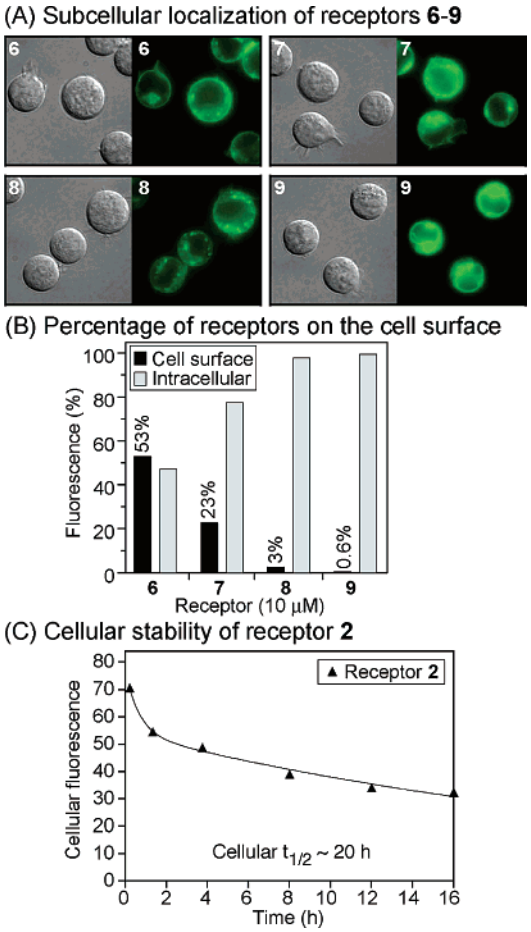

Figure 4. Subcellular localization of synthetic receptors in living Jurkat lymphocytes. Panel A: DIC (left images) and epifluorescence micrographs (right images) of cells treated for 1 h with green fluorescent receptors **6**−**9** (10 μM). Panel B: quantification of cell surface fluorescence by quenching of exposed fluorophores with cell-impermeable sodium dithionite. Cells treated, as shown in panel A, were washed with sodium dithionite (5 mM) for 10 s at 22 °C, and residual fluorescence was quantified by flow cytometry. Panel C: quantification of the cellular half-life of receptor **2**. Cells were treated with **2** (10 μM) for 1 h, resuspended in receptor-free media for the times indicated, and **2** was detected on the cell surface with anti-DNP/PrA-AF488.

predominately intracellular. Remarkably, the amide analogue **9** was essentially completely associated with different internal membranes, which explains the inability of this analogue to mediate significant cellular uptake of the anti-DNP IgG ligand. In contrast to the fluorescent *N*-alkyl derivatives of 3β-cholesterylamine (**6**−**8**), this *N*-acyl analogue (**9**) nearly completely co-localized with a red fluorescent probe of the cellular golgi apparatus and nuclear membrane, indicating a unique intracellular destination of this ineffective receptor (data shown in Figure S4 of the Supporting Information).

The fraction of receptors **6**−**9** at the cell surface was quantified by irreversible quenching of surface-exposed NBD fluorophores. This was accomplished by brief treatment of cells with the relatively cell-impermeable reducing agent sodium dithionite (sodium hypodisulfite, NaO_2S-SO_2Na), which rapidly reduces the nitro functionality of extracellular NBD headgroups to the amine, enabling quantification of differences in cell surface and intracellular fluorescence.[43,44] Quantitative dithionite quenching assays demonstrated substantial differences in receptor subcellular localization (Figure 4, panel B) that paralleled

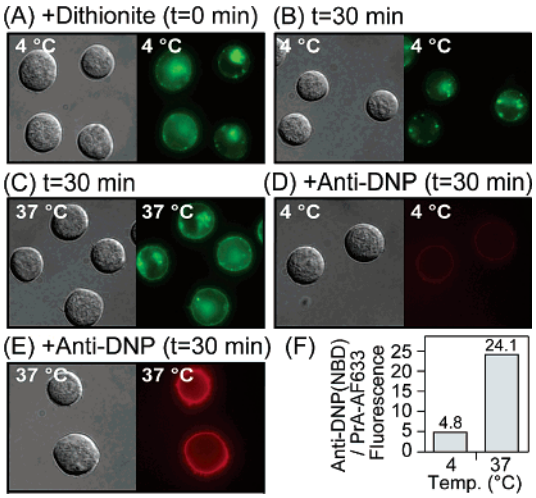

Figure 5. Cycling of receptor **6** between the cell surface and intracellular endosomes in Jurkat lymphocytes. Panels A−E: DIC (left) and epifluorescence (right) micrographs of cells captured after treatment with **6** (10 μM, 1 h) followed by quenching of cell surface fluorophores at 4 °C with sodium dithionite (30 mM, 5 min). Cells were washed with ice-cold media (t = 0 min, panel A), split into two portions, and return of green fluorescence to the cell surface was examined as a function of time and temperature (panels A−C). Cells were again cooled to 4 °C prior to detection of NBD headgroups at the cell surface with red fluorescent anti-DNP/PrA-AF633 by epifluorescence microscopy (panels D and E, t = 30 min) and flow cytometry (panel F, t = 30 min). NBD fluorescence is shown in panels A−C; PrA-AF633 fluorescence is shown in panels D−F.

the magnitude and kinetics of IgG uptake observed with receptors **2**−**5**. Thus, the relative distribution of synthetic receptors between the cell surface and intracellular membranes is a major factor controlling the efficiency of these compounds as mediators of ligand uptake.

To evaluate the cellular stability of receptor **2**, the half-life of this compound on living Jurkat lymphocytes was quantified by addition of a soluble antibody. Cells were treated with this receptor for 1 h (10 μM); the unincorporated receptor was removed by washing cells with fresh media, and the abundance of **2** on the cell surface as a function of time was detected with a green fluorescent anti-DNP/PrA-AF488 complex. Curve fitting of the flow cytometry data shown in Figure 4 (panel C) revealed a cellular half-life of 20 h. This value is similar to the ~24 h half-life of natural folate receptor proteins that are anchored to the cell surface by covalently attached GPI lipids.[45]

Synthetic Receptors Rapidly Cycle between the Plasma Membrane and Intracellular Endosomes. Many natural cell surface receptors undergo dynamic plasma membrane recycling.[4] In this process, free receptors and receptor/ligand complexes rapidly traffic between the cell surface and intracellular endosomes. Receptors typically dissociate from ligands in these acidic compartments and cycle back to the cell surface up to several hundred times, whereas free ligands are often sorted to degratory lysosomes. To examine whether the green fluorescent receptor **6** might undergo similar dynamic cycling, a fluorescence-quenching assay with sodium dithionite was employed. As shown in Figure 5, Jurkat cells were treated with receptor **6** (10 μM) and cooled to 4 °C to stop plasma membrane recycling.

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Fluorescence at the cell surface was quenched by treatment with ice-cold sodium dithionite to reduce the nitro group of **6**, and cells were washed with cold media to remove this reducing agent (Figure 5, panel A). This cell culture was split into two equal portions; one portion was maintained at 4 °C (Figure 5, panel B) for 30 min, and the other portion was warmed to 37 °C for 30 min to reactivate plasma membrane recycling (Figure 5, panel C). Significant return of green fluorescence back to the plasma membrane was observed only in the sample warmed to 37 °C (compare panels B and C of Figure 5). Moreover, NBD headgroups that returned to the cell surface were detected with the NBD-binding red fluorescent anti-DNP/PrA-AF633 complex. In the cells warmed to 37 °C, a 5-fold increase in receptor **6** at the plasma membrane was observed (Figure 5, panels D−F). These results demonstrate that the intracellular fraction of synthetic receptors is in dynamic exchange with the cellular plasma membrane, consistent with the synthetic receptors accessing an active plasma membrane recycling pathway.

To quantify the rate of plasma membrane recycling, the oregon green-derived receptor **10** was employed. Unlike the more strongly cell-associated DNP and NBD-based receptors, this compound (**10**) binds proteins in cell culture media and can be rapidly and efficiently depleted from cell surfaces by washing cells with "back exchange" media containing bovine serum albumin (BSA, 1%) and methyl-β-cyclodextrin (Me-β-CD, 2 mM). This concentration of Me-β-CD has been shown to affect neither cellular endocytosis nor plasma membrane recycling.[46] The oregon green fluorophore of **10** was also chosen for these studies because, unlike the structurally similar carboxyfluorescein, the fluorescence of oregon green is not appreciably quenched in the acidic environment of endosomes.[47] Cells treated with **10** (1 μM) exhibited bright green fluorescence both at the cell surface and in the intracellular (endosomal) compartments; this distribution of cellular fluorescence was similar to that of cells treated with the structurally related receptor **7** (data not shown). Cells were treated with **10** and cooled to 4 °C to stop dynamic plasma membrane recycling. These cells were washed four times with ice-cold back exchange media to extensively deplete **10** from the cell surface without affecting the population of this compound in intracellular endosomes. Back exchange media prewarmed to 37 °C was subsequently added to reactivate plasma membrane recycling. This temperature jump resulted in rapid return of **10** to the plasma membrane by exocytosis, and the added back exchange media efficiently removed the surface-exposed recycled receptors. Analysis of cellular fluorescence as a function of time provided the fluorescence decay data shown in Figure 6. Fitting of these data with a one-site exponential decay model yielded a recycling half-life of 2.8 ± 0.8 min, indicating rapid recycling of synthetic cell surface receptors. The fluorescent lipid C_6-NBD-sphingomyelin is known to exhibit similar recycling kinetics in mammalian cells.[48]

Synthetic Receptor/Ligand Complexes Cofractionate with Lipid Raft Components of Cellular Plasma Membranes. Another hallmark of many natural cell surface receptors is the proposed association of these biomolecules with cholesterol and

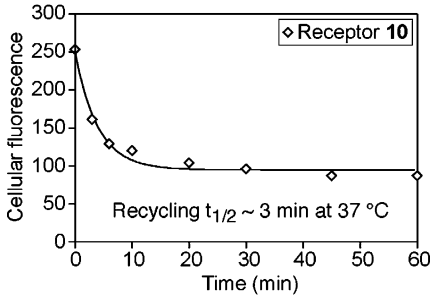

Figure 6. Quantification of efflux kinetics of receptor **10** from endosomes to the plasma membrane of Jurkat lymphocytes by back exchange. Cells treated with **10** (1 μM, 1 h) were cooled to 4 °C and cell surface fluorophores removed (>90%) by repeated washing with ice-cold back exchange media containing BSA (1%) and methyl-β-cyclodextrin (2 mM). Cells were resuspended in back exchange media at 37 °C to resume recycling; cellular fluorescence was quantified by flow cytometry, and loss of fluorescence was evaluated with a one-site exponential decay model.

sphingolipid-enriched lipid raft subdomains of cellular plasma membranes.[49] These subdomains are generally biochemically characterized as low-density fractions of the plasma membrane insoluble in buffers containing nonionic detergents, such as Triton X-100 at 4 °C.[29] Ganglioside GM1 (**1**) is a prototypical example of a small lipid raft-associated cell surface receptor, and complexes of this glycolipid with fluorescent cholera toxin protein are often used as markers for lipid rafts.[50] In addition, macromolecular receptors, including B-cell receptors, T-cell receptors, and growth factor receptors, are thought to associate with these membrane subdomains.[49] In certain cases, binding of ligands to receptors initiates formation of lipid rafts.[51] This ligand-mediated concentration of specific membrane lipids has been proposed to recruit intracellular raft-associated kinases to activate cellular signaling pathways.[31]

To determine whether synthetic cell surface receptors (**2**−**5**) might cofractionate with ganglioside GM1 (**1**) in lipid rafts, sucrose density gradient ultracentrifugation was employed to fractionate cellular membranes containing receptor/ligand complexes (Figure 7). As positive and negative control experiments, Jurkat cells were treated with green fluorescent conjugates of cholera toxin B subunit and transferrin proteins. Unlike binding of cholera toxin to ganglioside GM1 (**1**), binding of transferrin to the transferrin receptor on the cell surface does not result in association with lipid rafts.[50] As shown in Figure 7, a low-density fluorescent membrane fraction (#5) was observed upon treatment of cells with the cholera toxin B subunit, but this fraction was not observed upon treatment with transferrin, consistent with previous studies of lipid raft association of these proteins.[50] Application of this analysis to receptors **2**−**5** (1 μM) revealed that only receptors **2** and **3** enabled the isolation of this fluorescent low-density membrane fraction (#5). These results paralleled the magnitude of protein uptake mediated by synthetic receptors **2**−**5** at the receptor concentration studied (Figure 3, panel B). Unlike the IgG-bound receptors, fractionation of cells treated with the green fluorescent receptor **6** alone did not reveal any significant lipid raft association of the free receptor (data not shown). Free receptors may be associated with less well-ordered "lipid shell" domains of cellular mem-

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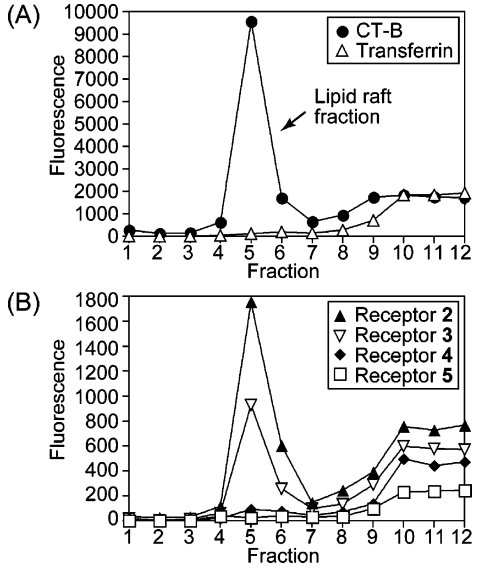

Figure 7. Association of receptor/ligand complexes with putative lipid raft fractions of plasma membranes of Jurkat lymphocytes. Panel A: cells were treated with the AF488-labeled protein ligand for 30 min at 4 °C. Panel B: cells were treated with receptors **2**−**5** (1 μM) for 1 h at 37 °C followed by an anti-DNP/AF488 conjugate for 30 min at 4 °C. Cells were fractionated by ultracentrifugation for 20 h at 4 °C in a 2−40% sucrose step gradient containing 0.2% Triton X-100. CT-B: cholera toxin B subunit.

branes.[52] These results suggest additional mechanistic similarities between uptake of IgG-bound synthetic receptors and uptake of cholera toxin mediated by ganglioside GM1 (**1**).

Discussion

We report the synthesis of mimics of small cell surface receptors. These synthetic receptors project DNP and NBD antibody-binding motifs from the surface of mammalian cells, capture anti-DNP IgG ligands, and trigger efficient endocytosis of these macromolecules. We demonstrate that synthetic receptors constructed from *N*-alkyl derivatives of 3β-cholesterylamine can access membrane trafficking pathways that rapidly cycle between the plasma membrane and intracellular endosomes. Access to these pathways may relate to the ability of these compounds to mimic cholesterol, a major component of both mammalian plasma membranes and endosomes[53] that plays key roles in endocytic trafficking.[54] Remarkably, the extent of receptor localization on the cell surface was found to be critically dependent on the structure of the linker between the protein-binding headgroup and the membrane anchor. Insertion of β-alanine subunits into this linker increased the population of synthetic receptors on the cell surface, resulting in much higher ligand uptake efficiency. The most effective receptor, **2**, bearing two β-alanine linker subunits, partitioned equally between the cell surface and internal endosomal membranes, as evidenced by fluorescence quenching assays with structurally similar **6**. Removal of these β-alanines resulted in substantially greater association of synthetic receptors with internal endosomal membranes. Although insertion of β-alanines may also favorably impact the affinity of the IgG ligand for the receptor on the cell surface by improving the accessibility of the protein-binding headgroup, differences in the extent of trafficking of receptors to internal membranes appear to predominately control the efficiency of these compounds as mediators of ligand uptake. The impact of changes in receptor subcellular localization was particularly evident when comparing the *N*-alkyl derivatives **4** and **8** with the analogous *N*-acyl derivatives **5** and **9**. These amide analogues (**5** and **9**) were much less effective receptors due to their unique localization at the internal membranes of the golgi apparatus and nuclear membrane of living cells. Differences in trafficking between these *N*-alkyl and *N*-acyl derivatives of 3β-cholesterylamine may relate to structural similarities of *N*-acyl derivatives to natural cholesteryl esters. These esters are known to localize at intracellular membranes of the golgi apparatus.[55]

Synthetic receptor **2**, bearing two β-alanine linker subunits between the DNP headgroup and 3β-cholesterylamine, stably associated with living Jurkat lymphocytes. After removal of the unincorporated receptor from cell culture media, this receptor was detected on the cell surface with a cellular half-life of 20 h. This stability is similar to that of natural folate receptors that are attached to the cellular plasma membrane by GPI lipids ($t_{1/2} \sim 24$ h).[45] Fluorescence quenching assays with the structurally similar fluorescent receptor **6** and endosomal efflux assays with receptor **10** revealed that these compounds undergo continuous trafficking (recycling $t_{1/2} \sim 3$ min) between the cell surface and intracellular endosomes. To maintain membrane balance, these synthetic receptors presumably make a round trip between the cell surface and endosomes in approximately 10 min. Other synthetic membrane probes, such as C_6-NBD-sphingomyelin, have been shown to similarly cycle between these membranes.[48] Certain macromolecular receptors also rapidly cycle between the cell surface and intracellular endosomes. For example, transmembrane LDL receptors complete this round trip in 10 min, cycling several hundred times in their 20 h life span.[56] IgG-bound receptors **2** and **3** also cofractionated with cholera toxin bound to the natural receptor ganglioside GM1 (**1**) in detergent-resistant lipid raft fractions of cellular plasma membranes, suggesting mechanistic similarities between these synthetic and natural systems. A simple model of synthetic receptor-mediated endocytosis summarizing these results is shown in Figure 8. The actual series of events in living cells is highly complex at the molecular level and presumably involves the interplay of multiple endosomal compartments.[4]

Synthetic mimics of mammalian cell surface receptors may have applications as cellular probes, as tools for drug delivery, and as mediators of cellular uptake of therapeutically important ligands. Because subtle molecular changes substantially affect the subcellular localization of 3β-cholesterylamine derivatives, these and related[57] compounds may provide useful probes of cellular mechanisms that regulate the segregation, localization, and sorting of membrane-associated biomolecules. Synthetic cell surface receptors may also enable novel approaches for cellular targeting and drug delivery. Cellular targeting applications may be facilitated by the structural similarity of these compounds to cholesterol, which may allow packaging of synthetic receptors

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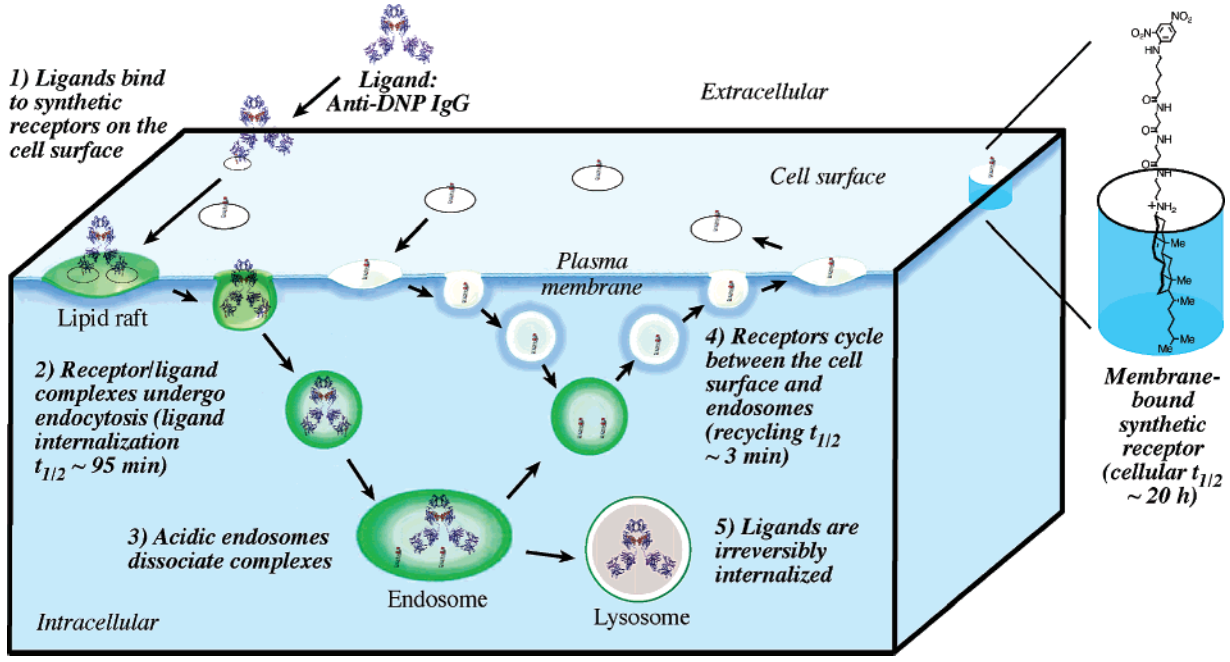

Figure 8. Simple model of synthetic receptor-mediated endocytosis. Synthetic receptors embedded in the cellular plasma membrane rapidly cycle between the cell surface and intracellular endosomes. Binding of ligand (IgG) results in association with lipid rafts and uptake of the complex by endocytosis. Dissociation in endosomes frees the receptor to return to the cell surface. The protein ligand is sorted to late endosomes and lysosomes.

in LDL particles for selective delivery to tumor cells.[58] In addition, because cells can become resistant to drugs by altering the expression of transporters or receptors at the plasma membrane, synthetic receptors that promote the internalization of drugs might provide novel drug delivery strategies. Other potential applications of synthetic receptor targeting might focus on eliminating autoreactive antibodies, tumor-promoting growth factors, or inflammatory cytokines involved in numerous diseases. Treatment with appropriately designed synthetic receptors might enable diverse cell types to remove these therapeutically important proteins from circulation. Synthetic receptors that function on cell surfaces have significant potential as biological modulators and probes.

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Supporting Information Available: Additional supporting figures (S1−S4) and Experimental Section. This material is available free of charge via the Internet at http://pubs.acs.org.

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