A Synthetic Membrane-Anchored Antigen Efficiently Promotes Uptake of Antifluorescein Antibodies and Associated Protein A by Mammalian Cells

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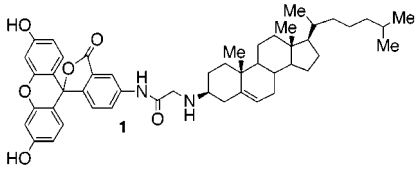
The efficient delivery of small molecules, proteins, and DNA to living cells is critical for the effectiveness of therapeutics and molecular probes. Although most small-molecule drugs enter cells though passive diffusion across low-polarity cell membranes, macromolecules are typically not cell-permeable and require specific active transport mechanisms to gain access to intracellular receptors.[1] The cellular uptake of both natural and nonnatural molecules can be enhanced by numerous methods including modification with cationic peptides,[2] proteins,[3] peptoids,[4] and lipids,[5] encapsulation with liposomes,[6] dendrimers,[7] and siderophores,[8] and complexation with cationic polymers.[9] The efficiency of these methods varies substantially, however, and improved methods that enhance the cellular uptake of macromolecules are needed in diverse areas from basic cell biology to drug delivery and genetic therapy.



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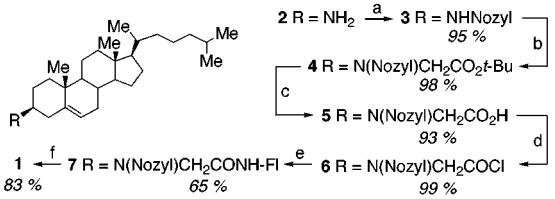
We describe here a novel synthetic ligand (**1**), termed a "memtigen" (membrane-anchored antigen), that enables dose-dependent uptake of proteins by mammalian cells as a consequence of non-covalent interactions at cellular plasma membranes. Compound **1** is a derivative of cholesterol designed to function as a fluorescent membrane anchor with high affinity for both cellular plasma membranes and commercially available antifluorescein antibodies. 3β-Cholesterylamine was employed as the membrane anchor component because cholesterol is an abundant membrane-associated steroid,[10] and protonated amines such as the headgroup of **1** favorably interact with anionic phospholipids under physiological conditions to increase affinity for plasma membranes.[11] 5-Aminofluorescein was incorporated to provide a fluorescent tag bearing high affinity for antifluorescein immunoglobulins (IgG) (e.g. $K_d = 0.7$ nM for monoclonal antifluorescein 4-4-20 binding to fluorescein-biotin). This system was chosen for investigation because molecular recognition between fluorescein and antifluorescein IgG proteins has been extensively characterized by structural,[12] computational,[13] spectroscopic,[14] kinetic,[13,14] thermodynamic,[15] and mutagenic[16] methods. Furthermore, studies of antifluorescein IgG proteins bound to fluorescein-conjugated lipids on model biomembrane monolayers, bilayers, and vesicles have been described.[17]

Scheme 1



[a] 2-Nitrobenzenesulfonyl chloride, DIEA, 0 °C, 1 h. [b] $BrCH_2CO_2t$-Bu, K_2CO_3, DMF, 85 °C, 12 h. [c] HCO_2H/Et_2O (1:1), 65 °C, 3 h. [d] $SOCl_2$, CH_2Cl_2, reflux, 2 h. [e] 5-Aminofluorescein (NH_2-Fl), THF, 25 °C, 3 h. [f] PhSH, K_2CO_3, DMF, 25 °C, 12 h.

Compound **1** was prepared as shown in Scheme 1 from 3β-cholesterylamine (**2**), which was synthesized following a previously reported method.[18] To determine whether **1** interacts with cellular plasma membranes, compound-treated Jurkat lymphocytes were examined by epifluorescence microscopy. Treatment of cells with **1** (10 μM) revealed intense green fluorescence at the periphery of 100% of living cells (Figure 1B). This staining pattern was analyzed by comparison with cells bearing red fluorescent plasma membranes from treatment with sulfosuccinimidyl biotin, which acylates amino groups on the cell surface, followed by addition of cell-impermeable texas red-conjugated streptavidin.[19] Comparison of red and green fluorescence confirmed that **1** is persistently and nearly exclusively localized at the cellular plasma membrane (compare Figure 1, parts B and D).

The avidity of antifluorescein IgG for **1** in cellular plasma membranes was assessed by the addition to cells of nonfluorescent IgG complexed with red fluorescent conjugates of commercially available Protein A (PrA) from *Staphlococcus aureus*. PrA comprises a 57 kDa protein that binds to the invariant Fc fragment of rabbit-derived IgG proteins with a dissociation constant of ca. 60 nM.[20] Pretreatment of cells with **1** followed by addition of rabbit polyclonal antifluorescein IgG and Alexa Fluor-594-conjugated PrA afforded red fluorescence at the plasma membrane of 100% of living cells within 10 min of addition of the IgG-PrA complex (Figure 1G).

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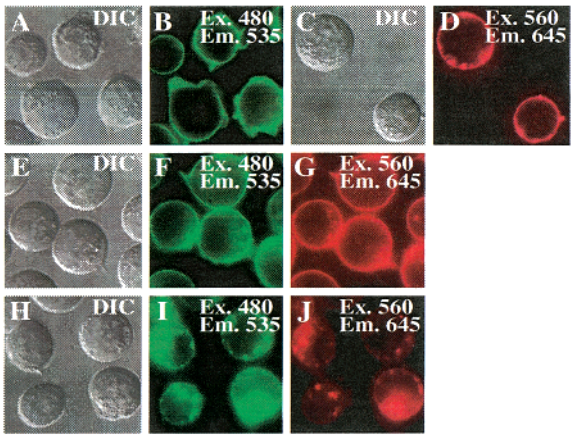

Figure 1. Epifluorescence and differential interference contrast (DIC) microscopy of compound-treated Jurkat lymphocytes. (A, B) Treatment with **1** (10 μM) for 1 h. (C, D) Control cells bearing plasma membranes labeled with biotin and Texas Red-conjugated streptavidin at 4 °C. (E−G) Cells treated with **1** (10 μM) for 1 h followed by antifluorescein IgG (0.05 mg/mL) and Alexa Fluor 594-conjugated Protein A (0.01 mg/mL) for 10 min at 37 °C. (H−J) Cells treated as shown in E−G for 4 h. Fluorescence excitation (Ex.) and emission (Em.) wavelengths (nm) are explicitly shown.

Remarkably, treatment of cells with **1** and the IgG-PrA complex for 4 h engendered both red and green fluorescence in the interior of >99% of the cells examined, indicating that the proteins and **1** were internalized (Figures 1I, 1J, and 2). No intracellular or membrane-associated fluorescence was observed when the antibody complex and **1** were premixed prior to addition to cells, possibly due to aggregation of the amphipathic complex formed.

Flow cytometry was employed to quantitate uptake of antifluorescein IgG bound to Alexa Fluor-633-conjugated PrA by Jurkat lymphocytes (Figure 2). Since this technique does not distinguish membrane-associated from intracellular fluorescence, cells were washed twice with 5-aminofluorescein (10 μM) prior to analysis to competitively displace any noninternalized protein. Flow cytometry revealed that **1** effects dose-dependent uptake of fixed concentrations of the IgG-PrA complex at ligand concentrations as low as 100 nM. This effect was blocked by competition with 5-aminofluorescein, confirming that the IgG interacts specifically with **1** (Figure 2C). Omission of **1**, the IgG, or the PrA conjugate yielded no significant cellular red fluorescence (Figure 2C).

Mammalian cells employ both receptor-mediated and nonreceptor-mediated endocytosis to internalize specific small molecules, macromolecules, and particles. In addition, viruses, toxins, and symbiotic microorganisms exploit endocytic pathways to gain entry into cells.[21] This process is dependent on time, temperature, pH, and energy.[21] We hypothesized that protein uptake mediated by **1** might involve endocytosis promoted by the high effective concentration of proteins immobilized at the plasma membrane. Hence, conditions that block endocytosis were examined. Protein uptake was suppressed by addition to the media of sodium azide,[22] an inhibitor of energy-dependent cellular processes, decreasing

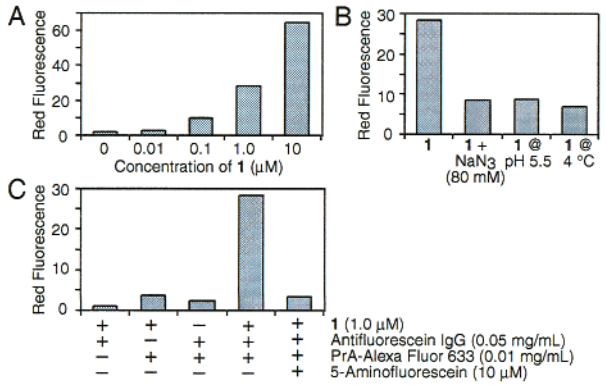

Figure 2. Analysis of protein uptake in Jurkat lymphocytes by flow cytometry. (A) Dose-response from treatment with **1** (1 h) followed by antifluorescein IgG (0.05 mg/mL) and Alexa Fluor 633-conjugated Protein A (0.01 mg/mL) for 4 h at 37 °C. (B) Inhibition of endocytosis in cells treated with proteins and **1** (1 μM). (C) Competition and omission control experiments. Each bar represents the median fluorescence of 10 000 living Jurkat lymphocytes.

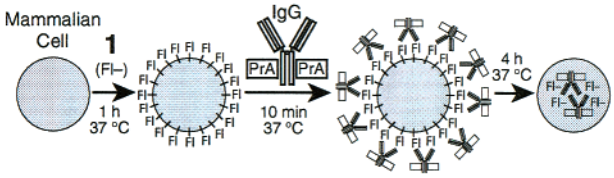

Figure 3. Schematic of compound-mediated protein uptake by mammalian cells. Compound **1** is first incorporated into the plasma membrane. Addition of antifluorescein IgG and PrA results in protein plasma membrane association and subsequent internalization.

the pH of the media to 5.5 with acetic acid, or decreasing the temperature to 4 °C (Figure 2B). Thus, the mechanism of protein internalization promoted by **1** meets the basic criteria for an endocytic process.[21]

Figure 3 illustrates this novel strategy for the delivery of IgG and PrA conjugates to >99% of viable mammalian cells, which includes adherent cell lines such as Chinese Hamster Ovary (CHO) cells. In summary, a novel plasma membrane-associated synthetic ligand promotes dose-dependent uptake of antifluorescein IgG and associated PrA in mammalian cells by an endocytic mechanism. This strategy may enable regulated intracellular delivery of numerous cell-impermeable molecules conjugated or noncovalently linked to antibodies or Protein A.

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Supporting Information Available: Experimental procedures, characterization data for new compounds, micrographs of uptake of Texas Red-conjugated antifluorescein IgG, and micrographs of protein uptake in adherent CHO cells (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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