Molecular Recognition

1D Saturation Transfer Difference NMR Experiments on Living Cells: The DC-SIGN/ Oligomannose Interaction**

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Dedicated to Professor Luis Castedo on the occasion of his 65th birthday

Molecular recognition by specific targets is at the heart of the drug discovery process. Recently, it has been demonstrated that NMR screening is ideal for finding ligands that bind to a receptor.[1] Although modern NMR experiments and technologies permits the exploration of the interaction process using nanomole quantities of the receptor, in some cases the availability of the target protein might pose a problem in the detection of the molecular recognition event, especially in the case of membrane-bound proteins. On this basis, we hypothesized that receptor-rich living cells could be used as a first step to screen the binding of different molecules to some types of receptors. The study of the role of the carbohydrate-binding C-type lectin and lectin-like receptors in the immune system is a topic of current interest. In particular, the dendritic-cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) receptor[2] mediates the binding and internalization of a large array of pathogens including HIV,[3] Ebola virus,[4] SARS-CoV,[5] *Leishmania* amastigotes,[6] *Mycobacterium tuberculosis*,[7] *Schistosoma mansoni*, *Helicobacter pylori*,[8] and *Candida*[9] in a Man- or Fuc-dependent manner.[10] Moreover, DC-SIGN plays an important role in the establishment of the initial contact between the dendritic cell and naive T lymphocytes through recognition of ICAM-3,[2] and also mediates dendritic cell trafficking through interactions with endothelial ICAM-2.[11] In this context, *Saccharomyces cerevisiae* mannan has been shown to compete with most of these DC-SIGN-dependent pathogen recognitions.[12] Herein, we demonstrate that the direct interaction between the DC-SIGN receptor and *S. cerevisiae* mannan may be easily detected by simple 1D saturation transfer difference (STD) NMR experiments[13] that employ living cells directly, without the necessity for isolating the protein receptor. This evidence may open new avenues in the drug discovery process, since NMR spectroscopy may also provide key structural information.

The cell line K562 transfected with the prototype DC-SIGN, namely K562-CD209 (DC-SIGN+),[12] and mock-transfected K562 (DC-SIGN−) were employed. These two cell types differ only in the expression of the receptor DC-SIGN on the cell surface. Thus, the untransfected cells were used as a control. The K562-CD209 DC-SIGN expression level was determined by flow cytometric analysis (see the Supporting Information). The *S. cerevisiae* mannan was obtained from Sigma and its average molecular weight was estimated to be 100 kDa by DOSY NMR experiments[14] (see the Supporting Information).

To perform the STD NMR experiments, about 5×10^6 cells were counted, washed, and dissolved in deuterated phosphate-buffered saline (PBS), and mannan (5 mg) was dissolved in the same deuterated PBS. Typical NMR tube volumes were 600 μL. Additional experiments with a commercial beta-glucan preparation, which does not bind to DC-SIGN, were also performed as blank experiments. All the experiments were repeated three times, and carried out using different batches. The estimated concentration of DC-SIGN in the NMR tube was approximately 0.1 μM.[15,16]

NMR control experiments were recorded in all cases. As illustrated in Figure 1, the STD control spectrum (Figure 1 d) of mannan confirmed that the on-resonance irradiation ($\delta =$ 6.8 ppm, aromatic region) did not affect the mannan signals.

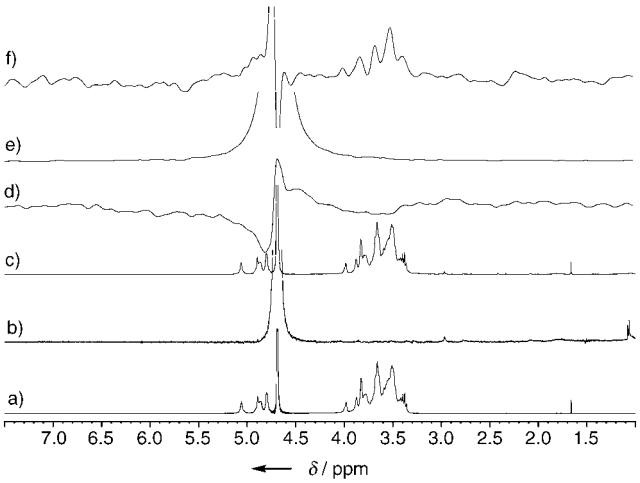

Figure 1. a) ^{1}H NMR spectrum of mannan in PBS; b) ^{1}H NMR spectrum of K562-CD209 cells in PBS, enhanced 20×; c) ^{1}H NMR spectrum of mannan with K562-CD209 cells in PBS; d) STD reference spectrum of mannan: number of scans (NS) = 256, on-resonance frequency = 6.8 ppm, off-resonance frequency = 100 ppm, total saturation time = 2 s, enhanced 100×; e) STD reference spectrum of K562-CD209 cells under the same experimental conditions as (d), enhanced 600×, signals enhanced 16×; f) STD spectrum of mannan with K562-CD209 cells: NS = 64, on-resonance frequency = 6.8 ppm, off-resonance frequency = 100 ppm, signals enhanced 800×. All the samples (cells and mannan) were dissolved in deuterated PBS at pH 7.3 containing $CaCl_2$ (1 mM). Total sample volumes were 600 μL.

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Identical results were obtained with saturation at $\delta = 1.3$ ppm (aliphatic side-chain region). Control NMR data of the living cells were acquired in an analogous manner (Figure 1 b and e). The cells used to obtain these spectra were then centrifuged and the pellet was dissolved in the mannan solution used to generate the spectra in Figure 1 a and d.[17] The resulting reference spectrum is shown in Figure 1 c, while its corresponding STD NMR spectrum is displayed in Figure 1 f. The mannan signals are observed unambiguously. Thus, irradiation at the aromatic or aliphatic regions protons of the DC-SIGN receptor protein expressed in living cells results in transfer of magnetization to the polysaccharide protons. The control spectra support the notion that the observed process is specific for the interaction of living cells of K562-CD209 (DC-SIGN+) with mannan. Indeed, when the same set of experiments was repeated with mock-transfected K562 cells (DC-SIGN−) instead of K562-CD209 (DC-SIGN+) cells, no mannan signals were detected in the STD NMR experiment (Figure 2 f). Additional reference experiments were performed using beta-glucan as the ligand. In this case, and as expected for a molecule that does not bind to DC-SIGN, no STD signals were observed with either cell line (data not shown), which supports the NMR-based detection of the specific interaction between the K562-CD209 cells and mannan.

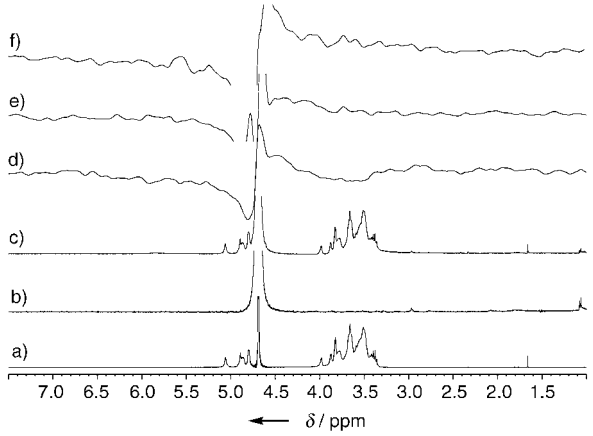

Figure 2. a) ^{1}H NMR spectrum of mannan in PBS; b) ^{1}H NMR spectrum of K562 (DC-SIGN−) cells in PBS enhanced 4×; c) ^{1}H NMR spectrum of mannan with K562 (DC-SIGN−) cells in PBS enhanced 10×; d) STD reference spectrum of mannan: NS = 256, on-resonance frequency = 6.8 ppm, off-resonance frequency = 100 ppm, total saturation time = 2 s, enhanced 600×; e) STD reference spectrum of K562 (DC-SIGN−) cells under the same experimental conditions as (d), signals enhanced 400×; f) STD spectrum of mannan with K562 (DC-SIGN−) cells: NS = 64, on-resonance frequency = 6.8 ppm, off-resonance frequency = 100 ppm, signals enhanced 800×.

Direct detection of interactions between a ligand and a membrane protein by employing living cells could open new possibilities in the screening of compound libraries in a fast and efficient way. By using our current experimental setup for this K562-CD209/mannan system robust data have been obtained with 32 scans, which correspond to a total experimental time of 3 minutes, and includes a recycling of the mannan solution from a previous experiment. The cells were checked by optical microscopy before and after the NMR experiments to determine their biological stability, and cell viability was also evaluated by the Trypan Blue exclusion method. Both assays demonstrated that cell viability was not significantly affected during the NMR experiments (see the Supporting Information).

In conclusion, receptor–ligand interactions with integral membrane proteins may be investigated by 1D STD NMR spectroscopy as the initial step in screening processes, which use living cells directly in a manner similar to that previously reported for detecting virus–ligand and platelet–ligand interactions.[16] This protocol represents a significant advance in molecular recognition studies, since it eliminates time-consuming purification processes, especially in the case of receptors that are difficult to isolate.[18]

Experimental Section

Cells: K562 cells were cultured in RPMI media supplemented with 10% fetal calf serum (FCS), 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES, 25 mM), and glutamine (2 mM; complete medium). DC-SIGN-expressing K562 transfectants (K562-CD209) have been previously described,[12] and were cultured in complete medium supplemented with G418 (300 μg mL^{-1}, Gibco).

NMR experiments: All the experiments were recorded on a Bruker 500-MHz instrument at 283 K. A basic STD sequence was used, with the on-resonance frequency variable between $\delta = 6.8$ and 1.3 ppm.[13] The off-resonance frequency was maintained at 100 ppm. A train of 40 Gaussian-shaped pulses of 50 ms each was employed, with a total saturation time of the protein envelope of 2 s. All the samples (cells and mannan) were dissolved in deuterated PBS at pH 7.3 containing $CaCl_2$ (1 mM) previously exchanged with D_2O. Total sample volumes were 600 μL.

Flow cytometry and antibodies: Cellular phenotypic analysis was carried out by indirect immunofluorescence. Monoclonal antibodies used for cell-surface staining included 9E10 (anti-c-Myc) and MR-1 (anti-DC-SIGN, CD209).[12] All incubations were performed in the presence of human immunoglobulin G (50 μg mL^{-1}) to prevent binding through the Fc portion of the antibodies. Flow cytometric analysis was performed with an EPICS-CS instrument (Coulter Científica, Madrid, Spain) using log amplifiers.

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[17] The NMR solution stays relatively clear for about 10 minutes. After this time, the cells start to sediment. However, the high sensitivity of the STD NMR experiment[1] allows one to overcome this point without major problems.
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