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Detection of Ligand Binding to Nucleotide Sugar Transporters by STD NMR Spectroscopy

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Fifty percent of all drugs on the market target integral membrane proteins,[1] however, crystallization and structure determination of these proteins for use in the drug discovery process, particularly for highly hydrophobic multiple-membrane-spanning proteins, has proven problematic. In fact, integral membrane proteins represent less than 1% of all available crystal structures.[1] The hydrophobic nature and poor solubility of multiple-membrane-spanning proteins also complicates protein–ligand interaction studies. A much-used approach involves generating recombinant soluble forms of the catalytic/receptor domain. However, this approach is only effective when the catalytic/receptor domain functions independently of the membrane. In these instances, the contribution of the membrane environment in protein function is ignored. Ligand interactions with membrane-anchored proteins, such as the interaction of binding ligands to the human sweet receptor,[2] $\alpha_{IIb}\beta_3$ integrin, which was reconstituted in proteoliposomes[3] and present on platelets[4] have been studied by saturation transfer difference (STD) NMR experiments. There have been no reports however, of STD NMR being used to assess ligand binding to nucleotide sugar transporters whose function is inextricably linked to the membrane environment.

Here we report the direct interrogation of an isolated cellular organelle (Golgi-enriched Fraction; GeF) that incorporates nucleotide sugar transporters for ligand-binding studies with STD NMR spectroscopy (Figure 1). GeFs can be easily isolated and directly used in STD NMR experiments without the need for membrane protein solubilisation, purification and/or reconstitution into artificial liposomes. Further, by using isolated cellular organelles, the binding of ligands to integral membrane proteins that are not exclusively associated with the cell surface can be studied.

STD NMR spectroscopy is an ideal tool to study the interaction of larger-sized targets with low-molecular-weight ligands, and has been previously used to investigate ligand interactions with whole virus particles,[5] platelets,[4] intact cells,[6] sepharose-immobilized recombinant protein[7] and more recently virus-like particles.[8,9] The large molecular weight of bulky particles makes them particularly attractive for STD NMR spectroscopy studies because the inherently large line width enables saturation of the particle without affecting the ligand signals. Additionally, the larger correlation time of bulky particles results in efficient spin diffusion and consequently stronger saturation transfer.

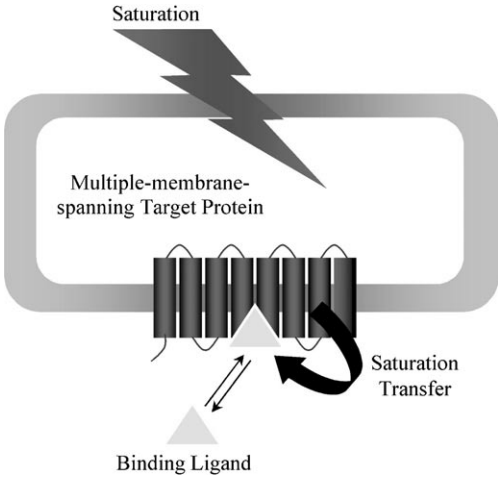

Figure 1. The interrogation of a Golgi-enriched fraction (GeF) to study ligand binding to a type III transmembrane transport protein by STD NMR spectroscopy.

The cytidine-5′-monophosphate (CMP)-sialic acid transporter (CST) was used as the model multiple-membrane-spanning protein in this study. The CST is a Golgi-resident type III transmembrane protein with 8–10 predicted membrane-spanning domains that catalyses the transport of CMP-sialic acid (commonly CMP-*N*-acetylneuraminic acid; CMP-Neu5Ac) into the Golgi apparatus of eukaryotic cells.[10–12] The CST plays a central role in the sialylation of glycoproteins and glycolipids that are destined for the surface of healthy cells,[13] however, the overexpression of sialic acid on tumour cells directly correlates with their metastatic potential.[14,15] Significantly, a reduction in cancer cell sialylation by inhibiting the CST leads to a decrease in metastasis.[16] Therefore, the CST represents an attractive target for regulating cancer cell sialic acid expression, and hence is a candidate for drug discovery. There are currently no 2D or 3D crystal structures available for the CST.

The CST was overexpressed in *Pichia pastoris* under the control of the methanol-inducible AOX1 promoter. *P. pastoris* does not possess an endogenous CST,[17] and therefore represents an ideal system for heterologous CST expression. Western blot analysis (Figure 2A) showed that recombinant CST was only present in GeFs that were isolated from methanol-induced *P. pastoris* cells (GeF-I; Figure 2A, lane 3); no CST was detectable in GeFs that were isolated from methanol-uninduced *P. pastoris* cells (GeF-UI; Figure 2A, lane 2) or in GeFs that were isolated from pPICZ-B-CST nontransformed *P. pastoris* cells (GeF-wt; Figure 2A, lane 1).

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

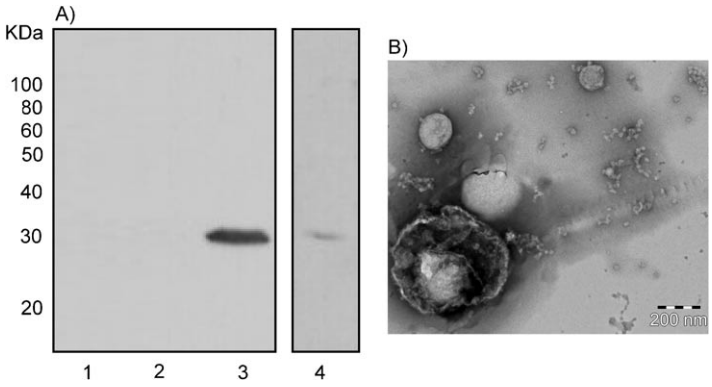

Figure 2. A) Western blot analysis of mouse anti-His_6 (primary) and HRP-conjugated anti-mouse (secondary) antibodies. Lane 1, GeF-wt (20 μg); lane 2, GeF-UI (20 μg); lane 3, GeF-I (20 μg); lane 4, GeF-UI (200 μg). B) Transmission electron micrograph of a GeF-I preparation (6 $\mu g\,mL^{-1}$) stained with 2% uranyl acetate. Golgi-enriched vesicles are intact and vary in size between 200–500 nm in diameter.

Isolated GeF-I preparations were subjected to STD NMR spectroscopy by using CMP-Neu5Ac and CMP as the binding ligand. The ^{1}H NMR spectra of CMP-Neu5Ac and CMP are shown in Figure 3A and E, respectively. The STD NMR spectra of GeF-I:CMP-Neu5Ac and GeF-I:CMP complexes (Figures 3B and F, respectively) clearly reveal that both ligands interact with the GeF-I preparation. In order to ascertain if these interactions were specific for the recombinant CST, control STD NMR spectra were acquired by using GeF-UI (Figures 3C and G) and GeF-wt (Figures 3D and H) preparations in complex with CMP-Neu5Ac and CMP. STD NMR effects for CMP-Neu5Ac when complexed with GeF-UI (Figure 3C) were evident, although with a 55% decreased intensity compared to that observed for the CMP-Neu5Ac:GeF-I complex (Figure 3B). No binding of CMP-Neu5Ac to GeF-wt preparations was observed, (Figure 3D), and trypsin treatment of GeF-I preparations prior to complexation with CMP-Neu5Ac resulted in an almost complete reduction in binding (see the Supporting Information). These observations suggest that some recombinant CST is associated with the GeF-UI preparation, probably due to low-level background expression in the absence of inducer. The AOX1 promoter offers tight regulation; however, when cultures are grown at high cell density prior to induction, the promoter can undergo de-repression resulting in "leaky" protein expression.[18] The low-level expression of recombinant CST in the absence of inducer was confirmed by analysing the equivalent quantity of GeF-UI that was used in STD NMR experiments (200 μg) by Western blot (Figure 2A, lane 4). Electron microscopy revealed that Golgi-enriched vesicles are intact and vary in size between 200–500 nm in diameter (Figure 2B).

As seen for the GeF-UI:CMP-Neu5Ac complex (Figure 3C), STD NMR effects were also observed for the GeF-UI preparation that was complexed with CMP (Figure 3G); however,

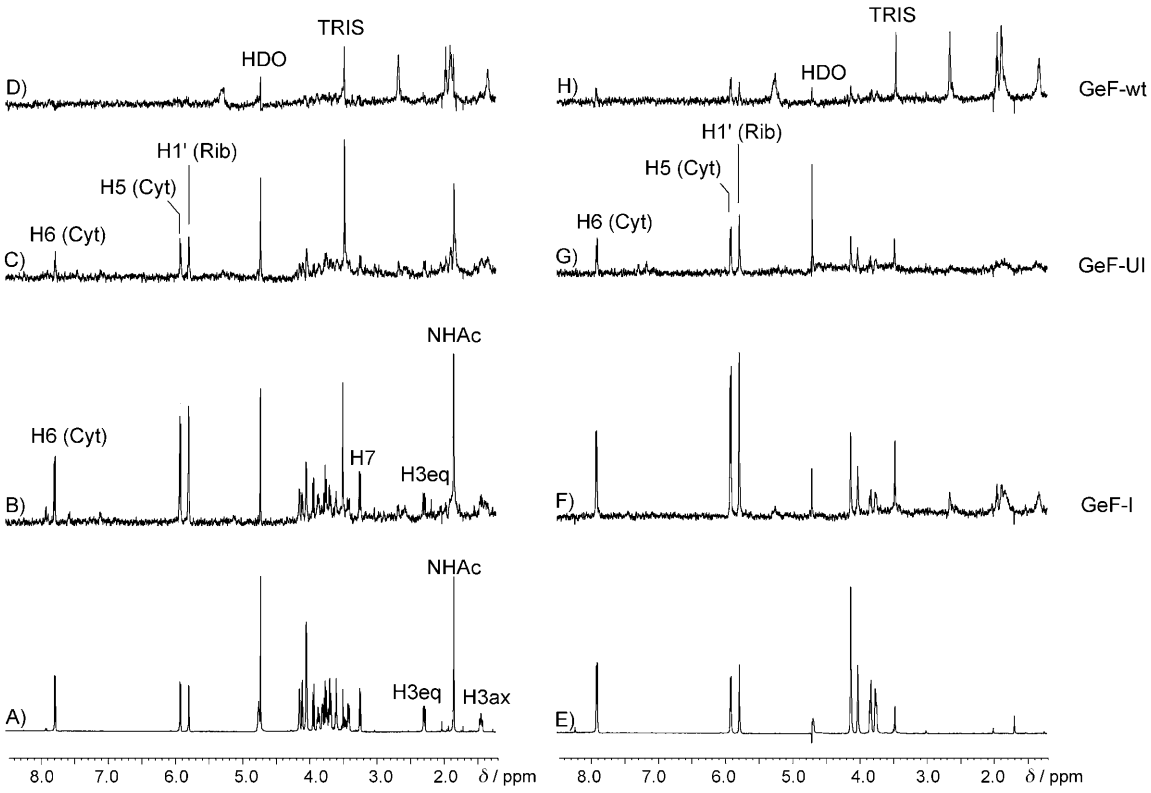

Figure 3. ^{1}H NMR spectra of CMP-Neu5Ac (A) and CMP (E). STD NMR spectra of CMP-Neu5Ac and CMP in the presence of GeF-I (B and F, respectively), GeF-UI (C and G, respectively) and GeF-wt (D and H, respectively). All spectra were recorded at 285 K, 600 MHz in deuterated Tris buffer (10 mM, pH 7.5), $MgCl_2$ (2 mM) containing GeFs equivalent to 200 μg of protein. the protein–ligand ration was set to 1:100. The on-resonance frequency was set to −1.00 ppm and the off-resonance to 300 ppm. The residual water signal was removed by applying a WATERGATE sequence. Epitope maps were constructed by calculating the relative STD NMR effects according to the formula: $A_{STD} = (I_0 \times I_{sat})/I_0 = I_{STD}/I_0$ (Supporting Information).

unlike the GeF-wt:CMP-NeuAc complex (Figure 3D), in which no STD NMR signals were detected, STD NMR effects for the GeF-wt:CMP complex (Figure 3H), which represented approximately 15% of the signal intensity seen for the GeF-I:CMP complex, were observed. This result strongly suggests that CMP is being bound by endogenous proteins within the GeF-wt preparation; this highlights the sensitivity of STD NMR for monitoring ligand interactions with proteins that are associated with isolated cellular organelles. Importantly, the subtraction of the GeF-wt:CMP from the GeF-I:CMP spectra clearly reveals significant STDD effects that are attributable to the specific binding of CMP to recombinant CST present in *P. pastoris* GeF-I preparations (Supporting Information).

P. pastoris, like other yeasts, lacks an endogenous CST,[17] but is presumed to possess other NSTs. We were therefore interested in assessing the potential of STD NMR to monitor interactions of nucleotide sugars with endogenous NSTs associated with the *P. pastoris* GeF-wt preparation. STD NMR spectra that were acquired upon complexation of UDP-Glc, UDP-Gal, UDP-GalNAc, GDP-Fuc and PAPS individually with GeF-wt showed direct binding of all nucleotide sugars to the GeF-wt preparation, probably to their corresponding NST (Supporting Information). Interestingly, addition of an equimolar amount of CMP to the GeF-wt:UDP-Gal complex reduced the STD NMR signal intensities that corresponded to UDP-Gal by 56%. This strongly suggests that CMP is displacing UDP-Gal from its binding site (possibly the UDP-Gal transporter), which is associated with the GeF-wt preparation, thus accounting for the observed binding of CMP to GeF-wt (Figure 3H). These results clearly show that not only can the specific binding of ligands to recombinant CST be assessed, but binding to endogenous NSTs, and hence ligand selectivity, can also be monitored.

Taken together, our method has the potential to provide unique binding data that is not accessible by other methods; this permits the high-throughput screening and design of novel and specific CST inhibitors that might lead to the development of novel anti-metastasis drugs. In summary, the STD NMR methodology reported herein provides an efficient technique for probing recombinant and endogenous multiple-membrane-spanning proteins with ligands. Importantly, the contribution of the membrane environment in protein function is not ignored; in fact it is a fundamental component of the analysis.

Experimental Section

Detailed experimental procedures for this article are available in the Supporting Information.

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