Origin and features of mammalian chitinases

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N-AZIDOACETYL MANNOSE AMINE
MEDIATED CHEMICAL TAGGING OF GANGLIOSIDES

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Peracetylated \(N\-\alpha\)-azidoacetylmannosamine is metabolized by cells to CMP-azidosialic acid. It has earlier been demonstrated that in this way azidosialic acid-containing glycoproteins are formed that can be labeled on the cell surface by a modified Staudinger ligation. We here firstly demonstrate that the same procedure also results in formation of azidosialic acid-containing gangliosides. Deoxymannojirimycin, an inhibitor of \(N\)-glycan processing in proteins, lowers by about 25\% the total cell surface labeling in Jurkat cells. Inhibition of ganglioside biosynthesis with \(N\)-(adamantanemethyloxy)pentyl-deoxynojirimycin reduces by about 75\% cell surface labeling. In conclusion, exposure of cells to peracetylated \(N\-\alpha\)-azidoacetylmannosamine allows in vivo chemical tagging of gangliosides.

**INTRODUCTION**

Glycoconjugate-metabolizing glycosyltransferases and glycosidases have become important drug targets in recent years. Nature provides numerous lead compounds, primarily polyhydroxylated alkaloids (denominated iminosugars), which exert important biological activities by inhibiting specific glycosidases [1, 2]. Effective therapeutic strategies based on interfering with glycoprocessing enzymes have recently been described. Two iminosugar-based drugs are now used in the clinic. Miglitol (\(N\)-hydroxyethyldeoxynojirimycin), inhibiting the intestinal glycosidases sucrase and maltase, is used for the treatment of diabetes mellitus type II [3]. Miglustat (\(N\)-butyldexyüznojirimycin), inhibiting the glycosyltransferase glucosylceramide synthase, is in use for the treatment of Gaucher disease [4-6]. Glucosylceramide synthase, the transferase responsible for the assembly of glucosylceramide from UDP-glucose and ceramide, is a key enzyme in the biosynthesis of neutral glycosphingolipids and sialic acid-containing gangliosides. More recently, the reduction of ganglioside levels has been identified as therapeutic approach for diabetes mellitus type II [7-9]. Partial inhibition of glucosylceramide synthase therefore also appears an attractive therapeutic target for prevention and treatment of diabetes mellitus type II. As part of our efforts in obtaining effective glucosylceramide synthase inhibitors, we searched after means to monitor the inhibitory effect of selected iminosugars on ganglioside biosynthesis in living cells. Bertozzi and coworkers earlier developed a strategy for in vivo labeling of cell surface glycoproteins [10-13]. Their approach is based on the finding that \(N\-\alpha\)-azidoacetylmannosamine is accepted by the CMP-sialic acid biosynthesis machinery. The resulting CMP-azidosialic acid in turn is recognized by sialic acid transferases, leading to the biosynthesis and cell surface expression of azidosialic acid containing \(N\)-linked glycoproteins. The azide can next be chemoselectively tagged by a modified Staudinger ligation. To promote formation of azidosialic acid, Bertozzi and coworkers established that...
peracetylated N-α-azidoacetylmannosamine is vividly taken up by cells and efficiently metabolized to N-α-azidoacetylmannosamine by cytosolic esterases [10]. The realization that gangliosides, like GM3, also contain a sialic acid residue at the non-reducing end, led us to explore whether the strategy developed by Bertozzi would also have merit in cell surface labeling of gangliosides. We here demonstrate the validity of this with the finding that Jurkat cells cultured in the presence of peracetylated N-α-azidoacetylmannosamine express azidosialic acid-containing glycosphingolipids at levels at least as high as azidosialic acid-containing N-linked glycoproteins. We further show that cell surface labeling of azidosialic acid-containing glycoproteins and gangliosides can be suppressed independently by the proper selection of iminosugars, respectively those that inhibit N-linked glycan processing mannosidases or those that inhibit glucosylceramide synthase.

**MATERIALS & METHODS**

**CHEMICALS**
Peracetylated N-azidoacetylmannosamine was synthesized as previously described [11]. Phosphine-biotin was synthesized as earlier described [14]. N-(adamantanemethyloxy)pentyl deoxynojirimycin was synthesized as reported earlier [15]. All other chemical solvents and reagents were of analytical grade, obtained from commercial suppliers and used without further purification unless stated otherwise.

**CELL CULTURE CONDITIONS**
Jurkat cells were grown and maintained in RPMI 1640 medium with 2 mM L-glutamine (Biowhittaker, Baltimore, U.S.A.) containing 10% FCS (Gibco, Carlsbad, U.S.A.) at 5% CO₂. Cells were seeded at approximately 1.5 x 10^5 ml in 5 ml flasks for flow cytometry or 50 ml flasks for lipid analysis. Cell viability was assessed during maintenance and before and after the various stages of labeling using trypan blue dye exclusion.

Both peracetylated N-azidoacetylmannosamine and N-(adamantanemethyloxy) pentyl deoxynojirimycin were added to cell cultures from stock solutions in DMSO. The final concentration of DMSO in the culture medium was 1% (v/v). Deoxymannojirimycin (Sigma-Aldrich, Nieuwegein, The Netherlands) was added from stock solution in ethanol. As negative controls equal volumes of the appropriate solvent were added to the cell culture.

**LABELING OF CELL SURFACE AZIDES**
After three days of incubation in the presence of 50 μM peracetylated N-azidoacetylmannosamine, cells were collected by centrifugation at 1500 rpm for 10 minutes, washed three times in cold PBS and resuspended in PBS containing
2% (v/v) foetal calf serum. The cells were distributed in a 6-well plate in 1 ml, after which an equal amount of 0.5 mM biotin-phosphine in PBS was added. Following incubation at room temperature for 3 hours under mild shaking, the cells were collected by centrifugation and washed three times in cold PBS. The cells were either labeled with streptavidin – FITC for the purpose of flow cytometry or total lipids were isolated as described below. FITC-labeling was accomplished by incubation of cells with 1 ml of 1:1000 streptavidin – FITC (Gibco, Carlsbad, U.S.A.) in PBS for one hour in the dark at 4°C, after which the cells were washed three times in cold PBS. Flow cytometry was performed using a FACSscan (Beckton Dickinson, Palo Alto, USA) with settings optimized for FITC fluorescence.

**Isolation of Gangliosides and Ganglioside Ligation Product**

Lipids were extracted with chloroform/methanol (1:1 (v/v)) and phase separation was performed according to Bligh and Dyer [16]. The aqueous phase was evaporated to dryness under N2. The samples were desalted on a SPE C18 column (Bakerbond, Deventer, The Netherlands). In short, the dried fractions were dissolved in 1 ml of water containing 0.1 M NaCl (pH 4.5). The solution was applied on the column, which had been pre-equilibrated with 2 ml of the same watery solution. Subsequently, the column was desalted with 30 ml of water, after which the lipids were eluted with 20 ml of a mixture containing equal volumes of chloroform and methanol. The eluens was evaporated to dryness under N2.

**In Vitro Staudinger Ligation of Metabolically Labeled Gangliosides**

Desalted gangliosides were dissolved in a 500 μl of a mixture of equal volumes of chloroform and methanol containing phosphine-biotin in a final concentration of 1 mM. Next, 100 μl of water was added and the reaction was allowed to proceed overnight at room temperature under mild stirring, after which the organic solvents and the water were evaporated to dryness under N2.

**Ganglioside Detection**

Gangliosides were detected by analysis of the acidic glycolipid fraction obtained after Folch extraction using chloroform/methanol/water (65:25:4) as solvent [17]. Gangliosides were quantified following release of oligosaccharides from glycosphingolipids by ceramide glycanase detection [18]. The oligosaccharides were labeled at their reducing end with the fluorescent compound anthranilic acid (2-aminobenzoic acid), prior to analysis using normal-phase high-performance liquid chromatography.

Glucosylceramide synthase activity in living cells was determined using as substrate fluorescently labeled C6-NBD-ceramide [19]. Briefly, cells were incubated with 150 mM lipid and harvested at different time points. Lipids were extracted, separated by thin layer chromatography and NBD-ceramide and NBD-
glucosylceramide were quantified [19]. Endogenous cell surface GM3 was visualized by flow cytometry using monoclonal anti-GM3 antibody (Seikagaku, Tokyo, Japan) and FITC-conjugated secondary antibodies according to the procedure described earlier [20].

RESULTS

CELL SURFACE LABELING OF AZIDOSIALOSIDES
Jurkat cells were cultured for 3 days in medium containing 50 mM peracetylated N-α-azidoacetylmannosamine (Ac₄ManNAz). Labeling of cell surface azidosialosides was performed as described in Materials and methods. Cells were harvested and washed in labeling buffer and labeled for 3 hours with phosphine-biotin. Next, cells were incubated with streptavidin-FITC, washed and resuspended for flow cytometry analysis. Intense labeling of the cell surface was obtained by this procedure. The presence of Ac₄ManNAz did not influence the rate of cell proliferation. Cell viability, as assessed by trypan blue exclusion, was not affected by the procedure. Very similar results with obtained with marine B16 melanoma cells, cultured in DMEM containing 10% FCS, 100 units/ml of penicillin and 0.1 mg/ml of streptomycin at 10% CO₂ (not shown).

DISTINCTION OF AZIDE-CONTAINING N-LINKED GLYCOPROTEINS AND GANGLIOSIDES
To distinguish between the presence of azide moieties in N-linked glycoproteins and gangliosides, cells were cultured in the presence or absence of 1 mM Deoxymanojoirimycin (DMM) and 10 mM N-( adamantanemethyloxy) pentyl deoxynojirimycin (AMP-DNM). DMM specifically inhibits processing of high mannose type N-linked glycan to sialic acid-containing complex type structures. We demonstrated earlier that 1 mM DMM prevents formation of complex type-glycan in glycoproteins in various cell types [21]. Using radioactive motioning labeling, we observed that the presence of 1 mM DMM in the culture medium of Jurkat cells also completely blocked conversion of EndoH-sensitive glycan to resistant structures in newly formed glucocerebrosidase molecules, indicating effective inhibition of glycan processing (not shown). The presence of 1 mM DMM in the culture medium did not reduce the cell surface concentration of the ganglioside GM3 as detected by flow cytometry using antibody directed to the ganglioside.

AMP-DNM specifically inhibits the first step in glycosphingolipid biosynthesis catalyzed by glucosylceramide synthase (IC50: ~150 nM). Incubation of Jurkat cells with 10 mM AMP-DNM also completely inhibits in these cells the conversion of C6-NBD-ceramide to C6-NBD-glucosylceramide and subsequent glycosphingolipids (see Figure 1). We also observed by flow cytometry analysis that after 3 days culture of Jurkat cells in the presence of 10 mM AMP-DNM, cell surface ganglioside GM3 is reduced by 80% (not shown). AMP-DNM is known not
to interfere with glycoprotein biosynthesis and processing [22].

Culturing of Jurkat cells for 3 days in the presence of 1 mM DMM and 0.05 mM Ac$_2$ManNAz resulted in a 24.0% reduction of fluorescent cell surface labeling (Figure 2). In a second independent experiment the reduction of fluorescence by DMM was 27%. The presence of 10 mM AMP-DNM led to an 86.5% reduction of fluorescence (Figure 2). In the second independent experiment the reduction of fluorescence by AMP-DNM was 68%. The combined presence of DMM and AMP-DNM led to almost complete (95%) loss of fluorescence in all experiments. Very similar observations were made with murine melanoma cells (not shown). Our findings suggest that a very large proportion of the cell surface azidosialosides in Jurkat cells are found in sialic acid containing glycosphingolipids.

Figure 1. Inhibition of glucosylceramide synthesis in Jurkat cells by AMP-DNM. Jurkat cells were incubated for 1 hr with C6-NBD-Cer and formation of C6-NBD-GlcCer and C6-NBD-SM (sphingomyelin) after 4 hrs was monitored as described in Materials and methods. Lysosomal degradation of C6-NBD-GlcCer was prevented by the presence of 1 mM conduritol β-epoxide. Cells were exposed during incubation and chase with indicated amounts of AMP-DNM. Cellular NBD-sphingolipid is put as 100%.

DEMONSTRATION OF FORMATION OF AZIDO-GM3

To further substantiate that azido-gangliosides are indeed formed in Jurkat cells exposed to Ac$_2$ManNAz, we isolated gangliosides and analyzed their oligosaccharides released by ceramide glycanase treatment and fluorescent labeling with anthranilic acid. In Jurkat cells, the gangliosides almost exclusively consist of GM3 molecules. Figure 3 shows that two trisaccharides derived from GM3 were detected. The retention time of the first peak coincides with the normal sialic acid-galactose-glucose-AA generated from GM3. Incubation with phosphine reagent did not influence its chromatographic behavior. The second peak shifted in its retention time after incubation with the phosphine reagent, indicating that it indeed contains an azide moiety.
Figure 2. Cell surface labeling of cells cultured with Ac4ManNAz in the absence or presence of DMM and AMP-DNM. Jurkat cells were labeled with Ac4ManNAz, incubated with phosphine-biotin and next streptavidin-FITC as described Materials & Methods. Cell surface labeling was determined by FACS. A. Example of the effect of AMP-DNM. Overlay histogram showing labeled cells cultured in the absence of AMP-DNM, on the right, and the downward shift in cells cultured with 10 M AMP-DNM, dotted in the middle. Cells cultured in absence of Ac4ManNAz, but exposed to the Staudinger reagent are shown on the left. Note the log- scale on the x- axis. B. Overview of impact of AMP-DNM (10 M) and DMM (1 mM) on labeling of Jurkat cells cultured in presence of Ac4ManNAz.

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<th>Fluorescence intensity (%)</th>
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<tr>
<td>No addition</td>
<td>100</td>
<td>5.1</td>
</tr>
<tr>
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Figure 3. Analysis of oligosaccharides derived from GM3. Demonstration of azidoGM3 formation in cells cultured with Ac4ManNAz. Jurkat cells were labeled with Ac4ManNAz and glycosphingolipids were isolated as described in Materials and methods. Oligosaccharides were removed from glycosphingolipids by ceramide glycanase digestion, labeled with anthranilic acid and separated by HPLC as described in Materials and methods. Oligosaccharides were reacted with phosphine-biotin or incubated identically without the agent. The dotted line, a double peak of two closely related molecular species (a1 and a2), displays the oligo-saccharides not exposed to the Staudinger reagent. The solid line represents the chromatogram of oligo-saccharides exposed to the Staudinger reagent derived, revealing a selective shift of a2 to b.
DISCUSSION

Our study reveals that exposure of Jurkat cells to Ac$_4$ManNAz results in formation of chemically tagged ganglioside, in particular of the most abundant ganglioside GM3. The proportion of azidoGM3 is about 60% of total GM3, suggesting a very efficient incorporation of azidosialic acid in gangliosides. This is not entirely surprising since different forms of sialic acid occur in nature, species that are either acylated or glycolated at the N-atom. Apparently, the azide group modification in sialic acid is equally well tolerated by the ganglioside biosynthetic machinery. It is of interest to note that the proportion of cell surface tagged glycoproteins is actually lower than that of gangliosides. Again this is not entirely surprising if one considers the estimated ratio of ganglioside to glycoprotein molecules at the cell surface. Gangliosides are largely located at the cell surface. Since nearly all ganglioside is GM3 in Jurkat cells, these cells contain per gram wet weight about 200 nanomole sialic acid associated to glycolipid. Assuming that about 1% of all cellular protein is cell membrane glycoprotein with an average mass of 50 kDa, Jurkat cells would contain about 20 nanomole glycoprotein per gram wet weight. To explain the observed ratio of FITC-labeled ganglioside to glycoprotein of 3:1, it would at least require on average 3 sialic acids per membrane glycoprotein. The pioneering work of Bertozzi has led to a convenient procedure to chemically tag sialic acid-containing glycoconjugates that can be subsequently labeled at the cell surface. Our study reveals that this also includes gangliosides besides glycoproteins. The approach may have interesting applications. First, it offers a novel tool to screen synthetic and natural compounds that interfere in ganglioside biosynthesis and/or trafficking to the cell surface. Second, given the postulated role for GM3 in modulating insulin receptor mediated signaling it may be of interest to analyze the impact of various synthetic tags on this process that is impaired in type 2 diabetes mellitus.

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