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Analysis of glycosyl phosphatidylinositol-anchored proteins by two-dimensional gel electrophoresis

The aim of this study was to characterize mammalian glycosyl phosphatidylinositol (GPI)-anchored proteins y two-dimensional gel electrophoresis using immobilized pH gradients. Analysis was performed on detergent-resistant membrane fractions of baby hamster kidney (BHK) cells, since such fractions have previously been shown to be highly enriched in GPI-anchored proteins. Although the GPI-anchored proteins were readily separated by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), these proteins were undetectable on two-dimensional (2-D) gels, even though these gels unambiguously revealed high enrichment of known hydrophobic proteins of detergent-resistant membranes such as caveolin-1 and flotillin-1 (identified by Western blotting and tandem mass spectrometry, respectively). Proper separation of GPI-anchored proteins required cleavage of the lipid tail with phosphatidylinositol-specific phospholipase C, presumably to avoid interference of the hydrophobic phospholipid moiety of GPI-anchors during isoelectric focusing. Using this strategy, BHK cells were observed to contain at least six GPI-anchored proteins. Each protein was also present as multiple isoforms with different isoelectric points and apparent molecular weights, consistent with extensive but differential *N*-glycosylation. Pretreatment with *N*-glycosidase F indeed caused the different isoforms of each protein to collapse into a single spot. In addition, quantitative removal of *N*-linked sugars greatly facilitated the detection of heavily glycosylated proteins and enabled sequencing by nanoelectrospray-tandem mass spectrometry as illustrated for the GPI-anchored protein, Thy-1.

Keywords: Raft / Two-dimensional gel / Mass spectrometry / Proteomics / Thy-1 / Glycosyl phosphatidylinositol
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1 Introduction

Communication between a mammalian cell and its environment is primarily regulated by proteins of the plasma membrane. These not only include transmembrane proteins but also proteins that are anchored to the bilayer by a glycosyl phosphatidylinositol (GPI) moiety. This class of proteins has attracted much attention lately due to their role in signal transduction [1], development [2], infection [2, 3] and disease [4]. Interestingly, GPI-anchored proteins are not distributed randomly on the plasma membrane but appear to cluster transiently in specialized microdomains of the plasma membrane, now referred to as lipid rafts [5, 6]. The ability of these proteins to interact in a dynamic fashion with lipid rafts has been proposed to be important for the regulation of their activity.

Interestingly, in an era of proteomics, no large-scale screening/identification of GPI-anchored proteins has been performed. Also, very few studies of membrane fractions by 2-D gel electrophoresis reveal the presence of mammalian GPI-anchored proteins. We were interested in mapping the endogenously expressed GPI-anchored proteins in a cell line commonly used in cell biology, the baby hamster kidney (BHK) cells. Our analysis revealed that these proteins could not be separated by 2-D gel electrophoresis using immobilized pH gradients even when analyzing a membrane fraction that is highly enriched in GPI-anchored proteins. Only after removal of the lipid moiety by phosphatidylinositol-specific phospholipase C (PI-PLC), could these proteins be properly resolved. All GPI-anchored proteins were heavily *N*-glycosylated and separated as multiple, often faint, p*I* and M_r isoforms. After removal of *N*-linked sugars, GPI-anchored proteins migrated as single spots easily detectable and amenable to sequencing by nanoelectrospray-tandem mass spectrometry.

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Abbreviations: BHK, baby hamster kidney; **DRMs**, detergent-resistant membranes; **GPI**, glycosyl phosphatidylinositol; **PI-PLC**, phosphatidylinositol phospolipase C; **TM**, total membrane

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2 Materials and methods

2.1 Cell culture and reagents

Monolayers of BHK cells were grown and maintained as described [7]. Glasgow minimal essential medium (GMEM; Sigma, St. Louis, MO, USA) was supplemented with 5% fetal calf serum (FCS) and 2 mM L-glutamine. PI-PLC was a gift from M. G. Low (Columbia University, New York, NY, USA). Triton X-100 Ultra Pure was purchased from Pierce (Rockford, IL, USA), and *N*-glycosidase F from Boehringer Mannheim (Mannheim, Germany). Mouse anti-caveolin-1 monoclonal antibodies (clone 2297) were purchased from Transduction Laboratories (Lexington, KT, USA).

2.2 Purification of detergent-resistant membranes (DRMs)

Protocols for detergent extraction and separation by flotation on sucrose gradients were adapted from previously described procedures [8, 9]. Briefly, approximately 2×10^8 cells were solubilized at 4°C for 30 min in 5 mL of lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) in the presence of Complete, a cocktail of protease inhibitors (Boehringer Mannheim). The lysate was adjusted to 41.5% sucrose, placed on the bottom of six SW40 tubes (1.75 mL per tube) from Beckman Instruments (Fullerton, CA, USA). Samples were overlaid with 8 mL of 35% sucrose and 2 mL of 15% sucrose (in 10 mM Tris-HCl, pH 7.4) and centrifuged for 18 h at 35 000 rpm (4°C). Eleven fractions of 1 mL were collected from the top. Two low density fractions, corresponding to the 15–35% sucrose interface, were pooled and concentrated down to a volume of 0.5 mL (200 μg of protein) using Amicon filters (10 kDa cut-off; Beverly MA, USA).

2.3 Preparation of a BHK total membrane (TM) fraction

A BHK post-nuclear supernatant (PNS) was prepared as follows. Cells were gently homogenized in 250 mM sucrose, 3 mM imidazole, pH 7.4, by passage through a 22G injection needle. The PNS, obtained after centrifugation (2500 rpm in 15 mL Falcon tubes) was further ultracentrifuged for 30 min (4°C) in TLS55 tubes (Beckman Instruments) at 55 000 rpm. The supernatant was discarded and the membrane pellet was resuspended in PBS.

2.4 PI-PLC and *N*-glycosidase F treatment

BHK TMs and DRMs were adjusted to 0.5% Triton X-100 and treated for 2 h at 37°C with the PI-PLC (1 U/mL). For subsequent *N*-glycosidase F treatment, the PI-PLC-treated DRMs were adjusted to 1% SDS and 1% β-mercaptoethanol, and heated for 5 min at 95°C. The denatured samples were then diluted fivefold with 40 mM phosphate buffer, pH 7, containing 1% Triton X-100, 10 mM EDTA, 1% β-mercaptoethanol, 2.5 mM PMSF and Complete. *N*-glycosidase F was then added (10 U/mL final concentration) and the incubation was allowed to proceed for 20 h at 37°C.

2.5 2-D gel electrophoresis

Prior to 2-D gel analysis all samples were precipitated with 6% thrichloroacetic acid in the presence of 375 μg of sodium deoxycholate as a carrier. 2-D gel electrophoresis was performed with nonlinear immobilized pH gradient strips, pH range 3–10 (Pharmacia, Uppsala, Sweden) as described [10]. Precipitated membrane fractions were resuspended in 350 μL lysis buffer (8 M urea, 2 M thiourea, 4% w/v CHAPS, 20 mM Tris-base, 30 mM dithioerythritol (DTE), 2% carrier ampholytes, pH 4–8) for 2 h at room temperature, under constant shaking. Samples were loaded by an in-gel reswelling procedure and strips were allowed to rehydrate for at least 8 h. Isoelectric focusing was performed at 15°C, with a total focusing time of 65 kVh. After isoelectric focusing, immobilized pH gradient (IPG) strips were equilibrated for 12 min in 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 2% SDS, and 2% DTE. This was followed by a second equilibration step of 5 min with 2.5% iodoacetamide instead of DTE. The second dimension was performed using 9–15% acrylamide gradient gels. 2-D gels were then silver-stained, as described [10]. When gels were prepared for sequencing, the glutaraldehyde fixation step was omitted from the silver staining procedure and gels were understained, so as to barely see the spots of interest. All gels were digitized using a ScanJet 6100C/T (300 dpi), and subsequently analyzed using the Melanie II 2-D gel software.

2.6 Sequencing by nanoelectrospray-tandem mass spectrometry

The protein spots were excised from silver-stained 2-D gels and digested in-gel with trypsin [11]. The extracted peptide mixture was analyzed by tandem mass spectrometric sequencing as described [12] using a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a nanoelectrospray ion source.

3 Results and discussion

GPI-anchored proteins have been described extensively as lipid raft components [5, 6]. A useful biochemical characteristic of these microdomains is that they are resistant to solubilization in Triton X-100 at 4°C and can thus be purified as low-density DRMs by flotation on a sucrose density gradient. We made use of this procedure to pre-

pare a membrane fraction that was highly enriched in GPI-anchored proteins (DRM fraction). In view of 2-D gel analysis, the final precipitated BHK DRM fraction was solubilized according to a protocol developed by Pasquali *et al.* [10] to improve the solubilization of membrane and other hydrophobic proteins. The solubilized sample was then loaded on the nonlinear IPG strip and 2-D electrophoresis was performed [13, 14]. As can be seen when comparing Figs. 1 and 2, the protein profile of a DRM fraction differed greatly from that of a total BHK membrane fraction. For example, DRMs were enriched, as expected, in caveolin-1, a marker of a specialized type of raft, *i.e.*, caveolae (for review see [15]), both by silver stain (Fig. 2A) and by Western blot analysis (Fig. 2B). Caveolin-1 is a highly hydrophobic protein and thus its presence indicates that transmembrane/hydrophobic proteins could be efficiently resolved using this procedure. This was further confirmed by the identification, using nanoelectrospray-mass spectrometry (Table 1), of another hydrophobic protein known to be present in DRMs, namely flotillin-1 [16]. Neither of these proteins could be identified in the total BHK membrane fraction, illustrating the efficiency of the DRM enrichment procedure.

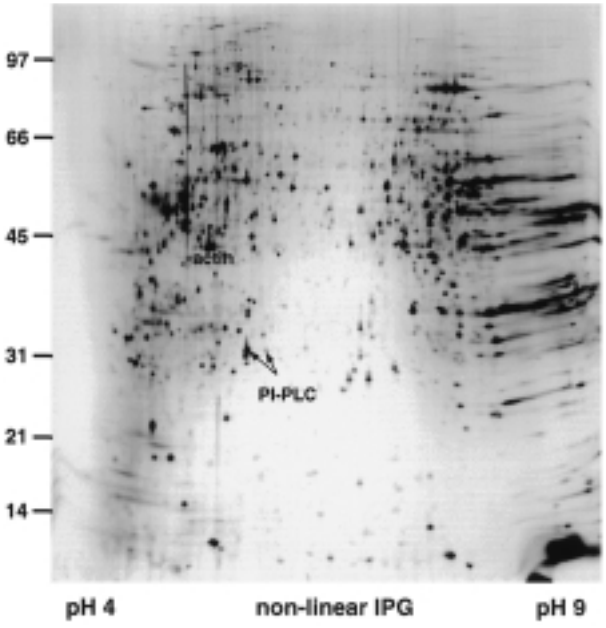

Figure 1. Silver-stained 2-D gel of a BHK total membrane fraction. 100 μg of a total BHK membrane fraction was treated with PI-PLC prior to 2-D electrophoresis.

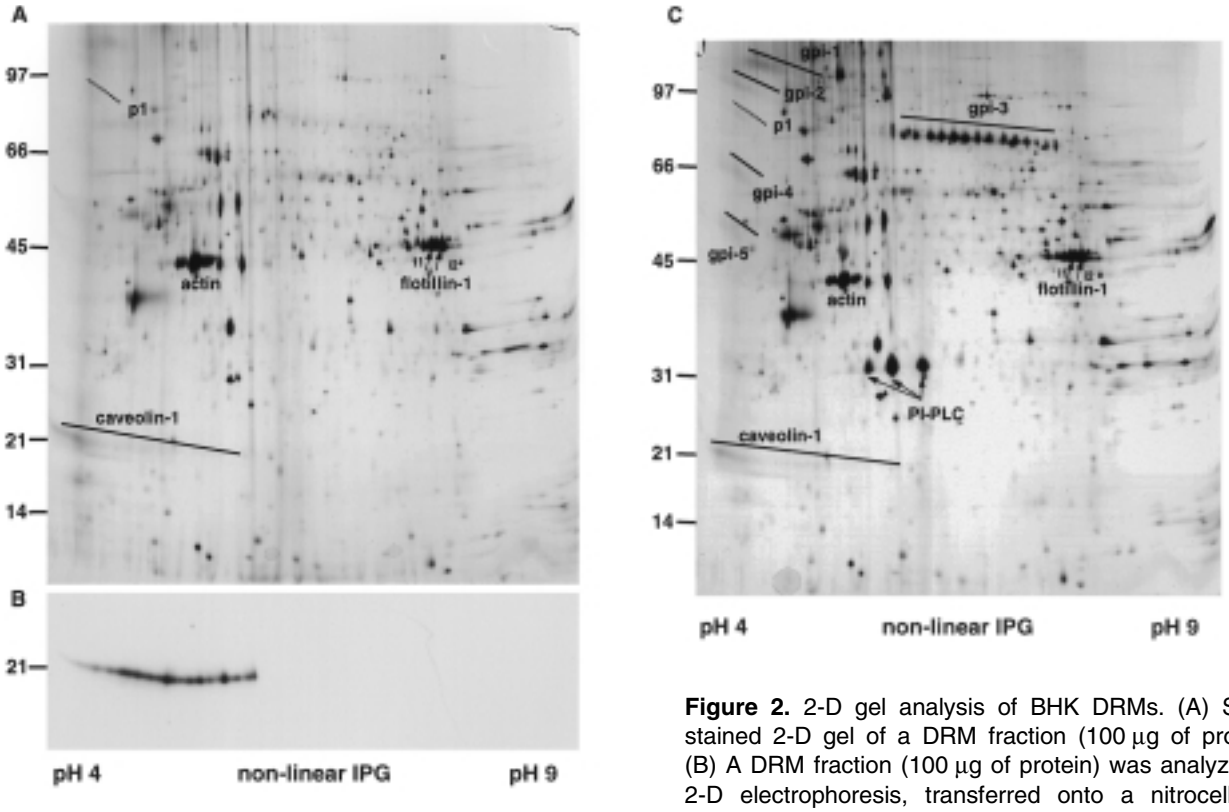

Figure 2. 2-D gel analysis of BHK DRMs. (A) Silver-stained 2-D gel of a DRM fraction (100 μg of protein). (B) A DRM fraction (100 μg of protein) was analyzed by 2-D electrophoresis, transferred onto a nitrocellulose membrane and then Western-blotted using an anti-caveolin-1 antibody. (C) Silver-stained 2-D gel of a PI-PLC-treated DRM fraction (100 μg of proteins loaded). The larger absolute amount of PI-PLC in (C), when compared to Fig. 1, reflects the fact that the TM fraction was initially more concentrated than the DRM fraction. Note that GPI-anchored proteins, labeled gpi-1 to gpi-5, are only detected in the PI-PLC-treated DRMs. p1, present in both (A) and (C) is a glycosylated protein (Fig. 3) which is not GPI-linked.

Table 1. Proteins identified by nanoelectrospray-tandem mass spectrometry

Spot No.	Protein identified	Molecular mass (kDa)[b]	Predicted p*I*	Peptide ion (monoisotopic mass)	Sequences determined (BHK)	Residues (human homolog)
i1	Flotillin-1 O75955[a]	47.3	7.08	1499.6	VFVLPCIQQIQR	29– 40
				1752.8	LPQVAEEISGPLTSANK	361–377
				1303.6	VTGEVLDIL**S**[c]R	393–403
i2	Flotillin-1 O75955	47.3	7.08	1499.6	VFVLPCIQQIQR	29– 40
				1214.6	ISLNTLTLNVK	41– 51
				1752.8	LPQVAEEISGPLTSANK	361–377
				1644.8	AQLIMQAEAEAESVR	303–317
gpi-6	Thy-1 P04216	17.9	8.97	1588.4	VTSLTACLVDQ**N**[c]LR	22– 35

a) SWISS-PROT accession number
b) Derived from cDNA
c) All BHK sequences determined were identical to their human homologs, except for a single aa in flotillin-1, and in Thy-1, indicated in bold.
d) Cys is CAM Cys

In order to map the spots on the gel shown in Fig. 2C that correspond to GPI-anchored proteins, we further purified BHK GPI-anchored proteins from DRMs as previously described [9]. Briefly, DRMs were first subjected to Triton X-114 phase partitioning [17]. The detergent phase, enriched in membrane components, was then treated with PI-PLC to release the protein from the lipid moiety of the GPI anchor. Upon a second round of Triton X-114 phase partitioning, the GPI-anchored proteins (now without the lipid) were specifically redistributed to the soluble phase. 2-D gel analysis of the PI-PLC-released fraction led to the identification of multiple GPI-anchored proteins (gel not shown, but proteins labeled in Fig. 2C). It became apparent, however, that none of these GPI-anchored proteins could be matched to existent protein spots on the starting DRM fraction (Fig. 2A). This unexpected observation raised the possibility that GPI-anchored proteins might not be resolved on 2-D gels unless the hydrophobic glycerophospholipid moiety was removed. In order to test this hypothesis, a total DRM fraction was treated with PI-PLC prior to precipitation, and then analyzed by 2-D electrophoresis. As can be seen in Fig. 2c, PI-PLC treatment did not affect the overall protein pattern, when compared to Fig. 2A, but led to the specific appearance of a number of spots, corresponding to GPI-anchored proteins and to PI-PLC itself. Our observation suggests that care should be taken when analyzing available 2-D maps of DRMs, since GPI-anchored proteins are likely to be absent from these maps, even though these proteins are now known to be important components of lipid rafts. Note that the spots corresponding to GPI-anchored proteins were absent from the gel shown in Fig. 1 despite the fact that the total BHK membrane fraction had been treated with PI-PLC, indicating that GPI-anchored proteins, in this particular cell type, are of relatively low abundance.

A striking feature of the identified GPI-anchored proteins is that they all migrated either as multiple highly resolved spots (Fig. 2C; gpi-3) or as faint smears in the acidic

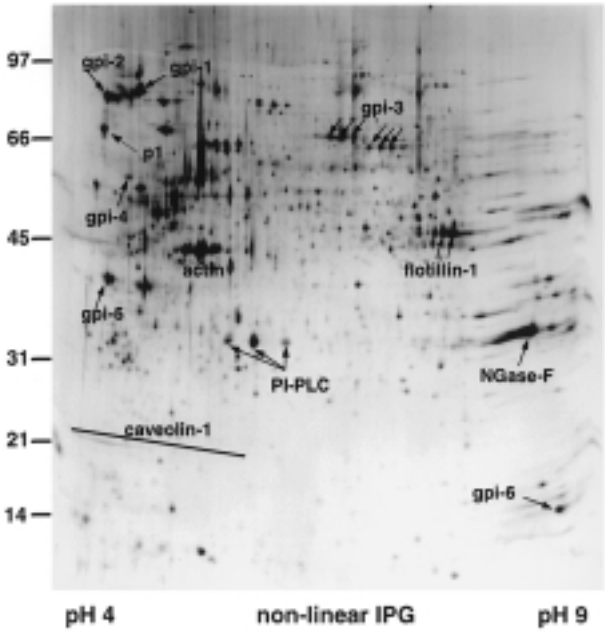

Figure 3. Silver-stained 2-D gel of a deglycosylated DRM fraction. The sample (100 μg) was treated with PI-PLC (1 U/mL) and then *N*-deglycosylated, prior to gel electrophoresis. Deglycosylated products were identified as protein spots specifically appearing on this gel after *N*-glycosidase F treatment (compare to Fig. 2C) which could be reproduced in three independent experiments. Polypeptides were assigned according to their expected shift in p*I* and M_r upon deglycosylation. Confirmation of these assignments will require sequencing by mass spectrometry.

region of the gel. These p*I* isoforms most likely correspond to glycosylation isoforms since GPI-anchored proteins are known to be *N*-glycosylated [18], a post-translational modification that was shown to be important for their proper targeting [19]. The presence of these various glycosylation isoforms and the fact that these proteins are generally of low abundance impairs their identification by in-gel tryptic digest/tandem mass spectrometry, due to a high acrylamide-to-protein ratio, shielding of proteolytic cleavage sites, and to the presence of numerous differentially modified peptides [20]. These various factors result in poor peptide map yields. In an attempt to circumvent these problems, we have submitted a PI-PLC-treated DRM fraction to *N*-glycosidase F treatment. As can be seen in Fig. 3, gpi-1, gpi-2, gpi-4, and gpi-5 were each redistributed to single, highly resolved spot and shifted to a lower M_r, and a more basic p*I*, confirming that the various p*I* isoforms observed in Fig. 3 were due to *N*-linked sugar modifications. The assignment of these four proteins after deglycosylation will, however, require confirmation by sequencing (see legend, Fig. 3). Gpi-3 in contrast to the other gpi-proteins, migrated after *N*-glycosidase F treatment as two separate rows of multiple p*I* isoforms, each of significantly lower M_r than the gpi-3 isoforms in Fig. 2C. Whether this is due to incomplete deglycosylation or additional post-translational modifications remains to be determined. Interestingly, *N*-glycosidase treatment led to the appearance of a new abundant spot in the highly basic, low molecular weight range of the gel (Fig. 3). Tandem mass spectrometry analysis of this spot led to the determination of a 14-amino acid sequence (Fig. 4) which unambiguously identified this protein as the hamster homolog of the GPI-anchored protein Thy-1 (Table 1). Since Thy-1 could not be detected on the gel in Fig. 2C, this observation clearly illustrates that removal of *N*-linked sugars strongly increases the detection threshold of GPI-anchored proteins on silver-stained gels, thereby facilitating mass spectrometry sequencing of these proteins and, most likely, of *N*-glycosylated proteins in general.

4 Concluding remarks

Low recovery of hydrophobic proteins on 2-D gels is a major limitation in proteome research. Although recent progress has been made in the analysis of integral membrane proteins by 2-D gel electrophoresis [10, 21], highly hydrophobic proteins such as those bearing multiple transmembrane segments, remain intrinsically difficult to separate by isoelectric focusing [22]. The present work shows that GPI-anchored proteins constitute an additional class of membrane attached proteins, which are poorly resolved by 2-D gel electrophoresis due to the presence of the hydrophobic glycerophospholipid. This

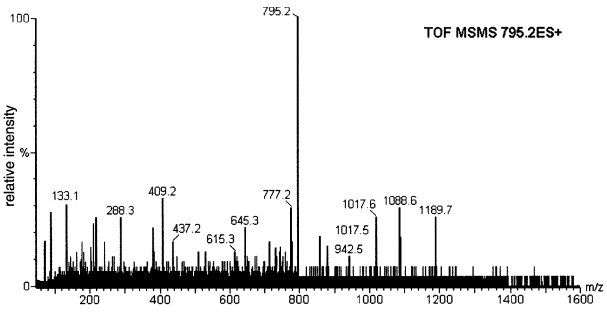

Figure 4. Nanoelectrospray-tandem mass spectrometry identification of deglycosylated Thy-1. The sequence of the peptide was identified using the program PeptideSearch. Search parameters were: sequence tag (645.3)V(I/L)CAT(1189.7); peptide mass, 1588.4 Da; cleavage agent, trypsin; cysteine is carbamidomethyl-cys; mass accuracy, 2 Da, search by "*y*"-type ions. The peptide sequence VTSLTACLVNQNLR from mouse Thy-1 matched the found peptide. A mass higher by 1 Da was measured for the molecular ion and some of the fragment ions, as compared to the theoretical list of fragment ions from the mouse peptide: the sequence for the hamster Thy-1 peptide is VTSLTACLVDQNLR.

lipid moiety presumably leads to aggregation of the protein during isoelectric focusing and/or prevents extraction from the IPG strip after isoelectric focusing. Migration in the second dimension is not incriminated since GPI-anchored proteins can readily be analyzed by 1-D SDS-PAGE [23]. To allow proper analysis on 2-D gels, the lipid moiety must first be removed from the protein by PI-PLC treatment. Since GPI-anchored proteins are often highly *N*-glycosylated, identification is greatly improved by the additional removal of *N*-linked sugars. The proteins then generally migrate as single easily identifiable spots that can be subjected to sequencing by tandem mass spectrometry. Our work, in addition, suggests that 2-D electrophoresis analysis of deglycosylated samples may greatly facilitate the identification of heavily glycosylated cell surface markers by mass spectrometry, a major challenge in proteomics today.

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