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Combined biochemical and cytological analysis of membrane trafficking using lectins

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ABSTRACT

We have tested the application of high-mannose-binding lectins as analytical reagents to identify *N*-glycans in the early secretory pathway of HeLa cells during subcellular fractionation and cytochemistry. Post-endoplasmic reticulum (ER) pre-Golgi intermediates were separated from the ER on Nycodenz–sucrose gradients, and the glycan composition of each gradient fraction was profiled using lectin blotting. The fractions containing the post-ER pre-Golgi intermediates are found to contain a subset of N-linked α-mannose glycans that bind the lectins *Galanthus nivalis* agglutinin (GNA), *Pisum sativum* agglutinin (PSA), and *Lens culinaris* agglutinin (LCA) but not lectins binding Golgi-modified glycans. Cytochemical analysis demonstrates that high-mannose-containing glycoproteins are predominantly localized to the ER and the early secretory pathway. Indirect immunofluorescence microscopy revealed that GNA colocalizes with the ER marker protein disulfide isomerase (PDI) and the COPI coat protein β-COP. In situ competition with concanavalin A (ConA), another high-mannose specific lectin, and subsequent GNA lectin histochemistry refined the localization of *N*-glyans containing nonreducing mannosyl groups, accentuating the GNA vesicular staining. Using GNA and treatments that perturb ER–Golgi transport, we demonstrate that lectins can be used to detect changes in membrane trafficking pathways histochemically. Overall, we find that conjugated plant lectins are effective tools for combinatory biochemical and cytological analysis of membrane trafficking of glycoproteins.

Glycosylation is an important posttranslational modification affecting protein function in health and disease. The substantial majority of eukaryotic proteins and many lipids are glycosylated, and these oligosaccharides contain glucose (Glc),[1] mannose (Man), and *N*-acetylglucosamine (GlcNAc). The three main carbohydrate modifications are the addition of N- or O-linked oligosaccharides and glycosylphosphatidylinositol protein anchoring [1]. N-Linked glycosylation is initiated as the polypeptide is translocated into the lumen of the rough endoplasmic reticulum, with the transfer of a precursor $Glc_3Man_9GlcNAc_2$ moiety from a dolichol lipid donor to the asparagine residue in the peptide. Further processing occurs in the Golgi and *trans*-Golgi network, generating three main classes of N-linked glycans: oligomannose and complex- and hybrid-type sugars [1,2]. O-Linked glycosylation is believed to be initiated in the Golgi complex by the addition of *N*-acetylgalactosamine (GalNAc) to serine or threonine residues [1]. Subsequently, a stepwise enzymatic elongation yields several core structures that are further modified by sialylation, sulfation, acetylation, fucosylation, and polygalactosamine extension [3].

The recognition of N-linked glycans by intracellular lectins (nonenzymatic carbohydrate-binding proteins) plays an important role in protein folding and sorting in the biosynthetic pathway (reviewed in Refs. [2,4–6]). Within the confines of the endoplasmic reticulum (ER), glucosidase I removes the terminal glucose moiety, permitting glucosidase II to successively trim the remaining two glucose residues. The UDP-glucose:glycoprotein glucosyltransferase distinguishes folded and incompletely folded glycoproteins, reglycosylating only the latter [7]. Monoglucosylated glycoproteins are retained in the ER through interaction with the lectins calnexin

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[1] *Abbreviations used:* Glc, glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; ER, endoplasmic reticulum; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; CI–MPR, cation-independent mannose 6-phosphate receptor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RT, room temperature; PBS, phosphate-buffered saline; BCIP/NBT, 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium; PFA, paraformaldehyde; FITC, fluorescein isothiocyanate; DAPI, 4′,6′-diamidino-2-phenylindole; GNA, *Galanthus nivalis* agglutinin; BFA, brefeldin A; PDI, protein disulfide isomerase; DBA, *Dolichos biflorus* agglutinin; HPA, *Helix pomatia* agglutinin; ConA, concanavalin A; LCA, *Lens culinaris* agglutinin; PSA, *Pisum sativum* agglutinin; DSA, *Datura stramonium* agglutinin; LEL, *Lycopersicon esculentum* lectin; PNA, peanut agglutinin; RCA, *Ricinus communis* agglutinin I/II; BSLI/II, *Bandeira simplicafolia* lectin I/II; UEA-I, *Ulex europaeus* agglutinin I; GM130, Golgi matrix protein 130; EEA1, early endosome antigen 1; LAMP-1, lysosome-associated membrane protein 1; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate.

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and calreticulin. If the bound glycoprotein is incompletely folded, it will be a substrate for the UDP-glucose:glycoprotein glucosyltransferase; if correctly folded, it is deglucosylated [2,4]. Mannosidase I then removes a single mannose residue to form the "high-mannose" structure $Man_8GlcNAc_2$ that is recognized by another calcium-dependent lectin, ER–Golgi intermediate compartment (ERGIC)-53/58. ERGIC-53/58, a homologue of leguminous lectins with an affinity for mannose, then facilitates recruitment of the newly synthesized glycoproteins into COPII vesicles for exit from the ER [2]. Another type of ER resident lectin Htm1p/EDEM, which recognizes the Man_8 and an unknown number of glucose residues to target malfolded glycoproteins for ER-associated degradation, has been described previously [4]. Lectins such as the cation-independent mannose 6-phosphate receptor (CI–MPR) and VIP36 (which also exhibits homology to legume lectins) function later in the secretory pathway [2,4].

Conjugated plant lectins offer the potential to combine biochemical fractionation and histological techniques for the analysis of membrane trafficking. Glycosylation state (e.g., endoglycosidase sensitivity) has long been used as a biochemical marker for protein transport through the secretory pathway [8,9]. We present a variety of techniques that depend on lectin–carbohydrate binding to illustrate the utility of several carbohydrate-binding lectins to examine membrane trafficking events involving glycosylation. Based on the homology of ERGIC-53 to high-mannose specific plant lectins, we have tested the application of high-mannose-binding lectins as analytical reagents to identify *N*-glycans during transport through the ER and Golgi network of organelles using biochemical methods in conjunction with cytochemistry.

Materials and methods

Tissue culture

HeLa cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco).

Nycodenz–sucrose centrifugation

HeLa cells were grown to 70% to 80% confluency, harvested by scraping in ice-cold handling buffer (250 mM sucrose, 3 mM imidazole [pH 7.4], and 0.5 mM ethylenediaminetetraacetic acid [EDTA] containing protease inhibitors), and resuspended at 1×10^8 cells/ml prior to lysis using a ball-bearing homogenizer [10] with a clearance of 6 μm with the minimum number of strokes (usually five or six) required to achieve 70% lysis, as indicated by trypan blue exclusion. The homogenate was centrifuged at 4000*g* for 10 min at 4 °C to pellet nuclei and mitochondria. Postnuclear supernatant (1 ml) was loaded on top of a continuous 5% to 15% (w/v) Nycodenz (Axis–Shield) gradient at 4 °C in 10 mM Tris–Cl (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 75 mM sucrose, as described previously [11]. The gradient was spun to equilibrium at 90,000*g* for 20 h at 4 °C in a Beckman SW28 rotor. Fractions (1.5 ml) were collected from the top of the gradient and snap-frozen in liquid nitrogen until use. The protein concentration of 10 μl of each fraction was determined spectrophotometrically using a Bio-Rad Protein Assay Kit I.

SDS–PAGE and silver staining

Fractions (10 μl) were dissolved in reducing sample buffer and separated in 1.5-mm 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) mini-gels with a 4% stacking gel according to Laemmli [12]. Electrophoresis was performed in a Bio-Rad Mini-Protean system at 25 mA constant current using Bio-Rad SDS–PAGE molecular weight standards (high and low range). For visualization of protein bands, gels were silver-stained [13] as follows using ultra-pure 18-MOhm (Ω) water throughout. Gels were fixed in 40% methanol and 10% glacial acetic acid for 1 h at room temperature (RT). Fixed gels were washed 3×10 min in 40% methanol, sensitized for 1 min in 0.02% sodium thiosulfate, and washed 3×15 min in water. Gels were incubated for 20 min in 0.1% silver nitrate and 0.02% formalin and were washed twice for 20 s in water. Gels were developed in 3% sodium carbonate, 0.02% formalin, and 0.0002% sodium thiosulfate. Developed gels were rinsed for 20 s in water, and color development was stopped by immersion in 1% glacial acetic acid.

Immunoblotting and lectin blotting

Following electrophoresis, gels were blotted onto 0.45-μm-pore nitrocellulose membranes (BA 85, Schleicher & Schuell) by wet transfer. After transfer, the membranes were rinsed once in phosphate-buffered saline (PBS). For immunoblotting, the membrane was incubated with blocking buffer (PBS containing 5% Marvel milk powder and 1% Tween 20) for 1 h at RT. The primary antibody was added in blocking buffer and incubated for 1 h on a rocking platform. The membranes were washed five times for 5 min in PBS with 1% Tween 20 before horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma) was added in blocking buffer for 45 min. The membranes were washed as above and developed with BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium) substrate for the detection of alkaline phosphatase [14] or chemiluminescence reagent (ECL, Amersham) for signal detection.

To detect glycosylated proteins, the blots were first equilibrated for 1 h with 0.4% Teleostean gelatin (Sigma–Aldrich), 50 mM Tris–Cl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 (Sigma–Aldrich) prior to the addition of 2.5 μg/ml biotinylated lectins (Vector Laboratories) in the same buffer for 1 h at RT. The blots were washed five times for 5 min in PBS with 1% Tween 20 and incubated for 1 h with streptavidin–alkaline phosphatase conjugate (Vector Laboratories) diluted to 1:2500 in PBS. After repeat washing, the blots were developed with BCIP/NBT as described above. Blots were scanned and quantified by densitometry analysis with Image J software (National Institutes of Health). The total lectin signal for each gradient fraction was determined by densitometry and subjected to background intensity correction normalized to the same area. The percentage value for each gradient fraction was plotted so that it sums to 100 across the graph.

β-Hexosaminidase assay

Immediately after collection of fractions, 10 μl of sample was incubated with 50 μl of 0.6 mM 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide in 40 mM citric acid, 70 mM disodium hydrogen phosphate, and 0.1% Triton X-100 (pH 4.5). The sample was incubated at 37 °C for 30 min in the dark. The reaction was stopped by the addition of 200 mM sodium carbonate and 110 mM glycine (pH 10.0), and the extent of the reaction was determined by fluorescence spectroscopy at an excitation wavelength of 365 nm and reading the emission at 450 nm.

Triton X-114 extraction

Cells were partitioned using Triton X-114 as described previously [15].