Nuclear Pore Complex Glycoproteins Contain Cytoplasmically Disposed O-Linked *N*-Acetylglucosamine

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Abstract. A novel form of protein–saccharide linkage consisting of single *N*-acetylglucosamine (GlcNAc) residues attached in O-linkages directly to the polypeptide backbone has been described (Holt, G. D., and G. W. Hart, 1986, *J. Biol. Chem.*, 261:8049–8057). This modification was found on proteins distributed throughout the cell, although proteins bearing O-linked GlcNAc moieties were particularly abundant in the cytosolic and nuclear envelope fractions of rat liver. In the accompanying article (Snow, C. M., A. Senior, and L. Gerace, 1987, *J. Cell. Biol.*, 104: 1143–1156), the authors describe monoclonal antibodies directed against eight proteins localized to the nuclear pore complex. These proteins occur on the cytoplasmic and nucleoplasmic (but not lumenal) sides of nuclear membranes. In this report, we demonstrate that all members of this group of pore complex proteins bear multiple O-linked GlcNAc residues. Further, we show that the O-linked GlcNAc moieties are linked via serine (and possibly threonine) side chains to these proteins. Perturbing the O-linked GlcNAc residues either by covalently attaching galactose to them or by releasing them with β-*N*-acetylglucosaminidase strongly diminishes the immunoreactivity of the proteins with all of the monoclonal antibodies. However, the O-linked GlcNAc moieties are only part of the epitopes recognized, since O-GlcNAc–containing limit pronase fragments of nuclear pore complex proteins cannot be immunoprecipitated by these antibodies. These findings, taken together with those in the accompanying article, are a direct demonstration that proteins of the cytoplasm and nucleoplasm bear O-linked GlcNAc residues.

PREVAILING evidence suggests that the bulk of the well-studied types of carbohydrate moieties on glycoconjugates are either localized to the cell surface or within lumenal compartments of intracellular organelles (e.g., lysosomes, Golgi, and endoplasmic reticulum). In contrast, relatively little evidence has been obtained suggesting the existence of glycoproteins in the cytoplasmic and nucleoplasmic compartments of cells. Early studies involving carbohydrate analyses of nuclei and nuclear matrix fractions indicated the presence of glycoconjugates in these fractions (reviewed in Furukawa and Terayama, 1979; Stein et al., 1981; Stoddart, 1979), and the presence of cytoplasmic glycoproteins has also been suggested (Meyer and Burger, 1976). These data have received support in several more recent investigations (e.g., Kan and Pinto da Silva, 1986; Fedarko and Conrad, 1986; Nagakura et al., 1986). However, attempts to identify and characterize nucleoplasmic and cytoplasmic glycoproteins have been severely hampered by cross-contamination with other cellular compartments, and detailed biochemical analyses of most putative cytoplasmic and nucleoplasmic glycoproteins is lacking.

Recently the subcellular distribution of glycoproteins bearing terminal *N*-acetylglucosamine (GlcNAc)[1] residues was surveyed (Holt and Hart, 1986) by employing galactosyltransferase, an enzyme that transfers galactose residues from a high energy sugar donor to virtually any terminal GlcNAc moiety (Brew et al., 1968), to specifically label glycoproteins present in subcellular fractions of rat liver. It was demonstrated that while most subcellular fractions contain distinctive groups of proteins bearing O-linked GlcNAc, the bulk of the proteins bearing this linkage are in the cytosol and nuclear envelopes. A 62-kD protein of the nuclear pore complex that binds wheat germ agglutinin was recently described (Davis and Blobel, 1986), and was suggested to be an O-linked GlcNAc-bearing protein.

In the accompanying paper, Snow et al. (1987) describe a set of monoclonal antibodies that bind to different members of a group of eight structurally distinct proteins of the nuclear pore complex that share common epitopes. By immunoelectron microscopy performed on isolated nuclear envelopes, it was found that the proteins recognized by the monoclonal antibodies occur on the cytoplasmic and nucleoplasmic (but not

1. *Abbreviations used in this paper*: GalNAc, β-*N*-acetylgalactosamine; GlcNAc, β-*N*-acetylglucosamine.

lumenal) surfaces of nuclear membranes. In this report, the proteins and the epitopes recognized by these monoclonal antibodies are characterized further. We demonstrate that the proteins have multiple sites of O-linked GlcNAc addition, and that these residues are attached to protein via the hydroxyl side chains of serine (and possibly threonine). In addition, our data strongly suggest that O-linked GlcNAc moieties are part of the epitopes recognized by all of the monoclonal antibodies described. These data demonstrate the existence of a specific form of glycosylation, O-linked GlcNAc, which occurs on proteins in the cytoplasmic and nucleoplasmic compartments of the cell.

Materials and Methods

Nuclear Envelope Preparation and Labeling

Nuclear envelopes were prepared as described (Dwyer and Blobel, 1976; Gerace et al., 1982). The fraction of nuclear envelopes used in the experiments presented here corresponded to the D_2P fraction as defined by these authors. The following manipulations were performed either with ice-cold reagents or at 4°C unless noted. In some experiments, nuclear envelopes were prewashed with 0.5 M NaCl (as described in the accompanying paper, Snow et al., 1987) before labeling when it was desirable to remove contaminating chromatin. Labeling of the nuclear envelopes with galactosyltransferase was performed by a modification of a previously described technique (Holt and Hart, 1986). Nuclear envelopes were solubilized at a ratio of 100 U (1 U of nuclear envelopes is the amount derived from 1 A_{260} U of isolated rat liver nuclei containing 3×10^6 nuclei) per 100 μl of 2% Triton X-100, 0.5 M NaCl, and 50 mM Hepes, pH 6.8 (buffer H). The solubilized nuclear envelopes were stored on ice for 60 min and were subjected to occasional bursts of sonication to fully disrupt the sample. Insoluble material was removed by subjecting the samples to microcentrifugation for 4 min at 4°C. The supernatant was transferred to a second tube containing 6 μl of 100 mM Hepes, pH 7.3, 100 mM galactose, 50 mM $MnCl_2$, 2.5% Nonidet P-40, and 10% Aprotinin (Sigma Chemical Co., St. Louis, MO) per 100 U. Bovine colostrum galactosyltransferase (Sigma Chemical Co.), which had been previously auto-galactosylated as described (Holt and Hart, 1986) was added to the nuclear envelopes at a ratio of 175 mU per 100 U except where noted. Labeling was initiated with the addition of 5 μl of 1 mCi/ml of uridine 5′-diphospho-[^{3}H]galactose (Amersham Corp., Arlington Heights, IL) in 25 mM 5′-AMP. The specific activity of the radiolabeled sugar donor was 17.3 Ci/mmol or, if limit labeling of the sites of galactosylation was desired, the specific activity was adjusted to 1.73 Ci/mmol by adding the appropriate amount of cold uridine 5′-diphosphogalactose (Sigma Chemical Co.). Labeling proceeded for 30 min at 37°C unless noted, and then the reaction was terminated by bringing the solution to 50 mM EDTA. Using labeling conditions in which no cold uridine 5′-diphosphogalactose is added to the reaction mixture, we found that only a small percentage (<7–10%, see Results for details) of the possible sites of glycosylation by galactosyltransferase became occupied by [^{3}H]galactose. Protein-incorporated label was separated from unincorporated uridine 5′-diphospho-[^{3}H]galactose by fractionation according to size on columns of Sephadex G-50 (1 × 30 cm) run in 50 mM ammonium formate, 0.1% SDS, unless otherwise noted. Protein labeled in this manner was concentrated by precipitation in 8 vol of acetone at −20°C for 18 h.

Monoclonal Antibodies

Monoclonal antibodies were prepared as described in the accompanying paper (Snow et al., 1987). Antibodies were coupled to cyanogen bromide preactivated Sepharose 4B (Sigma Chemical Co.) at 5 mg antibody per ml packed beads. Monoclonal antibody–Sepharose conjugates were stored in PBS with 0.02% azide and then washed with buffer H immediately before use.

Immunoadsorptions

The following manipulations were performed either with ice-cold reagents or at 4°C. Nuclear envelopes were solubilized and cleared of insoluble material in buffer H as described above for galactosyltransferase labeling. The solubilized nuclear envelopes were then combined with an amount of immobilized antibody determined to be in excess of the amount necessary to fully clear the solution of its corresponding antigen(s), usually ~100 μl of packed antibody–Sepharose beads per 3×10^8 nuclear equivalents. The nuclear envelope/antibody–Sepharose mixtures were shaken for 18 h, and the supernatants were removed after sedimentation of the beads in a microcentrifuge for 7 s. The beads were washed four times with 10 vol of buffer H, and then once with 10 vol of 10 mM Tris-HCl, pH 7.4. The immunoadsorbed material was eluted two times by boiling the washed beads in 1.5 vol of 0.5% SDS, 50 mM Tris-HCl, pH 7.4. The eluates were then pooled and precipitated in 8 vol of acetone at −20°C for further manipulation or for direct quantitation.

Competition Experiments

Nuclear envelopes were labeled as described above such that only a small percentage of the possible sites of glycosylation by galactosyltransferase were occupied by [^{3}H]galactose. Labeled proteins were then immunoadsorbed directly from this solution (i.e., with unincorporated label still present) using RL2-Sepharose in the presence of various monosaccharides and glycoproteins. Immunoadsorbed materials were washed and eluted as described above, and the quantity of labeled protein bound to the monoclonal antibody was determined. For inhibitors of immunoadsorption, 1-M solutions of either GlcNAc or *N*-acetylgalactosamine (GalNAc) were used, or 5 mg/ml solutions of either GlcNAc or GalNAc coupled synthetically to BSA through a *p*-aminophenyl linkage (16 and 21 mol carbohydrate per mol BSA, respectively; Sigma Chemical Co.). For the latter class of inhibitors of immunoadsorption, fatty acid- and globulin-free BSA was used as a control inhibitor.

In a second set of competition experiments, nuclear envelopes were labeled as described above with cold uridine 5′-diphosphogalactose using one-tenth the amount of galactosyltransferase used above. It was necessary to reduce the amount of transferase so that the kinetics of galactosylation were slow enough to be analyzed. The nuclear envelopes with intermediate levels of galactosylation were combined with antibody–Sepharose for immunoadsorption, as described above.

Hexosaminidase Digestions

Nuclear envelopes were washed in 10 mM sodium cacodylate, pH 5.0, and then suspended at 100 U per 100 μl of the same buffer. The samples were then sonicated briefly. Protease cocktails Pic 1 and Pic 2 (Holt et al., 1985) were added at 1 μl/100 μl sample. Jack Bean β-*N*-acetylglucosaminidase (Sigma Chemical Co.) was added at 0.71 U/100 μl sample. Deglycosylation was initiated by incubating the samples at 37°C. For time-course experiments, the reaction was stopped by boiling aliquots for 5 min. For limit deglycosylation experiments, the reaction was continued for 22 h and then the reaction was stopped by boiling in gel sample buffer (see below). The extent of deglycosylation was monitored by radiolabeling the hexosaminidase-treated nuclear envelopes with galactosyltransferase, as described above. The transfer of [^{3}H]galactose to free GlcNAc released by the hexosaminidase was determined by paper chromatography according to the method of Porzig (1978).

Pronase Digestion of Nuclear Envelopes

Nuclear envelopes (3×10^8 nuclear equivalents) were washed in 0.2 M Tris-HCl, pH 7.8, 1.5 mM $CaCl_2$ and then suspended in 200 μl of this buffer. The solution was brought to 2% final concentration of Triton X-100 and stirred at room temperature for 1 h. To this was added 25 μl of 10 mg/ml Pronase-CB (75,500 proteolytic units/g; Calbiochem-Behring Corp., La Jolla, CA) that had been preincubated for 40 min at 50°C in the Tris/Ca buffer. The solution was stirred at room temperature for 15 min and then incubated without stirring for 20 h at 37°C. Additional Pronase-CB (25 μl) was added and the reaction was continued at 37°C for 20 h. The reaction was terminated by boiling the solution for 20 min. Insoluble material was removed by pelleting in a microcentrifuge. Portions of the supernatant were immunoadsorbed with RL2-Sepharose, as described above. Eluates from this immunoadsorption, as well as the RL2-Sepharose supernatant and nonimmunoadsorbed digests, were labeled with galactosyltransferase, also as described above. The products of the labeling were fractionated on Bio-Gel P-4 columns (−400 mesh, 1 × 175 cm) at 12 ml/h in 0.2 M ammonium acetate at 50°C. The columns were standardized with GlcNAc polymer standards, assayed by the method of Park and Johnson (1949), and their void and included volumes were established by the elution of dextran and galactose, respectively.

SDS PAGE and immunoblot analyses of nuclear envelopes are described in the accompanying paper (Snow et al., 1987).

Determination of the Number of Sites of Glycosylation per Protein

RL2-immunoadsorbed material from nuclear envelopes was subjected to labeling conditions for maximal galactose incorporation as described above. The labeled proteins were then separated by SDS PAGE. The resulting gels were then fixed and stained with Coomassie Blue according to the method of Fenner et al. (1975) for the quantification of stained protein. Stained bands were cut out from the gel background and the Coomassie Blue bound to the proteins was eluted in 25% aqueous pyridine. Dye elution was spectroscopically determined at 605 nm and was compared with BSA standards that were run and stained on the same gel. Thus, the moles of protein in a given band on the gel from nuclear envelope immunoadsorptions could be calculated. Next the gel slices from the immunoadsorptions were subjected to β-elimination in a small volume of 0.1 M NaOH for 24 h at 37°C. The samples were neutralized with dilute HCl and counted to determine the release of [^{3}H]galactose. Moles of galactose released was calculated from the specific activity of the uridine 5′-diphospho-[^{3}H]galactose. Simple division of moles galactose released by moles protein present in a gel slice of the immunoadsorptions gave the ratio of sites of glycosylation per protein molecule.

Determination of the Site of Glycosylation

RL2-immunoadsorbed material from nuclear envelopes was lightly galactosylated, as described above, and then separated by SDS PAGE. The protein(s) migrating with an apparent molecular mass of 63 kD were excised and eluted from gel slices (monitored by radioactivity), and precipitated by adding 8 vol of acetone at −20°C. The protein was subjected to β-elimination in 0.1 M sodium hydroxide, and 1 mM NaB^3H_4 (20 mCi, 17.9 Ci/mmol; Amersham Corp.) for 22 h at 37°C. Radiolabeled protein was separated from unincorporated label by fractionation on columns of Sephadex G-50 (1 × 30 cm) and precipitated in acetone, as described above. Exchangeable hydrogens were cleared from the labeled protein by resuspending the precipitated protein in water, incubating the solution for 1 h at 37°C, and then removing the water by lyophilization. This water extraction was repeated five times. The labeled protein was subjected to compositional analysis according to the Water's Picotag method (Johns Hopkins Protein Sequencing Facility). The radioactivity of the derivatized amino acids was also monitored on fractions of the derivatized samples as they eluted from the compositional analysis column. During the β-elimination, serine and threonine residues bearing O-linked glycosides are converted into alanine and α-aminobutyric acid, respectively (Tanaka and Pigman, 1965).

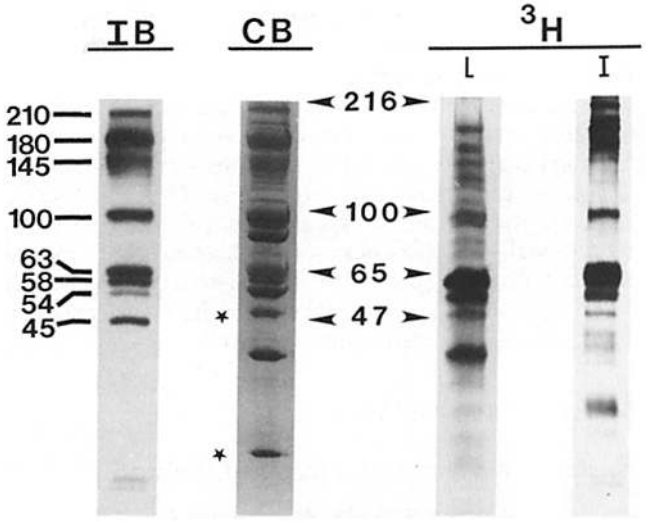

Figure 1. Correspondence between nuclear pore proteins immunoreactive with the monoclonal antibody, RL2, and the proteins which serve as substrates for galactosyltransferase. Solubilized nuclear envelopes were immunoadsorbed with RL2-Sepharose. Portions were subjected to differing extents of galactosylation with bovine colostrum galactosyltransferase and uridine diphospho-[^{3}H]galactose. These pretreated and untreated portions were separated by SDS PAGE. One lane of the untreated nuclear envelopes was transferred to nitrocellulose and subjected to blotting with soluble RL2 antibody (immunoblotting). (Lane *IB*) Nuclear envelopes immunoblotted with soluble RL2 antibody; (lane *CB*) immunoadsorbed sample stained with Coomassie Blue; (lane *[^{3}H] L*) immunoadsorbed sample galactosylated only lightly (40 pmol [^{3}H]galactose incorporated per 1.5 × 10^8 nuclear equivalents); (lane *[^{3}H] I*) immunoadsorbed sample galactosylated to an intermediate extent (105 pmol [^{3}H]galactose incorporated per 1.5 × 10^8 nuclear equivalents). The migration positions of the major glycoproteins are shown (×10^{-3}) at the left. In addition, the migration positions of molecular mass standards (center, ×10^{-3}) are indicated. The stars indicate the migration positions of heavy and light chains in the Coomassie Blue–stained lane.

Results

Monoclonal Antibodies Against Nuclear Pore Complex Proteins Recognize a Group of Polypeptides Which Are Glycosylated

The monoclonal antibodies described in the accompanying paper (Snow et al., 1987) react with different subsets of a group of eight nuclear pore complex proteins, ranging from all eight proteins (RL2) to a single polypeptide (RL11). We observed that the set of proteins recognized by RL2 on immunoblots of nuclear envelopes looked strikingly similar to the set of nuclear envelope glycoproteins labeled by galactosyltransferase described before (Holt and Hart, 1986). To determine whether the two groups coincide, solubilized nuclear envelopes were combined with RL2-Sepharose and the material that was immunoabsorbed was subsequently labeled by galactosyltransferase and uridine diphospho-[^{3}H]galactose. As shown in Fig. 1, the material immunoabsorbed by RL2 serves as an excellent substrate for galactosylation. Furthermore, RL2 immunoabsorbs the entire population of nuclear envelope proteins bearing GlcNAc residues; no detectable galactosylatable material remains in the supernatant of a nuclear envelope preparation after it has been cleared by excess RL2-Sepharose (data not shown). The major galactosylation products of the immunoabsorbed samples are very similar in migration to polypeptides detected by RL2 immunoblots (cf. lanes *IB* and *[^{3}H] I*, Fig. 1). However, the incorporation of increasing amounts of [^{3}H]galactose on the proteins decreases their mobility during SDS PAGE. For example, the protein(s) normally migrating with an apparent mass of 63 kD when lightly labeled are shifted to an apparent mass of ~67 kD when heavily labeled. Since >90% of the GlcNAc moieties galactosylated on nuclear envelope proteins consist of single GlcNAc residues attached directly to the polypeptide backbone in O-linkages (Holt and Hart, 1986), these data strongly suggest that the group of nuclear pore complex proteins recognized by the monoclonal antibody RL2 all have O-linked GlcNAc residues on them.

O-Linked GlcNAc Structures Are Attached via Serine Side Chains to Nuclear Pore Complex Proteins

The chemistry of O-glycosidic linkages was exploited in order to determine the amino acid to which the GlcNAc moieties on these nuclear pore complex glycoproteins are at-

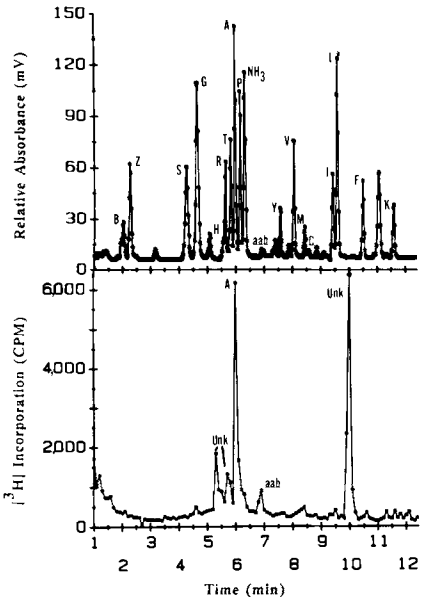

Figure 2. O-linked GlcNAc moieties are linked to nuclear pore proteins via serine side chains. RL2-immunoadsorbed nuclear envelopes were separated by SDS PAGE. The protein(s) migrating with an apparent molecular mass of ~63 kD on the gel was excised and eluted. The protein was then treated in a mild alkali solution with tritiated sodium borohydride. The resulting sample was subjected to amino acid compositional analysis using the Water's Picotag system. (*Top*) Amino acid compositional data for the 63-kD protein; (*bottom*) radioactivity elution profile for the same composition experiment as the top panel. The single letter abbreviations of amino acid elution peaks are indicated in both panels. aab, the elution position of α-aminobutyrate (the β-elimination product of threonine from which an O-linked carbohydrate was released); Unk, denotes peaks eluting with no known amino acid derivative, and probably representing radioactive contaminants in the NaB^3H_4, since these peaks are not labeled in other experiments. We have observed such anomalous peaks in all commercially available preparations of this reagent.

tached. Base-induced cleavage of O-linked glycosides (termed β-elimination) in the presence of sodium borohydride results in the reduction of the use of the amino acid as the site of attachment of the carbohydrate (Tanaka and Pigman, 1965). This leads to the production of alanine and α-aminobutyrate from serine and threonine, respectively, if these amino acids serve as sites of O-linked carbohydrate addition. To determine the β-elimination products of the RL2 antigens, immunoabsorbed proteins were first lightly labeled with galactosyltransferase and the products were separated by SDS PAGE. The protein migrating at ~63 kD after light galactosylation (see Fig. 1) was localized by the presence of [^{3}H]galactose and then excised and eluted from the gel. The protein was then subjected to β-elimination in a mild alkali solution (pH 13) containing NaB^3H_4, and the resulting ^{3}H-protein was subjected to amino acid compositional analysis (Fig. 2). As can be seen in this figure, the tritiated reduction product of a β-elimination experiment is largely [^{3}H]alanine. This indicates that serine serves as the major site of O-linked GlcNAc addition in this specific nuclear pore complex protein. Since there is a small amount of α-aminobutyrate in the NaB^3H_4 elimination experiments, the presence of some threonine linkages may also be supported by these data.

Table I. Determination of the Number of Sites of O-Linked GlcNAc Addition on Proteins Immunoadsorbed by RL2

Molecular mass of protein	Galactose/ protein	No. of determinations
kD	*mol*	
180	9.8 ± 0.6	2
100	2.0 ± 1.2	3
63	10.0 ± 1.8	3

RL2-immunoadsorbed samples from nuclear envelopes were subjected to exhaustive galactosylation with galactosyltransferase and uridine diphospho-[^{3}H]galactose. The products were separated by SDS PAGE and the resulting gel was stained with Coomassie Blue. The Coomassie Blue dye eluted from gel slices of the immunoadsorbed protein bands was determined and compared with a standard curve of dye elution from known concentrations of BSA which were also run on the same gels. Thus the pmol protein in a given band of immunoadsorbed sample were measured. The gel slices were then subjected to β-elimination in a mild base solution and the amount of [^{3}H]galactose was determined. From the amount of [^{3}H]galactose released, the pmol galactose present was determined according to the specific activity of the uridine diphospho-[^{3}H]galactose used in the labeling. The pmol galactose released were divided by the pmol protein present in the gel slices, giving the number of sites of glycosylation per protein.

Pore Complex Proteins Contain Multiple Sites of Glycosylation

To measure the amount of O-linked GlcNAc on the nuclear pore complex glycoproteins, RL2-immunoabsorbed material from nuclear envelopes was subjected to exhaustive galactosylation with galactosyltransferase and uridine diphospho-[^{3}H]galactose. The products were separated by SDS PAGE and the resulting gel was stained with Coomassie Blue to determine the moles of protein present in individual protein bands (see Materials and Methods and Table I). The gel slices containing protein bands were then subjected to β-elimination and the moles of [^{3}H]galactose released was determined, permitting an estimation of the number of sites of glycosylation per protein. As can be seen in Table I, major proteins immunoadsorbed by RL2 appear to have multiple sites of GlcNAc addition. The two major proteins migrating in the 58–63-kD range of the gel (which are increased in apparent molecular mass by 2–6 kD when they are extensively galactosylated and can no longer be separately resolved; see Fig. 1 for example) together have an average of 10 sites of O-linked GlcNAc addition per peptide. The 180-kD protein also appears to have ~10 sites of glycosylation, while the 100-kD band has ~2 sites.

Galactosylation Inhibits the Immunoadsorption of Nuclear Pore Complex Proteins

Since the monoclonal antibody RL2 immunoadsorbs a group of proteins that all appear to serve as substrates for galactosylation by galactosyltransferase (see Fig. 1), we examined whether the epitope recognized by RL2 contains O-linked GlcNAc moieties. First, we attempted to block immunoadsorptions with competing monosaccharides. It was found that, in comparison with immunoadsorption in the absence of inhibitors, RL2 immunoadsorbs 92 and 55% of the nuclear pore proteins in the presence of 1-M solutions of GalNAc and GlcNAc, respectively (data not shown). Thus the antibody apparently is not directed against the GlcNAc moieties on the pore complex proteins in a lectin-like fashion, since very high concentrations of monosaccharide poorly inhibit precipitation. This is substantiated by the failure of neo-

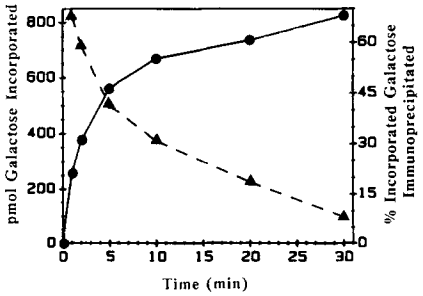

Figure 3. Galactosylation of nuclear envelopes blocks the immunoadsorption of nuclear pore proteins by RL2. Nuclear envelopes were subjected to increasing extents of galactosylation as described in Fig. 1, and then combined with RL2-Sepharose for immunoadsorption. The antibody supernatants and pelleted material were separated from unincorporated label by fractionation on columns of Sephadex G-50, and the total [^{3}H]galactose on proteins eluting in the columns' void volumes was determined. The percent incorporated galactose immunoadsorbed (- - ▲ - -) is expressed as the amount of [^{3}H]galactose-labeled protein immunoadsorbed by RL2-Sepharose divided by the total macromolecular label incorporated in protein at the given time point. The incorporation curve (— ● —) is expressed as the pmol [^{3}H]galactose incorporated per 1.5 × 10^8 nuclear equivalents.

glycoproteins (synthetically derived glycoproteins in which monosaccharides are chemically coupled to protein) such as GlcNAc-BSA and GalNAc-BSA, to block immunoadsorption. Greater than 90% of control precipitation by RL2 occurred in the presence of 5 mg/ml-solutions of either of these neoglycoproteins (data not shown; GlcNAc-BSA and GalNAc-BSA have monosaccharide/protein coupling ratios of 16 and 21, respectively). However, the fact that GlcNAc partially inhibits the precipitation while GalNAc does not suggests that RL2's binding to nuclear pore complex proteins may include GlcNAc as part of its epitope.

To further test this possibility, we attempted to block the antibody binding by modifying the O-linked GlcNAc moieties on the proteins with the attachment of galactose. Nuclear envelopes were subjected to increasing levels of galactosylation by galactosyltransferase and these preparations were then immunoadsorbed with RL2-Sepharose. As is shown in Fig. 3, there is an inverse relationship between the level of galactosylation of nuclear envelope proteins and the relative amount of labeled antigen immunoadsorbed by RL2. Interestingly, as the level of galactosylation of nuclear envelopes approaches saturation, the ability of RL2 to immunoadsorb its antigens is almost totally blocked (see below).

The different monoclonal antibodies described in the accompanying paper (Snow et al., 1987) which bind to various subsets of the eight glycoproteins apparently recognize spacially separate (and therefore distinct) epitopes on one of the proteins. Nevertheless, with all of the antibodies tested (RL1, RL2, RL3, RL4, and RL11), galactosylation of nuclear envelopes significantly inhibits the immunoadsorption of these nuclear pore complex glycoproteins (RL1, RL2, and RL3 shown in Fig. 4, others not shown). At comparatively low levels of galactosylation (Fig. 4, left, lanes *L*), each antibody binds to a set of [^{3}H]galactose-labeled antigens that is very similar to the proteins labeled by the antibody on immunoblots (see the accompanying paper). Furthermore, most or all of the mass of all relevant labeled antigen is bound to the antibodies with this light labeling condition (Fig. 4, left, cf. lanes *s* and *p*). As the level of [^{3}H]galactose incorporation is increased, the antibodies show markedly decreased efficiency for immunoabsorbing all of their antigens (Fig. 4, left, lanes *I*).

The immunoadsorption of total [^{3}H]galactose-labeled proteins was quantitatively measured for samples subjected to light, intermediate, and limit levels of galactosylation (Fig. 4, right). Clearly, increasing galactosylation of nuclear envelopes inhibits immunoadsorption to differing extents for individual antibodies. RL2 was the only antibody of those tested whose antigen binding was totally abolished at limit galactosylation, although all of the antibodies demonstrated significantly decreased abilities to immunoadsorb their respective subset of glycoproteins. These data taken together suggest that O-linked GlcNAc may serve as a portion of the epitopes recognized by all of the anti-nuclear pore complex antibodies described. However, the possibility that galactosylating this carbohydrate introduces allosteric effects

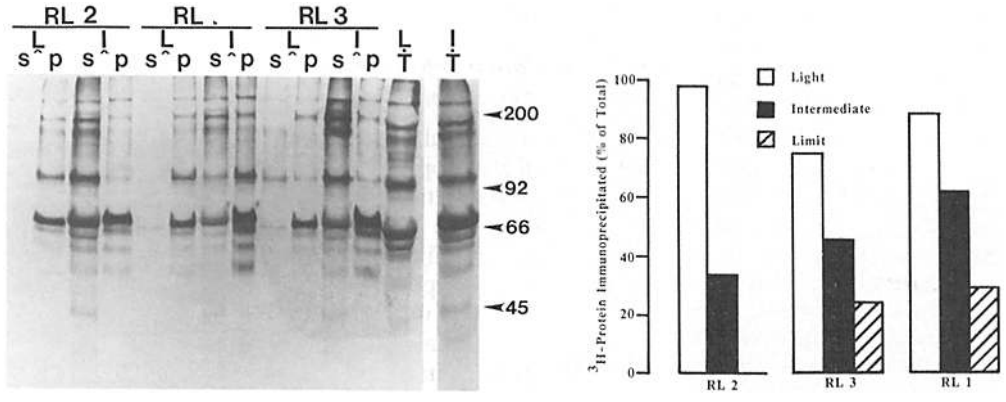

Figure 4. Galactosylation of nuclear envelopes blocks the immunoadsorption of nuclear pore proteins by many unique monoclonal antibodies. Nuclear envelopes were galactosylated and combined with Sepharose-immobilized monoclonal antibodies as described in Fig. 3. (*Left*) The antibody supernatants (*s*) and pelleted material (*p*) were then separated by SDS PAGE. The light (*L*), and intermediate (*I*) levels of galactosylation refer to incorporation ratios of ~68 and 236 pmol [^{3}H]galactose incorporated per 1.5 × 10^8 nuclear equivalents, respectively. The migration positions of molecular mass standards (×10^{-3}) are indicated. (*Right*) The light, intermediate, and limit extents of galactosylation refer to incorporation ratios of ~68, 236, and >700 pmol [^{3}H]galactose incorporated per 1.5 × 10^8 nuclear equivalents, respectively. The percent of the total labeled protein in each fraction was determined for the various antibodies as described in Fig. 3.

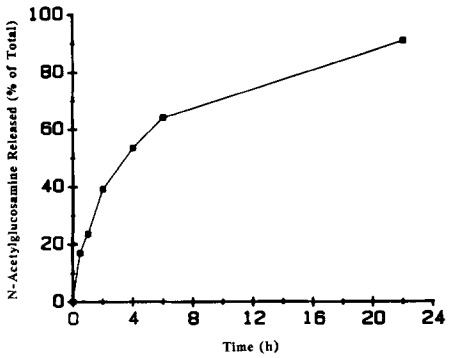

Figure 5. Hexosaminidase releases O-linked GlcNAc residues from nuclear envelopes. Nuclear envelopes were treated with β-*N*-acetylglycosaminidase for increasing lengths of time. The release of free GlcNAc moieties was monitored by galactosylating aliquots of the digests, and then determining the amount of [^{3}H]-*N*-acetyllactosamine generated by paper chromatography. In this figure, 100% of the total GlcNAc is the amount of GlcNAc present both still bound to protein and released as free GlcNAc in the 22-h timepoint aliquot.

which block immunoadsorption cannot be excluded by these data alone.

Hexosaminidase Digestion Prevents Binding of Anti-Nuclear Pore Complex Antibodies to Antigen

To further investigate the involvement of the O-linked GlcNAc moieties in the binding of the various anti-nuclear

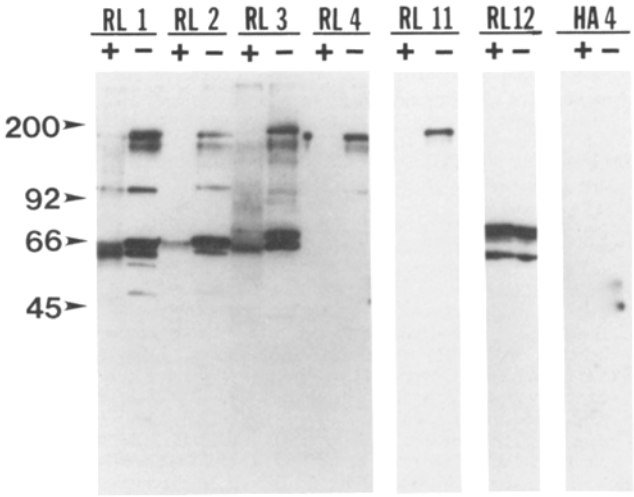

Figure 6. Hexosaminidase digestion inhibits binding of anti-nuclear pore antibodies to antigens. Hexosaminidase-treated (+) or mock treated (−) nuclear envelopes were subjected to immunoblotting, as described in Materials and Methods and Fig. 1, with various anti-nuclear pore antibodies. Assays of the digested nuclear envelopes used in this particular experiment indicate that ~92% of the total O-linked GlcNAc residues are removed from the proteins in this fraction. The digested nuclear envelopes were also blotted with RL12 (an anti-nuclear lamin A and C antibody), to show that there is no apparent proteolysis in the treated fractions, and with HA4 (an anti-plasma membrane marker), to show that there is no significant contamination of this membrane in the nuclear envelope preparation. The migration positions of molecular mass standards ($\times 10^{-3}$) are indicated.

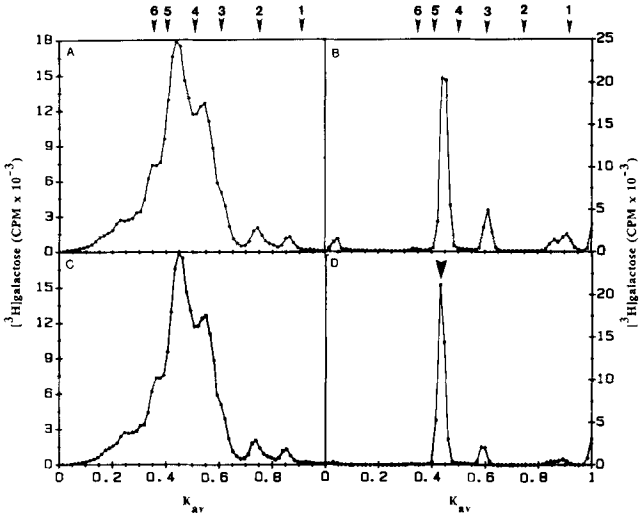

Figure 7. Anti-nuclear pore antibodies require extensive primary structure for immunoadsorption. Nuclear envelopes were treated exhaustively with pronase. Portions of the digest were subjected to immunoadsorption with RL2-Sepharose. Unabsorbed digest (*A*), RL2 precipitate (*B*), RL2 supernatant (*C*) and a mock elution of RL2-Sepharose (*D*) were each galactosylated with galactosyltransferase and uridine diphospho-[^{3}H]galactose. The products of this reaction were fractionated on columns of Bio-Gel P-4 (−400 mesh, 1 × 175 cm). The numbered arrows at the top of each column show the K_{av} for elution of chitin standards composed of a polymer of the indicated number of GlcNAc residues. The large arrowhead in *D* indicates the K_{av} for elution of uridine diphospho-[^{3}H]galactose. The K_{av}'s for elution of dextran (~40 kD apparent molecular mass) and galactose are 0 and 1, respectively.

pore antibodies to their antigens, we examined whether enzymatically removing the O-linked GlcNAc residues inhibits antigen recognition on immunoblots. Treatment of nuclear envelopes with β-N-acetylglucosaminidase for 22 h resulted in the cleavage of over 90% of the GlcNAc residues from protein in these preparations (Fig. 5). On immunoblots, limit hexosaminidase-treated samples exhibited greatly reduced reactivity with all of the monoclonal antibodies studied (Fig. 6). As in the galactosylation experiments described above, limit cleavage inhibited, to differing extents, the binding of each antibody to specific antigens. Binding of RL2, RL4, and RL11 are largely abolished, while binding of RL1 and RL3 are less inhibited. The loss of reactivity after hexosaminidase treatment is not due to proteolysis in these samples, since the gel bands labeled in lightly galactosylated nuclear envelopes (data not shown), as well as lamins A and C (Fig. 6, lanes RL12) are not detectably degraded by incubation with β-N-acetylglucosaminidase for 22 h at 37°C. Taken together with the results of the galactosylation experiments described above, these data strongly indicate that the O-linked GlcNAc residues on the glycoproteins are intimately involved in antibody binding.

RL2 Requires Protein Structure for Immunoadsorption

As shown above, the interaction of RL2 with its antigens in both immunoadsorption and immunoblotting appears to be very sensitive to perturbation in the O-linked GlcNAc moieties on the nuclear pore complex glycoproteins. To investigate whether protein structure also has an influence on RL2

binding, we examined whether RL2 epitopes are preserved after pronase digestion of the nuclear pore complex glycoproteins. Nuclear envelopes were exhaustively digested with pronase, and portions of the pronase digest were directly galactosylated (Fig. 7 *A*) or were immunoadsorbed with RL2 and then galactosylated (Figure 7, *B* and *C*). Exhaustively digesting nuclear envelopes with pronase leaves a mixture of glycopeptides that are heterogeneous in size when fractionated on columns of Bio-Gel P4 (Fig. 7 *A*). Since most of the GlcNAc residues apparently are not reduced to the size of single glycosylated amino acids, the sites of O-linked GlcNAc addition may be clustered such that they protect short sequences of intervening amino acids from proteolysis. Interestingly, RL2 is unable to immunoadsorb these pronase-resistant peptides, since no material detected by galactosylation was specifically bound to RL2-Sepharose compared with a control sample (Fig. 7, cf. *B* and *D*). These data indicate that RL2, and by analogy the other antibodies studied, require some secondary and/or primary protein structure in addition to serine and threonine-linked GlcNAc residues for binding their antigens.

Discussion

In this report, we have demonstrated that a group of nuclear pore complex proteins are all modified by a novel saccharide linkage. This glycosylation consists of GlcNAc residues attached in O-linkages predominantly to serine side chains along the polypeptide backbones, as shown by direct chemical analyses performed in this and previous papers (Torres and Hart, 1984, and Holt and Hart, 1986). These nuclear pore complex glycoproteins have multiple sites of carbohydrate attachment, including some with at least 10 O-linked GlcNAc moieties per polypeptide chain. In the accompanying paper, Snow et al. (1987) demonstrate by EM immunolocalization that the epitopes recognized by the monoclonal antibodies against these proteins are present on the cytoplasmic and nucleoplasmic surfaces of the nuclear pore complex. They are not detected on the lumenal sides of nuclear membranes. These results taken together show that a specific type of protein–carbohydrate linkage, O-linked GlcNAc, is present on certain proteins of the nuclear pore complex exposed to both the cytoplasmic and nucleoplasmic compartments.

Perturbing the O-linked GlcNAc moieties on the proteins by attaching galactose to them with galactosyltransferase strongly diminishes the immunoreactivity of these proteins with all of the monoclonal antibodies. The simplest interpretation of these data is that the O-linked GlcNAc moieties are actually part of the binding epitope. This is substantiated by the fact that binding of all of the antibodies to the glycoproteins on immunoblots is largely abolished by hexosaminidase treatment. However, it is important to note that none of the antibodies appears to be solely directed against the GlcNAc moieties. The monoclonal antibody RL2, which appears to be more sensitive to the integrity of the carbohydrates on the nuclear pore proteins than any of the other antibodies tested, cannot immunoadsorb limit pronase digest glycopeptides of nuclear envelope proteins. Thus, these data argue that RL2, and, by analogy, the other monoclonals, recognize O-linked GlcNAc groups together with some adjacent protein sequence to have high affinity interactions with nuclear pore proteins. Extensive tertiary protein structure is not required for the epitopes, since all of the antibodies react with SDS-denatured proteins on immunoblots. The precise composition of these epitopes and their similarity to the sites of attachment of O-linked GlcNAc will be determined in future studies.

The presence of O-linked GlcNAc moieties on proteins in the cytoplasm and nucleoplasm is in stark contrast with the prevailing evidence that the bulk of the well-studied types of glycosylation are either localized on glycoconjugates at the cell surface or within the lumenal compartments of intracellular organelles (e.g., lysosomes, Golgi, and the endoplasmic reticulum). Although studies have indicated the existence of glycoconjugates in the cytoplasmic (Meyer and Burger, 1976; Nagakura et al., 1986; Davis and Blobel, 1986; Jarvis and Butel, 1985) and nucleoplasmic (Furukawa and Terayama, 1979; Stein et al., 1981; Stoddart, 1979; Kan and Pinto da Silva, 1986) compartments, none of these studies conclusively show whether these glycoconjugates are glycoproteins or glycolipids, whether the glycosylation events they undergo occur via N- or O-linkages, or whether the glycoconjugates arose from single or multiple processing events.

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References

Brew, K., T. C. Vanaman, R. L. Hill. 1968. The role of α-lactalbumin and the A protein in lactose synthetase: a unique mechanism for the control of a biological reaction. *Proc. Natl. Acad. Sci. USA.* 59:491–497.

Davis, L. I., and G. Blobel. 1986. Identification and characterization of a nuclear pore complex protein. *Cell.* 45:699–709.

Dwyer, N., and G. Blobel. 1976. A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. *J. Cell Biol.* 70:581–591.

Fedarko, N. S., and H. E. Conrad. 1986. A unique heparan sulfate in the nuclei of hepatocytes: structural changes with the growth state of the cells. *J. Cell Biol.* 102:587–599.

Fenner, C., R. R. Traut, D. T. Mason, and J. Wikman-Coffelt. 1975. Quantification of coomassie blue stained proteins in polyacrylamide gels based on analyses of eluted dye. *Anal. Biochem.* 63:595–602.

Furukawa, K., and H. Terayama. 1979. Pattern of glycosaminoglycans and glycoproteins associated with nuclei of regenerating liver of rat. *Biochim. Biophys. Acta.* 585:575–588.

Gerace, L., Y. Ottaviano, and C. Kondor-Koch. 1982. Identification of a major polypeptide of the nuclear pore complex. *J. Cell Biol.* 95:826–837.

Holt, G. D., and G. W. Hart. 1986. The subcellular distribution of terminal N-acetylglucosamine moieties. Localization of a novel protein-saccharide linkage, O-linked GlcNAc. *J. Biol. Chem.* 261:8049–8057.

Holt, G. D., S. J. Swiedler, J. H. Freed, and G. W. Hart. 1985. Murine Ia-associated invariant chain's processing to complex oligosaccharide forms and its dissociation from the I-A^k complex. *J. Immunol.* 135:399–407.

Jarvis, D. L., and J. S. Butel. 1985. Modification of Simian virus 40 large tumor antigen by glycosylation. *Virology.* 141:173–189.

Kan, F. W. K., and P. Pinto da Silva. 1986. Preferential association of glycoproteins to the euchromatin reagions of cross-fractured nuclei is revealed by fracture-label. *J. Cell Biol.* 102:576–586.

Meyer, D. I., and M. M. Burger. 1976. The chromaffin granule surface. Localization of carbohydrate on the cytoplasmic surface of an intracellular organelle. *Biochim. Biophys. Acta.* 443:428–436.

Nagakura, K., H. Tachibana, Y. Kaneda, and T. Nakae. 1986. *Leishmania braziliensis*: localization of glycoproteins in promastigotes. *Exp. Parasitol.* 61:335–342.

Park, J. T., and M. J. Johnson. 1949. A submicrodetermination of glucose. *J. Biol. Chem.* 181:149–151.

Porzig, E. F. 1978. Galactosyltransferase activity of intact neural retinal cells from the embryonic chicken. *Dev. Biol.* 67:114–136.

Snow, C. M., A. Senior, and L. Gerace. 1987. Monoclonal antibodies identify a group of nuclear pore complex proteins. *J. Cell Biol.* 104:1143–1156.

Stein, G. S., R. M. Roberts, J. S. Stein, and J. L. Davis. 1981. Nuclear glycoproteins and glycosaminoglycans. *In* The Cell Nucleus. Vol. 9. Academic Press, Inc. New York. pp. 341–357.

Stoddart, R. W. 1979. Nuclear glycoconjugates and their relation to malignancy. *Biol. Rev. Camb. Philos. Soc.* 54:199–235.

Tanaka, K., and W. Pigman. 1965. Improvements in hydrogenation procedures for demonstration of O-threonine glycosidic linkages in bovine submaxillary mucin. *J. Biol. Chem.* 240:1487–1488.

Torres, C.-R., and G. W. Hart. 1984. Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. *J. Biol. Chem.* 259:3308–3317.