Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 6462–6466, September 1987
Cell Biology

A monoclonal antibody against a family of nuclear pore proteins (nucleoporins): O-linked *N*-acetylglucosamine is part of the immunodeterminant

(nuclear pore/protein transport/mRNA efflux)

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Communicated by Gilbert Ashwell, May 18, 1987 (received for review March 31, 1987)

ABSTRACT **Using nuclear envelopes from Chinese hamster ovary (CHO) cells as an antigen, a mouse monoclonal antibody (IgM; designated mAb CHON211) that specifically binds to components of the nuclear pore complex (nucleoporins) was isolated. Immunofluorescence localization of the antigen recognized by mAb CHON211 revealed a punctate pattern restricted to the nuclear envelope; this pattern changed dramatically during the cell cycle. When examined by electron microscopy, the antigen was largely restricted to the nucleoplasmic and cytoplasmic faces of the nuclear pore complex. Immunoblots showed that mAb CHON211 bound to a series of polypeptides enriched in the nuclear envelope fraction with apparent molecular masses ranging from 110 to 35 kDa. Using galactosyltransferase and the lectin wheat germ agglutinin as probes, we have previously shown that proteins bearing O-linked *N*-acetylglucosamine (GlcNAc) are restricted to the cytoplasmic and nucleoplasmic faces of the nuclear pore complex. The nuclear membrane proteins recognized by mAb CHON211 had properties very similar to those identified by galactosyltransferase and wheat germ agglutinin labeling. Removal of O-linked GlcNAc residues with β-hexosaminidase greatly reduced the binding of mAb CHON211, strongly suggesting that O-linked GlcNAc moieties are part of the immunodeterminant. This monoclonal antibody defines a new family of antigens that are restricted to the nuclear pore and bear a common modification: O-linked GlcNAc. mAb CHON211 will be useful for defining the role of the nucleoporins in nucleo–cytoplasmic exchange.**

The nuclear pore complex is associated with the nuclear envelope of all eukaryotic cells. The pores are 800 Å in diameter and occur at the point of fusion between the outer and inner membranes of the envelope (1). They are the only apparent morphologic route of communication between the cytoplasm and the nucleus. Electron microscopic examination of the nuclear pore suggests that it is a complex structure having an 8-fold axis of symmetry and a central "plug" (2). The precise role of the nuclear pore complex is unknown, although it is presumed to mediate nucleo–cytoplasmic exchange. Little is known about the biochemical makeup of the pore complex; only recently have some clues emerged regarding its structure. A class of glycoproteins has been characterized that contain *N*-acetylglucosamine attached in an O-glycosidic linkage to the polypeptide chain (3, 4). Using the lectin wheat germ agglutinin (WGA) and the enzyme galactosyltransferase as probes, we have shown that O-linked *N*-acetylglucosamine (GlcNAc) is present on proteins that are on the cytoplasmic and nucleoplasmic faces of the nuclear pore complex (5). This is particularly interesting because lectins have been suggested to inhibit RNA efflux from the nuclear envelope (6). In another study, WGA, which binds to terminal GlcNAc moieties of glycoproteins, blocked the uptake of a nuclear protein in *Xenopus* (7). Together these observations suggest that a class of glycoproteins in the pore complex, which contain covalently attached O-linked GlcNAc, plays a role in nuclear transport. A monoclonal antibody that reacts with a single 62-kDa protein associated with the nuclear pore complex has been described; the term "nucleoporin" was coined to indicate the presence of this complex in the pore (8). The 62-kDa protein was shown to bind lectin WGA and was suggested to contain covalently attached carbohydrate. Another report has also suggested that monoclonal antibodies that recognize the nuclear pore complex may be directed against a carbohydrate antigen on nuclear pore antigens (9).

To characterize the glycoprotein components of the nuclear envelope, we have isolated a monoclonal antibody (mAb CHON211) that binds specifically to a group of proteins that are components of the nuclear pore complex (nucleoporins). The finding that a family of proteins associated with the pore complex shares O-linked GlcNAc suggests that the carbohydrate plays a role in the assembly or functioning of the nuclear pore.

MATERIALS AND METHODS

Reagents and Routine Methods. Jack bean β-*N*-acetylhexosaminidase and bovine galactosyltransferase were obtained from Sigma. Unconjugated, ferritin-conjugated, peroxidase-conjugated, and rhodamine-conjugated WGA were obtained from Vector Laboratories (Burlingame, CA). Protein concentration was estimated by the Pierce protein assay using ovalbumin as a protein standard. UDP ^{3}H-labeled galactose (80 Ci/mM; 1 Ci = 37 GBq) was purchased from New England Nuclear.

Immunoblotting. Proteins from nuclei or nuclear envelope fractions were run on 8% $NaDodSO_4$ gels and electrophoretically transferred to nitrocellulose (Bio-Rad). The filters were blocked for at least 3 hr using 3% gelatin in 50 mM Tris·HCl, pH 7.5/500 mM NaCl (TBS). Incubation with supernatants were done at 4°C for 12 hr. The filters were then washed sequentially with TBS, TBS containing 0.05% Tween 20 (TTBS) twice, and then TBS. Peroxidase-conjugated goat anti-mouse IgG and IgM (0.4 μg/ml) was then added for 2 hr. Antigens were visualized using diaminobenzidine and H_2O_2.

Galactosyltransferase and WGA As Probes for O-Linked GlcNAc. Lectin blotting using WGA-peroxidase and galac-

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Abbreviations: WGA, lectin wheat germ agglutinin; GlcNAc, *N*-acetylglucosamine; mAb, monoclonal antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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tosyltransferase labeling of nuclei and nuclear envelopes using ^{3}H-labeled UDP galactose were done as described previously (5).

Hexosaminidase Treatment. Treatment with β-*N*-acetylhexosaminidases from jack bean and from *Streptococcus pneumoniae* was done at the indicated concentration in a buffer consisting of 50 mM citrate phosphate, pH 4.0/1% (vol/vol) aprotinin (in benzyl alcohol). The β-hexosaminidase from *Streptococcus pneumoniae* was a gift of G. Ashwell and was protease free.

Preparation of Nuclei, Nuclear Envelopes, and Subcellular Fractions. Rat liver nuclei were purified by the method of Blobel and Potter (10). Nuclear envelopes were derived from rat liver nuclei by a previously published method (11). The purity of these fractions was confirmed by phase-contrast microscopy and electron microscopy. Other subcellular fractions were isolated from rat liver as described previously (5).

Nuclei and nuclear envelopes were purified from CHO cells (3×10^9 cells) by the method of Conner *et al.* (12). Yield of nuclei was 720 μg of protein; yield of nuclear envelopes was 190 μg of protein from 480 μg of nuclei protein.

Immunization and Spleen Cell Preparation. A BALB/c 6-week-old female mouse was immunized with i.p. injection of water-in-oil emulsion of 60 μg (protein) of a CHO nuclei preparation with complete Freund's adjuvant (GIBCO) and immediately thereafter was injected with 10 opacity units per 0.25 ml of *Bordetella pertussis* in saline (gift from C. R. Manclark; Division of Bacterial Products, National Center for Drugs and Biologies, National Institutes of Health). Four weeks later, 100 μg of purified CHO nuclei envelope protein was injected i.v.

Cell Culture, Fusion, and Screening. CHO cells (cell line 10900; obtained from M. Gottesman, National Cancer Institute, National Institutes of Health) were maintained at 37°C in minimal essential medium alpha (α-MEM) (GIBCO) supplemented with 10% (vol/vol) fetal calf serum (FCS; GIBCO), 50 units of penicillin per ml, and 50 mg of streptomycin per ml. NRK cells were obtained from the American Type Cell Collection and cultured in DME medium supplemented with 10% FCS and penicillin/streptomycin (GIBCO).

RPMI medium (GIBCO) was supplemented with 1 mM sodium pyruvate/1.5 mM L-glutamine, and 15% FCS (Hyclone) was used for the growth of X63.Ag8.653 mouse myeloma (American Type Cell Collection) and all hybridomas. Cell fusion was done according to Park and Wakabayashi (13). Screening for immunoglobulin secretion was done using an enzyme-linked immunosorbent assay (ELISA). Monoclonal antibody subclass was identified by comparison with mouse immunoglobulin standards by immunodiffusion using a monoclonal typing kit (Miles).

Immunofluorescence-Cytochemistry. Cells were washed three times with phosphate-buffered saline supplemented with Ca^{2+} and Mg^{2+} (PBS+) and fixed for 20 min with 2% formaldehyde in PBS+. Cells were permeabilized with 100% methanol for 5 min and washed. After blocking for 1 hr with 2% bovine serum albumin (BSA) in PBS, supernatant was added to each monolayer and incubated for 3 hr. After washing, the cells were incubated for 1 hr with 100 μl of rhodamine-labeled anti-mouse IgG and IgM (50 μg/ml in 1% BSA/PBS).

Electron Microscopy. Rat liver nuclei were attached to polylysin-coated 35-mm plastic dishes and fixed in 3.7% formaldehyde in PBS+ for 30 min at 23°C. Nuclei were then incubated in a diluent composed of 4 mg of normal goat globulin per ml/0.1% saponin/1 mM EGTA/PBS. Immunoferritin antibody labeling was done using the ferritin bridge method (14).

RESULTS

Our goal was to identify nuclear pore-specific monoclonal antibodies from the spleen of mice immunized with CHO cell nuclear envelopes. Screening by indirect immunofluorescence of CHO cells allowed the selection of seven hybridomas secreting antibodies specifically staining the nucleus or nuclear envelope. We attempted to subculture these clones to obtain a population of cells secreting a single antibody. Most of the original clones, however, proved difficult to subculture; this may have been due to cytotoxicity of the secreted antibodies because the cells survived briefly but then stopped growing when antibody began to accumulate in the growth medium. Clone CHON211 survived this subculturing and was found to secrete an IgM. This clone was selected for subsequent studies because it exhibited a bright punctate perinuclear pattern when analyzed by indirect immunofluorescence on CHO cells (Fig. 1 *A* and *B*). All of the bright images in Fig. 1 corresponded to the nucleus of the cells seen in phase-contrast microscopy. mAb CHON211 also selectively labeled the nuclear periphery in a rat cell line, NRK fibroblasts (Fig. 1 *C* and *D*). The immunofluorescence pattern was highly punctate and appeared to be evenly spaced at the surface of the nuclear membrane (Fig. 1*D*). Therefore, we examined the localization of the antigen on isolated rat liver nuclei. Isolated nuclei also exhibited a bright punctate fluorescence pattern when treated with CHON211 and rhodamine anti-mouse (IgG and IgM) (Fig. 1*E*).

Additional morphological observations confirmed that the antigen recognized by mAb CHON211 was restricted to the nuclear pore. We noted that the pattern of localization of the antigen recognized by mAb CHON211 was different at various stages of the cell cycle (Fig. 2). The immunofluorescence signal was brightest in cells just after mitosis and became punctate and dispersed in cells in prophase when the nuclear envelope breaks down. When examined at the electron microscopic level, mAb CHON211 was found to selectively label the cytoplasmic and nucleoplasmic faces of the nuclear pore complex (Fig. 3). The label was always found close to the pore complexes but not at the pore proper; this presumably reflects steric hindrance or exclusion of the IgM molecule and "ferritin bridge." No ferritin label was found on the lumenal face of the nuclear envelope. This localization was found to be strikingly similar to our previous localization of WGA-ferritin on isolated rat liver nuclear envelopes; WGA-ferritin also selectively labels the cytoplasmic and nucleoplasmic faces of the pore complex (5) (Fig. 3*H*).

The nature of the proteins recognized by mAb CHON211 was examined by immunoblotting using purified rat liver fractions including (*i*) nuclei and (*ii*) nuclear envelopes. Consistent with the morphological findings, both nuclei and nuclear envelope fractions were enriched for the antigens recognized by mAb CHON211. Immunoblots done using the nuclear envelope fraction revealed a family of proteins having apparent molecular masses of 35, 40, 43, 62, 65, 69, and 110 kDa (Fig. 4). As shown in Fig. 5, a 62-kDa species is the major labeled protein seen when nuclei were probed with the antibody. The different labeling pattern seen with nuclei and nuclear envelopes was also observed using WGA and galactosyltransferase as probes (5) and is presumably due to a preferential loss of the 62-kDa species during isolation of nuclear envelopes. Immunoprecipitation of the antigen from CHO cells metabolically labeled with [^{35}S]methionine revealed a series of polypeptides having molecular masses of 39, 58, 62, and 69 kDa (data not shown).

The finding that mAb CHON211 bound to a family of proteins rather than a single polypeptide chain suggested that the antibody may recognize a shared determinant on the nucleoporins. Previously, using WGA and galactosyltrans-

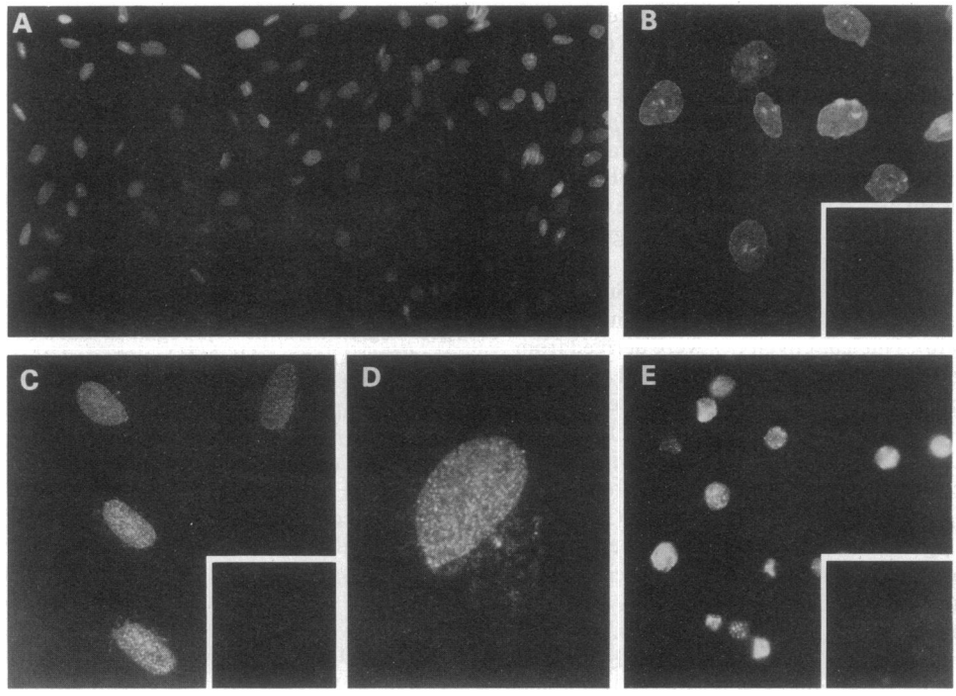

FIG. 1. Immunofluorescence microscopy of CHO and NRK cells using mAb CHON211. CHO cells (*A* and *B*) or NRK cells (*C* and *D*) were fixed and incubated with mAb CHON211 as described. Inserts in each case show the level of staining seen using control supernatants. *D* shows a high magnification of the pattern of immunofluorescence observed with mAb CHON211 in NRK cells. In *E* rat liver nuclei were fixed with 3.7% formaldehyde and incubated with mAb CHON211.

ferase, we have shown that a group of proteins possessing O-linked GlcNAc are selectively localized to the cytoplasmic and nucleoplasmic faces of the nuclear pore complex (5). We noted that these proteins had molecular properties strikingly similar to those recognized by mAb CHON211. The proteins of intact nuclei were then subjected to immunoblotting, galactosylation, or lectin blotting. Lectin blotting of nuclear proteins labeled a protein of similar apparent molecular mass. As shown in lane 3 of Fig. 5, when intact nuclei are labeled with ^{3}H-labeled UDP galactose and galactosyltransferase, a protein of 62 kDa is the major labeled species. We have previously demonstrated that >95% of the label transferred to terminal GlcNAc residues in sealed nuclei is present in O-glycosidically linked GlcNAc-β1-4-galactose (5). The ob-

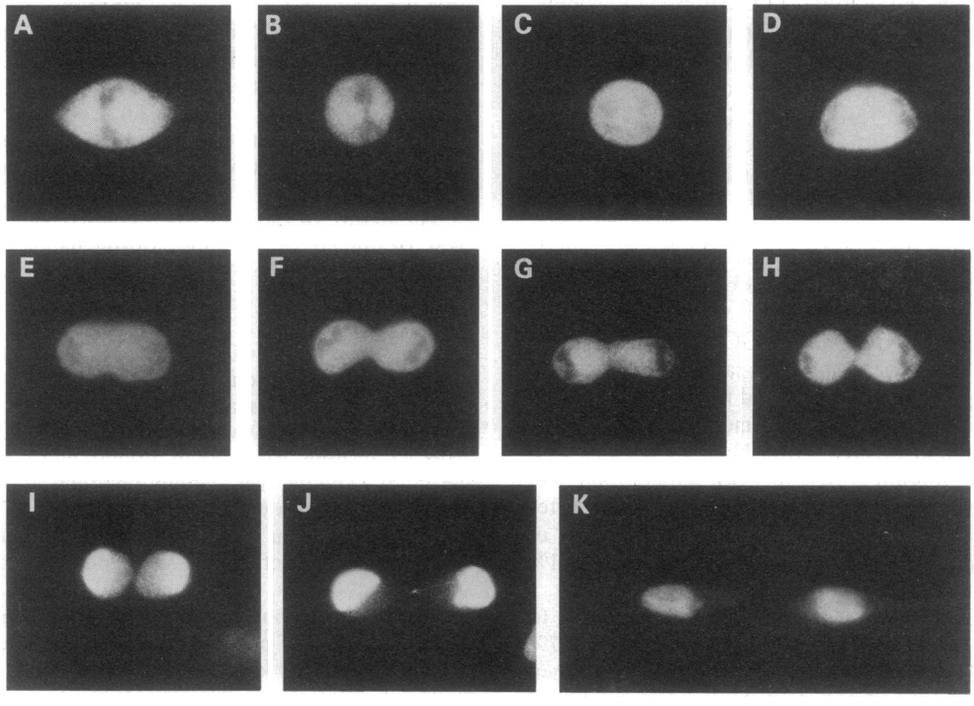

FIG. 2. Immunofluorescence localization of the antigen recognized by mAb CHON211 during various stages of the cell cycle. NRK cells were fixed and incubated with mAb CHON211 as described. Cells in various stages of the cell cycle were selected and photographed. The frames represent: metaphase (*A* and *B*), anaphase (*C*), early telophase (*D*), late telophase (*E–H*), daughter cells (*I–K*).

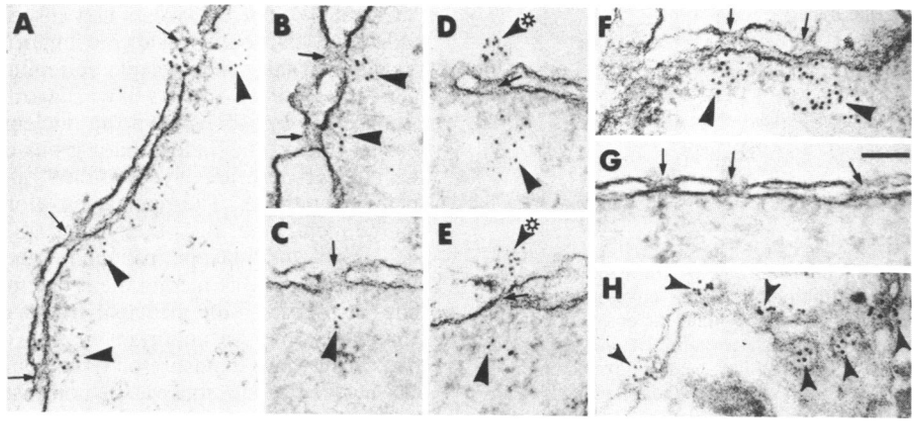

FIG. 3. Immunoelectron microscopic localization of mAb CHON211 in isolated nuclei. The ferritin bridge method (14) was used to label these nuclei using either mAb CHON211 supernatant (*A–F*) or a nonreactive control supernatant (*G*). Large arrowheads indicate clusters of ferritin cores; small arrows indicate the position of the nuclear pore complex. Asterisk arrowheads in *D* and *E* show ferritin on the cytoplasmic side of the pore complex. In *G*, the nuclei were labeled with WGA-ferritin, which binds to O-linked GlcNAc-containing glycoproteins of the nuclear pore complex (5). All magnifications are ×59,000; bar (in *G*), 0.1 μm.

served similarity between the 62-kDa species labeled by mAb CHON211 and the major 62-kDa O-linked GlcNAc glycoprotein of the nuclear membrane led us to ask whether the mAb CHON211 recognized proteins bearing O-linked GlcNAc. To determine whether the monoclonal antibody recognized a shared carbohydrate antigen, O-linked GlcNAc residues were modified by the addition of galactose residues (catalyzed by galactosyltransferase) or removed using jack bean β-hexosaminidase. We have previously shown that β-hexosaminidase removes the O-linked GlcNAc moiety from the nuclear pore proteins. The proteins were then subjected to immunoblotting with mAb CHON211 (Fig. 6). Galactosylation of the polypeptides (lane 2) did not significantly diminish antibody binding. Under these conditions, only 70% of the available GlcNAc residues were converted to Gal(β1–4)GlcNAc. However, treatment of nuclear envelopes with 1 unit/ml (lane 3) or 4 units/ml (lane 4) of β-hexosaminidase for 16 hr at 37°C destroyed the ability of mAb CHON211 to bind to the proteins. The polypeptides most resistant to β-hexosaminidase (35 and 39 kDa) were those most heavily labeled with galactosyltransferase and are presumed to be most heavily modified with O-linked GlcNAc. The enzyme preparation was used in the presence of protease inhibitor and, under these conditions, had no detectable protease activity using [^{35}S]methionine-labeled cell proteins or tosylarginine methyl ester as substrates. In addition, no proteolyses of the polypeptides were detected by amido-black staining (data not shown). Although terminal GlcNAc moieties were found to be required for antibody binding, the antibody does not appear to recognize only the carbohydrate. Neither ovalbumin, which contains terminal GlcNAc residues, nor the lectin WGA was found to compete for mAb CHON211 binding to the nucleoporins (data not shown). In other experiments, we have demonstrated the following: (*i*) after treatment with UDP [^{14}C]galactose and galactosyltransferase, ^{14}C-galactose-labeled nuclear antigens can be immunoprecipitated using mAb CHON211 and (*ii*) the antigens

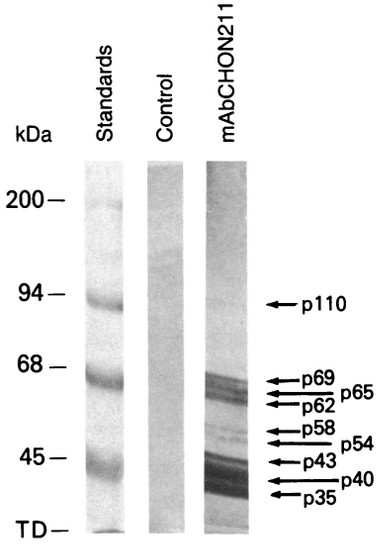

FIG. 4. Immunoblotting of nuclear envelope antigens using mAb CHON211. Nuclear envelope proteins were subjected to NaDodSO$_4$/PAGE and electrophoretically transferred to nitrocellulose as described. The filters were then incubated with mAb CHON211 or control supernatant. Molecular mass of protein standards is indicated on the left, and apparent molecular mass of labeled polypeptides is indicated on the right.

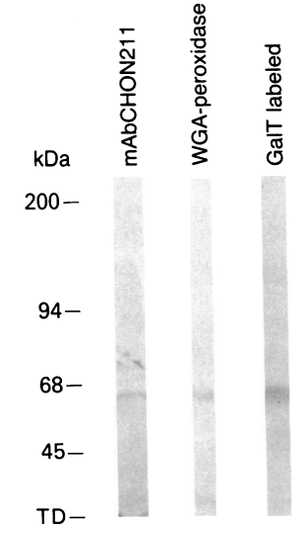

FIG. 5. Comparison between the 62-kDa antigen recognized by mAb CHON211 and polypeptides labeled by WGA-peroxidase and galactosyltransferase. Intact nuclei, labeled using galactosyltransferase (GalT) and ^{3}H-labeled UDP galactose or unlabeled, were transferred to nitrocellulose filters. One lane was immunoblotted with mAb CHON211. Another lane was blotted with the lectin WGA coupled to peroxidase. The ^{3}H-labeled UDP galactose-labeled sample was visualized by autoradiography. Migration position of protein standards is indicated on the left.

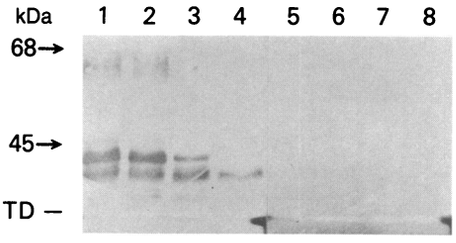

FIG. 6. Effects of treatment with galactosyltransferase and β-hexosaminidase on the antigenicity of nuclear envelope proteins recognized by mAb CHON211. Rat liver nuclear envelopes were treated with 2 units of galactosyltransferase and 0.4 mM unlabeled UDP galactose (lanes 2 and 6), 1 unit of jack bean β-hexosaminidase per ml (lanes 3 and 7), or 4 units (lanes 4 and 8) of jack bean β-hexosaminidase per ml. Other samples (lanes 1 and 5) were untreated. The modified proteins were then subjected to $NaDodSO_4$/PAGE and transferred to nitrocellulose. Lanes 1–4 were then blotted with mAb CHON211; lanes 5–8 were immunoblotted with control supernatant. TD, tracking dye.

can be metabolically labeled in CHO cells using [^{14}C]glucosamine (data not shown). Together these observations strongly suggest that O-linked GlcNAc is present on the nucleoporins and is a part of the determinant recognized by mAb CHON211.

DISCUSSION

We have described a mouse monoclonal IgM that binds specifically to a family of O-linked GlcNAc-containing polypeptides localized to the nuclear pore complex (nucleoporins). This antibody recognizes a group of polypeptide chains ranging in apparent molecular mass from 35 to 110 kDa, which appear to be identical to a group of proteins we have previously identified using the enzyme galactosyltransferase and lectin WGA. These O-linked GlcNAc-containing proteins were shown to be localized to the cytoplasmic and nucleoplasmic faces of the nuclear pore (5). Evidence presented here demonstrates that the monoclonal antibody recognizes proteins bearing this modification and that the O-linked GlcNAc is part of the immunodeterminant. Only one monoclonal antibody has previously been shown to react with the nuclear pore; this antibody recognizes a single 62-kDa species (8). The antibody we have described appears very different in its properties because it recognizes a number of polypeptides in addition to the prominent 62-kDa species. One possible explanation for this difference in specificities between the monoclonal antibodies is that the antibody described by Davis and Blobel (8) could be directed against the polypeptide chain. Alternatively, the antibody may indeed recognize the O-linked GlcNAc modification, but the other less abundant proteins bearing this linkage were not detected. The antibody we have described clearly depends upon the presence of O-linked GlcNAc moieties for binding to the nucleoporins. However, the evidence suggests that there is also a requirement for the polypeptide chain, because neither terminal GlcNAc-containing glycoproteins nor the lectin WGA competes for binding. It is likely that mAb CHON211 recognizes GlcNAc residues in an O-glycosidic linkage to Ser and/or Thr residues in a specific sequence motif. This polypeptide sequence motif may represent a recognition signal for the enzymes involved in the addition of O-linked GlcNAc. Therefore, mAb CHON211 defines a discrete family of antigens, restricted to the nuclear pore and bearing O-linked GlcNAc.

Current evidence suggests that carbohydrate residues are added to glycoproteins inside the lumen of eukaryotic cellular organelles (e.g., endoplasmic reticulum, Golgi apparatus) (15–20). However, reports have suggested that glycosylated proteins are also present in the nucleoplasm and the cytoplasm (21–25). The finding that a group of proteins present in the nuclear pore are glycoproteins raises questions about their biosynthesis. The nuclear envelope is composed of a double membrane; the outer membrane is contiguous with the rough endoplasmic reticulum and bears ribosomes. Crossing the double membrane is the nuclear pore complex that may serve as the principal site of nuclear–cytoplasmic communication. An important feature of the orientation of the pores is their exposure on either side to the cytoplasmic and nucleoplasmic spaces. In contrast, the carbohydrate residues of the glycoprotein components of the nuclear envelope having well characterized N-linked oligosaccharides are localized to the internal surfaces of the nuclear cisternae (26). The finding that O-linked GlcNAc is present on nuclear pore proteins in the cytoplasm and nucleoplasm suggests that the enzyme(s) involved in pore protein glycosylation may be localized in the cytoplasm (or nucleoplasm) rather than in the lumen of the endomembrane system.

The precise role of the nuclear envelope in the processes of membrane glycoprotein assembly and nucleocytoplasmic exchange remains unclear; the monoclonal antibody characterized here will be a useful reagent to investigate any effect of carbohydrate modification on the biosynthesis of the nucleoporins and the function(s) of the nuclear pore complex.

Note Added in Proof. Recently, monoclonal antibodies having specificities similar to mAb CHON211 have been described (27).

We thank Angelina Rutherford for technical assistance with the electron microscopy and acknowledge Dr. C. R. Manclark for the gift of *B. pertussis*. We also acknowledge Drs. William B. Jakoby, Gilbert Ashwell, and Barbara Wolff for helpful discussions.

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