Brief Definitive Report

Autoantibodies from Patients with Primary Biliary Cirrhosis Preferentially React with the Amino-terminal Domain of Nuclear Pore Complex Glycoprotein gp210

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Summary

Patients with primary biliary cirrhosis frequently develop autoantibodies directed to gp210, a major glycoprotein of the nuclear pore complex. This protein contains a large glycosylated cisternal domain, a single transmembrane segment, and a short cytoplasmic tail. It has been previously shown that autoantibodies from primary biliary cirrhosis patients exclusively react with the cytoplasmic tail. We demonstrate that autoantibodies against gp210 recognize at least two different epitopes. 4 out of 12 anti-gp210 positive sera reacted with the fragment consisting of the cytoplasmic tail, and 8 sera targeted a novel epitope located within the large glycosylated lumenal domain. Moreover, our data prove that carbohydrate moieties are an essential part of this novel epitope. We propose, therefore, that future screening assays should be performed with antigens possessing both epitopes to detect all sera with anti-gp210 specificity.

About one third of patients with primary biliary cirrhosis (PBC) have autoantibodies that recognize gp210, an integral glycoprotein of the nuclear pore complex (1–4). "Mature" gp210 is composed of three main domains. A large 1,783–amino acid amino-terminal domain is located in the perinuclear space between the outer and inner nuclear membrane. A short 20–amino acid hydrophobic segment spans the nuclear membrane and a 58–amino acid carboxy-terminal tail domain faces the cytoplasm (5, 6). It has been shown that gp210 contains high mannose-type oligosaccharides. Carbohydrate moieties contribute 5% of the mass of gp210 and are restricted to the amino-terminal domain (5). Since the three domains of gp210 greatly differ from each other structurally, it is of interest whether the autoantibodies directed against gp210 recognize a specific domain of this protein. Recently, it has been reported that autoantibodies from PBC patients exclusively recognize a short region within the cytoplasmic tail of gp210 (7). In the present study, we demonstrate that the anti-gp210 autoantibodies are directed against at least two antigenic determinants: one located in the cytoplasmic and another in the large lumenal domain of gp210 protein. Furthermore, the autoantibodies exclusively recognize the glycosylated form of the amino-terminal domain of gp210 protein, indicating that carbohydrate residues are an integral part of this novel epitope.

Materials and Methods

Patients. Sera were obtained from 43 patients with primary biliary cirrhosis and 20 healthy blood donors. The diagnosis of primary biliary cirrhosis was based on accepted clinical, biochemical, and histological criteria.

Chemical Reagents. Routine chemical reagents were obtained from either the United States Biochemical Corp. (Cleveland, OH) or Sigma Chemical Co. (St. Louis, MO).

Isolation of Nuclei and Nuclear Pore Complex–Lamina Fraction (NPC). To avoid proteolytic degradation of proteins, all isolation steps were performed at 4°C in the presence of protease inhibitors.

The nuclei were isolated as described previously (8). Briefly, HeLa cells were PBS washed, swollen for 10 min in hypotonic buffer, and spun down. The pellet was suspended again in hypotonic buffer and homogenized after addition of NP-40 and sodium deoxycholate to a final concentration of 1% and 0.5%, respectively. Then nuclei were pelleted through sucrose cushion.

Subsequently, the NPC was prepared according to the method described by Dwyer and Blobel (9). In short, the isolated nuclei were digested with DNase I at pH 8.5, then redigested with DNase I and pancreatic RNase at a neutral pH and extracted in two consecutive steps, first with high salt buffer and thereupon with buffer containing nonionic detergents. This yielded an insoluble pellet defined as the NPC. After treatment of the pellet with buffer containing high concentration of monovalent cations and nonionic detergents, proteins of the nuclear pores became soluble.

Purification of gp210 Protein. gp210 protein was purified by affinity chromatography on lentil lectin–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Nuclear pore proteins solubilized at concentration $\sim$2 mg/ml in high salt buffer (500 mM NaCl, 20 mM Hepes, pH 7.35, 1 mM MgCl$_2$, 1 mM DTT, and 1 mM PMSF) containing 2% Triton X-100 were loaded on preequilibrated lentil lectin–Sepharose. The column was then washed with at least 20 vol of high salt buffer containing 0.1% Triton X-100. gp210 protein was eluted with high salt buffer containing 1.2 M methyl-α-D-mannopyranoside and 0.1% Triton X-100.

Electrophoretic Separation of Proteins. Proteins of nuclear subfraction or purified proteins were separated by one-dimensional PAGE on 10 or 13% SDS slab gels, as described by Laemmli (10). After electrophoretic separation, proteins were detected by Coomassie blue R-250 or silver staining.

Immunoblotting. Proteins separated on slab gels were electrophoretically transferred onto membranes (Bio Rad Laboratories, Richmond, CA) and stained by Ponceau S. The blots were saturated with 3% BSA in PBS, then probed with patients sera diluted 1:500, extensively washed, and incubated with anti–human Ig coupled to horseradish peroxidase (HRP) at a 1:2,000 dilution. The reactive antigens were detected by HRP–hydrogen peroxide catalyzed oxidation of luminol under alkaline conditions using ECL Western blotting detection reagents (Amersham International, Little Chalfont, U.K.) and X-Omat S film (Eastman Kodak Co., Rochester, NY).

Limited Proteolysis of gp210 Glycoprotein. Isolated NPCs were resuspended at $\sim$0.5 mg/ml protein in 0.25 M sucrose, 20 mM Hepes, pH 7.35, 1 mM EDTA, and 1 mM DTT, and were incubated with papain immobilized on Sepharose (Pierce Chemical Co., Rockford, IL) at 4°C for 5, 15, or 30 min. The reaction was stopped by removal of papain-Sepharose and adding PMSF (1 mM), N-ethylmaleimide (2 mM), and leupeptin and pepstatin (1 μM each). The samples were centrifuged at 100,000 g for 1 h to yield pellets and supernatants.

Deglycosylation of gp210 Protein. Isolated NPCs or purified gp210 were suspended in 100 mM sodium citrate buffer, pH 5.5, 0.1% SDS, and 3 mM DTT. Protease inhibitors were added to a final concentration as follows: 0.5 mM PMSF, 0.7 μg/ml pepstatin, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.1 mM Pefabloc. Then endoglycosidase H (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added at a concentration of 50 mU/700 μg protein, and the incubation was performed for 14 h at 37°C. After deglycosylation, samples were ethanol precipitated and submitted for PAGE and immunoblotting.

Covalent Immobilization of gp210 Protein. Purified gp210 protein was immobilized on Mini-Leak-Medium beads (Kem.En-Tec, Copenhagen, Denmark) according to the manufacturer's protocol. Mini-Leak is a divinyl sulfone–activated matrix of spherical agarose especially designed for gentle immobilization of biomolecules. gp210 dissolved in PBS at a concentration of 0.5 mg/ml was added to the activated matrix and diluted with coupling buffer containing 30% polyethylene glycol 20,000 (PEG) to a final concentration of 10% PEG and incubated overnight. The supernatant was discarded, then the matrix was washed and incubated with 0.2 M ethanol amine/HCl, pH 9.0, for 4 h to block excess active groups. Coupling yield was determined by measurement of OD at 280 nm before and after coupling reaction.

Isolation of Anti-gp210 Antibodies by Immunoaffinity Chromatography. 300 μl of patient serum fivefold diluted with PBS was loaded on preequilibrated gp210 affinity column. The column was washed with PBS until the basic OD level has been reached. Then bound autoantibodies were stepwise eluted with 150 mM,

350 mM, and 1 M NaCl in 0.1 M phosphate buffer, pH 3.0, and immediately neutralized with 2 M Tris, pH 9.0.

Results and Discussion

We examined sera from 43 patients with PBC. 12 serum samples that recognized on immunoblots gp210 purified from HeLa cells were used for further experiments. gp210 was treated with papain, known to cleave this protein in a region of high papain sensitivity close to the hydrophobic transmembrane domain (5). By papain treatment, two polypetides were generated: an amino-terminal fragment with molecular mass of $\sim$170 kD and a carboxy-terminal peptide of $\sim$30 kD. These degradation products were then tested in immunoblotting experiments. 8 of 12 sera exclusively recognized the 170-kD amino-terminal fragment (Fig. 1 *a*, serum *B*), while 4 sera reacted only with the 30-kD peptide (Fig. 1 *b*, serum *A*). The 170-kD polypeptide contained carbohydrate moieties as evidenced by its ability to bind lentil lectin (not shown). To clarify whether these moieties play a role in the immune reaction, the gp210 was extensively treated with endoglycosidase H, known to specifically hydrolyze high mannose type N-linked oligosaccharides. This resulted in a marked reduction of the molecular mass of gp210 and completely abolished its binding to lentil lectin. Concomitantly, the reactivity of autoantibodies towards the antigen changed dramatically. All of the sera that reacted with the amino-terminal 170-kD fragment didn't recognize the deglycosylated form of gp210 (Fig. 2, sera *4–8*). On the other hand, the deglycosylation of gp210, as expected, didn't affect its reactivity with the four sera that targeted an epitope in the carboxy-terminal 30-kD fragment (Fig. 2,
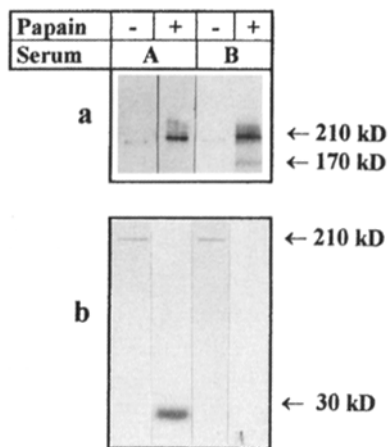

Papain	-	+	-	+
Serum	A		B	

Figure 1. Reactivity of anti-gp210–positive PBC sera with papain-generated fragments of gp210 protein. gp210 protein partially digested with papain immobilized on Sepharose was separated by SDS-PAGE and submitted for immunoblotting. To achieve optimal resolution of corresponding proteins, the high molecular weight fragment was separated on 10% polyacrylamide gels (*a*) and the small fragment was resolved on 13% polyacrylamide gels (*b*) 1.5 μg of untreated gp210 or 5 μg of papain digested gp210 was loaded pro lane. Human sera were used at a 1:500 dilution. Serum A doesn't react with the 170-kD fragment (*a*), but recognizes the 30-kD fragment. (*b*) Serum *B* reacts with the 170-kD fragment (*a*), but not with the 30-kD fragment (*b*).

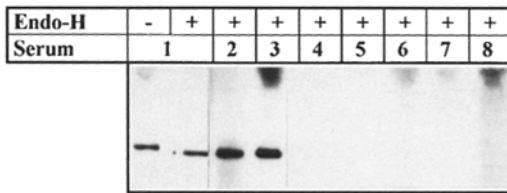| Endo-H	-	+	+	+	+	+	+	+	+
Serum	1	2	3	4	5	6	7	8

Figure 2. Reactivity of the anti-gp210 autoantibodies with deglycosylated form of gp210 protein. Only sera 1–3 are able to react with gp210 after endoglycosidase H treatment. As a positive control, untreated gp210 was used (lane *1*). Sera 1 and 5 correspond to sera A and B, respectively, in Fig. 1.
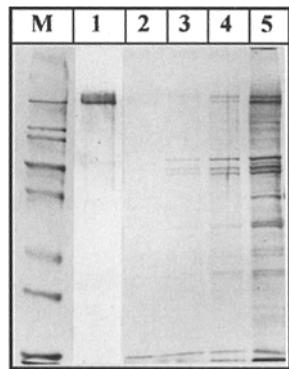

Figure 3. Purification of gp210 glycoprotein. Fractions of NPCs from HeLa cells were resolved by SDS-PAGE on a 10% slab gel, and proteins were stained with Coomassie blue R-250 (lanes *M* and *2–5*) or silver stained (lane *1*). (Lane M) Molecular weight markers: myosin, 200 kD; β-galactosidase, 116 kD; phosphorylase b, 97 kD; BSA, 66 kD, glutamic dehydrogenase, 55 kD; lactate dehydrogenase, 36 kD, carbonic anhydrase, 31 kD, trypsin inhibitor, 21 kD. (Lanes *1* and *2*) Affinity-purified gp210. (Lane *3*) Flowthrough fraction of lentil lectin–Sepharose. (Lane *4*) NPC extract loaded on lentil lectin–Sepharose. (Lane *5*) NPC proteins.

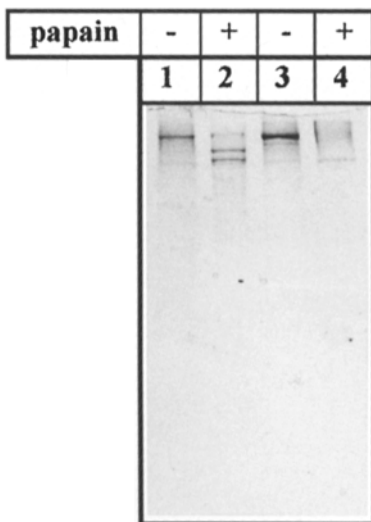| papain	-	+	-	+
	1	2	3	4

Figure 4. Glycosylated fragments of papain-digested gp210 protein. gp210 was papain digested for 5 or 15 min and loaded on lentil lectin–Sepahrose. Proteins eluted from the column were separated on 10% SDS slab gels and stained with Coomassie blue R-250. (Lanes *1* and *3*) Purified gp210 submitted for papain digestion. (Lane *2*) 180- and 170-kD fragments after 5 min digestion. (Lane *4*) 170-kD fragment after 15 min digestion.

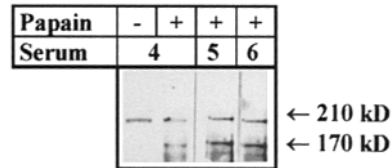| Papain	-	+	+	+
Serum	4	5	6

← 210 kD
← 170 kD

Figure 5. Reactivity of glycosylated fragments of gp210 with affinity-purified anti-gp210 antibodies. Papain-generated fragments of gp210 were purified on lentil lectin–Sepharose, loaded (3 μg) on 10% SDS-slab gels, and submitted for immunoblotting using affinity-purified anti-gp210 antibodies at a 1:500 dilution. Untreated gp210 (2 μg) was applied in lane *1* as a control. Antibodies 4–6 were isolated from corresponding sera 4–6 used in Fig. 2.

sera *1–3*). It is apparent that this autoepitope corresponds to that described by Nickowitz and Worman (7).

To unequivocally provide evidence that the protein fragment at 170 kD was recognized solely by specific anti-gp210 autoantibodies, PBC sera were submitted for fractionation on an immunoaffinity column. For this purpose, gp210 glycoprotein purified to homogeneity from HeLa cells (Fig. 3, lane *1*) was immobilized on activated agarose beads. This activated matrix is especially suitable for gentle coupling of biomolecules under mild conditions. Diluted PBC sera were loaded on preequilibrated immunoaffinity column. The bulk of proteins flowed through, and only anti-gp210–specific Igs were retained. The bound antibodies were eluted with salt in phosphate buffer at pH 3.0. The affinity-purified anti–gp-210 antibodies were used for immunoblotting. In these experiments, only the glycosylated fragments of papain-digested gp210 protein were used as antigen. As shown in Fig. 4, after short papain treatment of gp210 protein, two glycosylated fragments of ∼180 and 170 kD were purified by affinity chromatography. After longer digestion, only one glycosylated peptide of 170 kD was isolated. Immunoblotting tests using this peptide revealed that affinity-purified anti-gp210 antibodies react with the glycosylated 170-kD fragment of gp210 protein (Fig. 5).

These results, summarized in Table 1, clearly demonstrate that autoantibodies against gp210 recognize at least two different epitopes: one located in the cytoplasmatic tail and another positioned in the large amino-terminal do-

Table 1. *Specificity of the Anti-gp210 Antibodies*

			Recognized epitopes			
			170 kD		30 kD	
Patients	n	gp210 positive	Endo-H⁻	Endo-H⁺	Endo-H⁻	Endo-H⁺
PBC	43	12	8	0	4	4
Controls	20	0	0	0	0	0

Endo-H, endoglycosidase H.

main. Furthermore, these data prove that carbohydrate moieties are an essential part of this novel epitope.

There is an obvious discrepancy between our data and results that have been published by Nickowitz and Worman (7). However, since Nickowitz and Worman (7) have used a bacterially expressed gp210 fusion protein that, due to the properties of the expression system, lacks posttranslational modification, they failed to detect the glycosylated epitope. In their screening system, 25 of 309 PBC sera (8%) have been shown to also recognize a full-length gp210 fusion protein as its cytoplasmic part. It corresponds very well to our data, demonstrating that 4 of 43 PBC sera (9%) react with small cytoplasmic fragment of gp210.

Since the anti-gp210 antibodies are highly specific for PBC and its detection may be useful especially in diagnosing individuals without antimitochondrial antibodies we suggest the use of gp210 purified from cells of human origin to ensure optimal assay conditions.

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