Autoantibodies from Patients with Primary Biliary Cirrhosis Recognize a Restricted Region within the Cytoplasmic Tail of Nuclear Pore Membrane Glycoprotein Gp210

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Summary

Patients with primary biliary cirrhosis (PBC) frequently have autoantibodies against a 210-kD integral glycoprotein of the nuclear envelope pore membrane. This protein, termed gp210, has a 1,783-amino acid amino-terminal domain located in the perinuclear space, a 20-amino acid transmembrane segment, and a 58-amino acid cytoplasmic carboxy-terminal tail. We now demonstrate that autoantibodies from 25 patients with PBC that recognize gp210 react with the cytoplasmic carboxy-terminal tail while none react with unmodified linear epitopes in the amino-terminal domain. The epitope(s) recognized by autoantibodies from all 25 patients is contained within a stretch of 15 amino acids. The recognized amino acid sequence is homologous to the protein products of the *Escherichia coli mutY* gene and *Salmonella typhimurium mutB* gene with an exact identity of six consecutive amino acids, suggesting that anti-gp210 antibodies may arise by molecular mimicry of bacterial antigenic determinants.

Patients with liver and autoimmune diseases frequently have autoantibodies that recognize proteins of the nuclear envelope (1). The nuclear envelope is composed of the nuclear lamina, the nuclear pore complexes, and the nuclear membranes (for reviews see references 1–3). The lamina is a meshwork of intermediate filament proteins termed lamins and the pore complexes are composed of more than 100 different polypeptides. Three morphologically distinct but interconnected membranes are found in the nuclear envelope: the inner nuclear membrane, the outer nuclear membrane, and the nuclear pore membrane. The inner and outer nuclear membranes are separated from each other by the perinuclear space which is continuous with the lumen of the endoplasmic reticulum (ER). These two membranes are connected at numerous points by the nuclear pore membranes that also serve as the attachment sites for the pore complexes. Although the three nuclear envelope membranes are continuous, integral proteins are not randomly distributed between them and unique proteins reside in each of these membranes.

About one third of patients with primary biliary cirrhosis (PBC) have serum antibodies that recognize the protein components of the nuclear lamina or the nuclear membranes. Occasionally, patients with PBC have antibodies that recognize the nuclear lamins (4), although these autoantibodies are more commonly detected in patients with autoimmune hepatitis (5, 6) or systemic lupus erythematosus (7). Few patients with PBC have autoantibodies against the lamin B receptor (8),

a polytopic protein of the inner nuclear membrane (9, 10). As many as 25% of patients with PBC, and apparently none thus far described with other diseases, have serum antibodies that recognize a 200-kD protein of the nuclear envelope (4, 11–13) that has been identified as gp210 (14), an integral glycoprotein of the nuclear pore membrane (15). Gp210 is composed of an amino-terminal domain of 1,783 amino acids (after cleavage of the amino-terminal signal sequence) that is located in the perinuclear space, a 20-amino acid transmembrane segment that spans the nuclear pore membrane, and a cytoplasmic carboxy-terminal tail domain of 58 amino acids (16, 17). Residues in the amino-terminal domain are glycosylated (16). The transmembrane segment and cytoplasmic tail of gp210 both contain signals for targeting to the nuclear pore membrane (18) and the cytoplasmic tail may interact with proteins of the nuclear pore complex (16).

It is not known why patients with PBC develop autoantibodies against gp210 or whether these antibodies recognize a specific domain of this integral membrane protein. It is also unclear as to how this intracellular protein, or the autoantibodies that recognize it, are related to the pathogenesis of PBC. Identification of the epitope(s) of gp210 recognized by autoantibodies would provide information to help answer these questions. Identification of the recognized epitope(s) would also facilitate the development of simple assays to detect gp210 autoantibodies, such as those utilizing polypeptide antigens that have been clinically useful for detecting antimitochon-

drial antibodies (19–20). We have therefore identified the region of gp210 that contains the epitope(s) recognized by autoantibodies from patients with PBC.

Materials and Methods

Autoantibodies. 15 serum samples with antibodies shown to recognize a full-length gp210 fusion protein were obtained from a series of 159 patients with PBC followed at The Mount Sinai Medical Center in New York City (13). 10 serum samples containing antibodies that recognize purified gp210 (14) were provided by Dr. J.-C. Courvalin (Institut Jacques Monod, Université Paris 7, Paris, France) and obtained from a previously published series of 150 patients with PBC (4).

Plasmid Construction and Fusion Protein Expression. The pGEX-2T vector (Pharmacia LKB, Piscataway, NJ) was used to make plasmid constructs that express various regions of rat gp210 as glutathione-S-transferase (GST) fusion proteins (21). To generate cDNAs coding for the various regions of gp210, 25 cycles of PCR were performed as described (22) using the GeneAmp Kit (The Perkin Elmer Corp., Norwalk, CT) and the following parameters: denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 3 min. A full-length gp210 cDNA (18) provided by Dr. R. W. Wozniak (Laboratory of Cell Biology, The Rockefeller University, New York) was used as the template. Based on the previously published sequence of gp210 (16), PCR primers were designed to amplify portions of cDNA coding for the regions of the protein shown in Figs. 1 and 2. Restriction sites were engineered into the 5' ends of the PCR primers to facilitate cloning of the amplified products into the BamHI and/or EcoRI sites of pGEX-2T. Amplified products were purified from PCR reactions mixtures using either the GeneClean II Kit (Bio 101, Inc., Vista, CA) or phenol/chloroform extraction (23). The purified DNA was digested with the appropriate restriction enzymes, purified again, and ligated into pGEX-2T that was also digested with the appropriate restriction enzymes. *Escherichia coli* strains BB-4 or JM101 (Stratagene, La Jolla, CA) were transformed by standard methods (23) and the recombinant plasmids were analyzed by restriction digestion. All plasmids encoding gp210 carboxy-terminal domain fusion proteins were also analyzed by dideoxy chain termination

sequencing (24) using the Sequenase Version II Kit (United States Biochemical, Cleveland, OH). GST fusion proteins were expressed as described (21). Fusion proteins were purified on glutathione-Sepharose (Pharmacia LKB) as described (21), except for fusion proteins 2, 3, 4, and 5 (Fig. 1) for which whole bacterial cell lysates were used in immunoblotting experiments, as the proteins were hydrophobic and difficult to solubilize. Expression of the fusion proteins was confirmed by immunoblotting with antibodies that recognized the GST portions of the recombinant polypeptides.

Electrophoresis and Immunoblotting. SDS-PAGE was performed under reducing conditions according to Laemmli (25). Proteins were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) using a semi-dry blotting apparatus (Bio-Rad Laboratories, Richmond, CA). Immunoblotting with human serum, diluted from 1:100 to 1:1,000, was performed as previously described (8) using ^{125}I-labeled protein A (DuPont-NEN, Boston, MA) to detect the primary antibody. To obtain autoradiograms, immunoblots were exposed to XAR film (Eastman Kodak Co., Rochester, NY or Jersey Lab and Glove Supply, Livingston, NJ) at −80°C with intensifying screens for 14–48 h.

Sequence Analysis. Protein homology searches were performed using the computer facilities of the Advanced Scientific Computing Laboratory, National Cancer Institute Supercomputer Center (Frederick, MD) and version 7.B of the GCG sequence analysis software package (Genetics Computer Group, Inc., Madison, WI) (26).

Chemical Reagents. Routine chemical reagents were obtained from either Sigma Chemical Company (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA). Restriction enzymes and DNA ligase were from New England Biolabs (Beverly, MA).

Results

Immunoblotting of GST fusion proteins (Fig. 1 *a*) was performed to determine which domains of gp210 were recognized by antibodies in 25 serum samples from patients with PBC. Antibodies from all 25 patients recognized a fusion protein (Fig. 1, no. 6) that contained the 58 amino acids comprising the carboxy-terminal tail domain of gp210. None of the patients had autoantibodies that recognized the amino-terminal domain of gp210 (Fig. 1, nos. 1–5). The serum
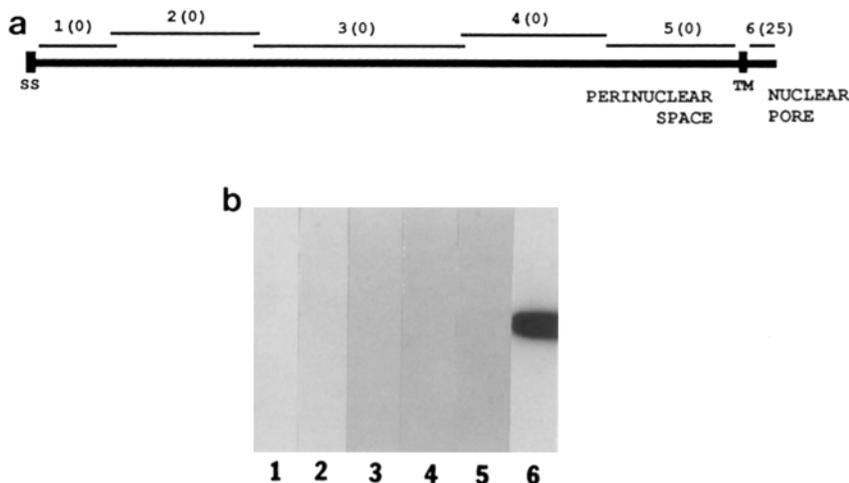
Figure 1. Autoantibodies from patients with PBC recognize the 58-amino acid cytoplasmic tail of nuclear pore membrane glycoprotein gp210. (*a*) Diagram of nuclear pore glycoprotein gp210 and regions expressed as GST fusion proteins. The cleaved amino-terminal signal sequence (*SS*) and single transmembrane domain (*TM*) are indicated, as are the regions that are located in the perinuclear space and that face the nuclear pore. Regions labeled 1 through 6 were expressed as GST-fusion proteins. The number of patients out of 25 with antibodies that recognized the respective fusion proteins is given in parentheses. Fusion proteins 1–6 contained the regions of gp210 between the amino acids indicated according to the numbering of Wozniak et al. (reference 16): Fusion protein 1, 26–222; fusion protein 2, 205–574; fusion protein 3, 561–1113; fusion protein 4, 1101–1481; fusion protein 5, 1461–1808; fusion protein 6, 1829–1886. The hydrophobic cleaved amino terminal signal sequence and transmembrane domain were not included in any of the fusion proteins. (*b*) Autoradiogram of a representative immunoblot showing autoantibody reactivity with fusion protein 6 and lack of reactivity with fusion proteins 1–5. This pattern was seen with serum samples from all 25 patients.

samples were not examined for antibodies against the transmembrane segment, which is integrated in the lipid bilayer, or the signal sequence, which is cleaved from mature gp210.

To identify a smaller epitope(s) within the carboxy-terminal tail domain of gp210, additional overlapping fusion proteins were expressed (Fig. 2 a). Antibodies from all 25 patients recognized fusion protein 11 (Fig. 2) but did not recognize fusion protein 7 (Fig. 2), demonstrating that the epitope(s) is contained within the last 34 amino acids of gp210. All of the samples contained antibodies that also recognized fusion protein 12 (Fig. 2), showing that the epitope(s) was contained within the last 18 amino acids of the carboxy-terminal domain. Only two samples contained antibodies that reacted with fusion protein 8 (immunoblots not shown) that overlapped with the amino-terminal side of fusion protein 12 (Fig. 2 a). From immunoblotting of fusion proteins 8, 9, 10, and 12, the epitope(s) recognized by all of the autoantibodies was shown to be present within a 15-amino acid stretch of sequence asp-arg-lys-ala-ser-pro-pro-ser-gly-leu-trp-ser-pro-ala-tyr. The first 9 amino acids of this sequence were sufficient for recognition by antibodies from 2 of 25 patients and the first 13 were sufficient for recognition by 23 samples. Although the fusion proteins contained portions of rat gp210, major

a

E. coli mutY product: QRPPSGLWGGLYCF
                                     || |||| |
HTVCAPRELASPLALTPHASPQHSPHYLASSPTAFNTLPS DRKASPPSGLWSPAYASH
       7 (0)

8 (2)

9 (23)

10 (25)

11 (25)

12 (25)

b

7 8 9 10 11 12

Figure 2. Autoantibodies from patients with PBC recognize a stretch of 15 amino acids in the cytoplasmic tail of gp210 that is homologous to a sequence present in an *E. coli* protein. (*a*) Amino acid sequence of the cytoplasmic tail of gp210 and the regions labeled 7 through 12 that were expressed as GST-fusion proteins are shown. The number of patients out of 25 with antibodies that recognized the fusion proteins containing regions 7–12 is given in parentheses. The underlined amino acids are the smallest stretch recognized by all 25 antibodies. Shown at the top right is a stretch of amino acids found in the protein product of the *E. coli mutY* gene and its homology with the region of gp210 recognized by the autoantibodies. Abbreviations for amino acids are: H, histidine; T, threonine; V, valine; C, cysteine; A, alanine; P, proline; R, arginine; E, glutamic acid; L, leucine; S, serine; Q, glutamine; Y, tyrosine; F, phenylalanine; N, asparagine; D, aspartic acid; K, lysine; G, glycine; W, tryptophan. (*b*) Autoradiogram of a representative immunoblot with one patient's serum showing autoantibody reactivity with fusion proteins 9, 10, 11, and 12 and lack of reactivity with fusion proteins 7 and 8. This pattern was observed for 21 of the 25 patients. Antibodies from two patients additionally recognized fusion protein 8. Antibodies from two others recognized fusion proteins 10, 11, and 12 but not fusion protein 9.

sequence differences in this region between rats and humans were unlikely as the autoantibodies reacted with nuclear pores when human cells were examined by immunofluorescence microscopy (data not shown).

Search of the translated GenBank showed that the 15-amino acid sequence recognized by all of the autoantibodies was homologous to a portion of the protein products of the *E. coli mutY* gene (Fig. 2) and the *Salmonella typhimurium mutB* gene (46.7% identity between each of them and the gp210 sequence). A stretch of six consecutive amino acids was identical in these three proteins (Fig. 2). Several other proteins were found that had sequence identities of 46.7% over a 15-amino acid stretch and two of these, the product of the human *c-myc* oncogene and human pro-galanin, had six different consecutive identical amino acids. One protein, carp urotensin II-α, had the same six consecutive amino acids common to gp210 and the bacterial polypeptides but only 40% overall identity over the 15-amino acid stretch.

Discussion

PBC is characterized by nonsuppurative inflammatory destruction of intrahepatic bile ducts that leads to cirrhosis (27, 28). Autoantibodies against the E2 subunits of mitochondrial pyruvate dehydrogenase and related enzyme complexes are found in about 90% of patients (19, 20, 29–32). Antibodies to nuclear proteins including histones (33), Sp-100 (34), nuclear lamins (4), the lamin B receptor (8), gp210 (4, 11–14), and others are also commonly found. Because of the presence of autoantibodies in almost all cases, PBC has been called a paradigmatic autoimmune disease (35). However, a paradox presented by PBC is how proteins that are present in all cells, and the autoantibodies that recognize them, relate to the pathological process of bile duct destruction (35).

Analysis of the epitopes of the intracellular protein antigens in PBC could provide insight into how specific autoantibodies arise and how autoimmunity underlies pathogenesis. In the present study, we have shown that autoantibodies from patients with PBC recognize a restricted region within the carboxy-terminal domain of nuclear pore membrane glycoprotein gp210. Previous work has demonstrated that this domain is located in the cytoplasm and faces the nuclear pore complex (16, 17). Although the fusion proteins used in this study may not display nonlinear or posttranslationally modified epitopes in the noncytoplasmic domain of gp210 with which autoantibodies may react in vivo, all of the serum samples examined nonetheless contained autoantibodies that recognized a restricted linear region of only 15 amino acids in the cytoplasmic tail. This differs from that seen with autoantibodies against lamin B, another nuclear envelope protein, where autoantibodies from 10 different patients with systemic lupus erythematosus reacted with at least five distinct linear epitopes along the length of the molecule (36).

Some investigators (37, 38) have proposed that molecular mimicry of bacterial antigens is responsible for antibody generation and pathogenesis in PBC. In molecular mimicry, an immune response is induced by a specific antigenic determinant of an infecting agent and this response is subsequently directed
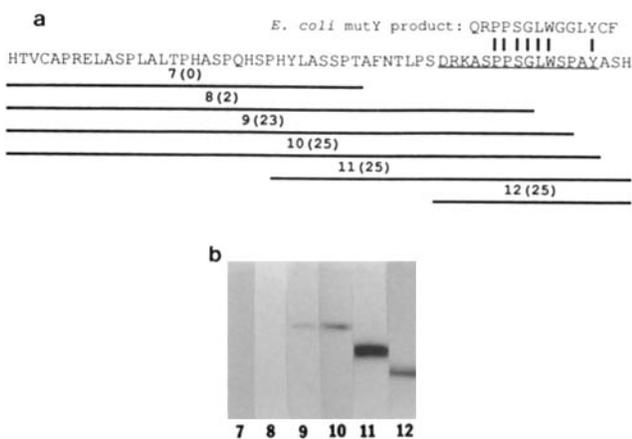
against an epitope on a host polypeptide leading to autoimmunity and possibly disease (38, 39). Molecular mimicry of a peptide sequence in myelin basic protein by a viral polypeptide determinant can cause an immune response leading to nervous system injury (40). Other examples of sequence similarities between microbial and host proteins in which disease may be caused by molecular mimicry include similarities between the sequences of adenovirus-12 E1B protein and A-gliadin (41) and *Klebsiella pneumonia* nitrogenase and HLA-B27 (42). Molecular mimicry, in the former case, may underlie the pathogenesis of celiac disease and, in the later case, ankylosing spondylitis and Reiter's syndrome.

Molecular mimicry has been postulated to be responsible for the generation of antimitochondrial antibodies in PBC because the autoepitope(s) of the E2 subunits of the mammalian mitochondrial enzymes (19, 20, 31) are homologous to the E2 subunits of the *E. coli* pyruvate dehydrogenase complex. Along these lines, patients with PBC have an increased incidence of *E. coli* urinary tract infections (43, 44). It has also been shown that HLA-DR-α, which is abnormally expressed on the surface of bile duct epithelial cells in PBC (45), shares homology with the E2 epitope(s), possibly relating the autoantibodies to the pathological injury (37, 38). Like the epitopes of the mitochondrial antigens recognized by autoantibodies from patients with PBC, the region of gp210 recognized by autoantibodies also shares sequence homology with an *E. coli* protein, the adenine glycosylase encoded by the *mutY* gene (46, 47). The homologous sequence is also present in the product of the similar *S. typhimurium mutB* gene (48). Although the identification of sequence similarity between the gp210 autoepitope(s) and bacterial gene products may be fortuitous, these results provide additional evidence that supports the hypothesis that molecular mimicry is responsible for the generation of autoantibodies in patients with PBC. Homologous bile duct cell surface antigens could hypothetically link this immune response to the patholog-

ical process in PBC, but candidate proteins have not yet been identified.

Another possibility that could result in the generation of autoantibodies against gp210 in patients with PBC is that polypeptides derived from the protein's cytoplasmic tail are abnormally expressed on the plasma membranes of certain cells. It is possible that the cytoplasmic tail of gp210 is degraded into peptides that are transported to the cell surface and then recognized as non-self. One example of the cell surface expression of peptides that arise from the degradation of proteins located in the nucleus or cytoplasm are those presented by MHC class I (49). These proteolytic peptides are transported into the ER lumen (49), and once in the ER lumen, the peptides, which are from 9 to 11 amino acids, bind to MHC class I proteins and are ultimately expressed on the plasma membrane (49). Although speculative, it is possible that a similar mechanism could be responsible for abnormal cell surface expression of gp210 peptides. If this were the case, our data would suggest that regions of a transmembrane protein residing in the ER lumen (perinuclear space) are protected from the proteolysis that generates such peptides.

In summary, autoantibodies against nuclear pore membrane glycoprotein gp210 in patients with PBC recognize a restricted region in the cytoplasmic tail. The autoepitope(s) is contained within a 15-amino acid stretch homologous to a portion of the *E. coli mutY* gene product, suggesting that gp210 autoantibodies could arise by molecular mimicry of a bacterial antigenic determinant. Alternatively, such antibodies may be generated because of abnormal expression of this epitope(s) on the cell surface in patients with PBC. Regardless of their mechanism of generation, anti-gp210 antibodies can be detected using small polypeptides. This will permit the development of clinically useful assays to detect gp210 autoantibodies that are highly specific for a diagnosis of PBC and present in up to 25% of these patients.

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