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Characterization of the Antibodies to p62 Nucleoporin in Primary Biliary Cirrhosis Using Human Recombinant Antigen

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Abstract Reactivity of sera from patients with primary biliary cirrhosis (PBC) with a 60 kDa component of nuclear pore complexes (NPCs), purified by affinity chromatography on wheat-germ agglutinin (WGA)-Sepharose, was previously detected. Recently, clinical significance of the anti-NPC antibodies in PBC became evident. In the light of recent reports, indicating the correlation of the anti-NPC antibodies with severity and progression of the disease, the characterization of the reactive antigens is becoming essential in the clinical management of patients with PBC. Since accurate autoantibody detection represents one of the fundamental requirements for a reliable testing, we have generated a human recombinant p62 protein and validated an immunoprecipitation assay for the detection of anti-p62. We also demonstrated that the generated human recombinant p62 nucleoporin was modified by N-acetylglucosamine residues. More than 50% of tested PBC sera precipitated ^{35}S-radioactively labeled p62 recombinant nucleoporin and 40% recognized this recombinant antigen by immunoblotting. We compared the reactivity of PBC sera with rat and human nucleoporin. The incidence of anti-p62 nucleoporin positive PBC sera increased by 15% when human recombinant antigen was used. The titer of autoantibodies in p62-positive PBC samples strongly varied. Preadsorption of the PBC sera with p62 recombinant protein completely abolished their reactivity with the antigen. In conclusion, this study unequivocally proves that autoantibodies reacting with the 60 kDa component of NPCs target p62 nucleoporin and, more importantly, provide a better antigen source for future evaluations of the clinical role of anti-p62 in PBC. J. Cell. Biochem. 104: 27–37, 2008.

Key words: ANA; autoimmunity; nuclear pore complexes; p62 nucleoporin; gp210 glycoprotein; nuclear envelope; human recombinant p62 nucleoporin

Patients with primary biliary cirrhosis (PBC) [Talwalkar and Lindor, 2003] generate a variety of autoantibodies, which are primarily directed against mitochondrial antigens [Walker et al., 1965; Van der Water et al., 1989]. However, in about 50% of patients there are also antibodies directed against nuclear components [ANA, anti-nuclear antibodies; for review, see Reference Invernizzi et al., 2005]. A number of nuclear structures have been recognized as specific targets of ANA in PBC. These include Sp100 and promyelocytic leukemia proteins, major components of nuclear bodies, which generate a nuclear dot pattern in indirect immunofluorescence (IIF) and constituents of the nuclear pore complexes (NPCs) that have been specifically associated with a perinuclear

Abbreviations used: AMA, anti-mitochondrial antibodies; ANA, anti-nuclear antibodies; ELISA, enzyme-linked immunosorbent assay; GlcNAc, N-acetylglucosamine; kDa; kilodalton; LBR, lamin binding receptor; NPC, nuclear pore complex; PBC, primary biliary cirrhosis; Sf9, *Spodoptera frugiperda*; Tpr, translocated promoter region; WGA, wheat-germ agglutinin.

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staining pattern [Lozano et al., 1988; Lassoued et al., 1990; Courvalin et al., 1990a; Wesierska-Gadek et al., 1995, 1996a,b; Miyachi et al., 1996, 2003; Invernizzi et al., 2005]. PBC-specific ANAs are associated with more severe disease and seem to have a prognostic role in the disease [Invernizzi et al., 2001, 2004; Wesierska-Gadek et al., 2006; Nakamura et al., 2007].

The frequency of the PBC sera staining the nuclear periphery strongly differs between reports and is in the range from 29% to 53% [Lozano et al., 1988; Lassoued et al., 1990; Courvalin et al., 1990a; Wesierska-Gadek et al., 1995, 1996a,b, 2006; Miyachi et al., 1996, 2003; Invernizzi et al., 2001, 2005]. The observed divergence is not surprising, considering the fact that in previous studies antigens isolated from different tissues (cervical carcinoma cells [Wesierska-Gadek et al., 1996a,b] vs. liver [Miyachi et al., 1996]) originating from different species (human [Wesierska-Gadek et al., 1996a,b] vs. rat [Miyachi et al., 1996]) were used for testing. A number of studies revealed that two major components of the NPC are targeted by PBC sera [Courvalin et al., 1990a; Lassoued et al., 1990; Wesierska-Gadek et al., 1995, 1996a,b; Miyachi et al., 1996]. First, gp210 protein, a mannose-substituted integral component of NPCs, was identified as a major target [Courvalin et al., 1990a; Lassoued et al., 1990; Wesierska-Gadek et al., 1995]. A few years later the reactivity of PBC sera with a 60 kDa component of NPCs has been reported by our group [Wesierska-Gadek et al., 1996a] and then confirmed by other laboratories [Miyachi et al., 1996, 2003]. The fact that the PBC sera also reacted with a 60 kDa constituent of human NPCs purified by affinity chromatography on wheat-germ agglutinin (WGA)-Sepharose indicated that the p62 nucleoporin is the reactive autoantigen [Wesierska-Gadek et al., 1996a].

To unequivocally prove the identity of the reactive antigen, we generated and characterized human recombinant p62 protein baculovirally expressed in insect cells. Moreover, we additionally used rat recombinant antigen and compared by immunoprecipitation the reactivity of PBC sera with human and rat ^{35}S-methionine labeled p62 nucleoporin. In the attempt to validate a novel detection method and to better define the anti-p62 reactivity of patients with PBC, we also developed an immunoprecipitation (IP) assay using this new recombinant protein.

Approximately half of PBC sera strongly reacted with human p62 recombinant nucleoporin and preadsorption experiments with His-tagged p62 recombinant protein completely abolished their reactivity with the antigen. These results unequivocally evidence that the p62 nucleoporin is the target for the previously described autoantibodies recognizing the 60 kDa component of NPC.

MATERIALS AND METHODS

Antibodies

Monoclonal anti-nucleoporin p62 antibodies (clone 53) from Transduction Laboratories (Lexington, KY) and anti-polyhistidine (clone HIS-1) from Sigma–Aldrich Co. (St. Louis, MO) were used.

Plasmids

We used following plasmids encoding rat p62 nucleoporin (pETp62) and encoding rat gp210.

Human Sera

Twenty non-selected sera of patients diagnosed with PBC were collected. The diagnosis of PBC was based on internationally accepted criteria [Kaplan and Gershwin, 2005]. Patients with serum positivity for hepatitis B surface antigen (HbsAg) and hepatitis C virus antibodies (anti-HCV) were not included in the present study. Additionally, 10 sera from normal healthy blood donors and from patients with diagnosed autoimmune hepatitis were used as controls.

Indirect Immunofluorescence for Serum Autoantibody Analysis

Serum anti-mitochondrial antibodies (AMA), ANA, and anti-smooth muscle antibodies (SMA) were determined blindly by an unbiased observer by IIF as previously described [Invernizzi et al., 2001; Wesierska-Gadek et al., 2006]. Briefly, sera were tested for the presence of AMA on rodent tissue preparations (rat kidney sections, normal $< 1:40$) and ANA on human Hep-2 cells (Kallestad, Houston, TX) and $HeLaS_3$ cells. $HeLaS_3$ cells grown on slides were fixed with ice-cold methanol/acetone-mixture (3:2) for 20 min. After PBS washing and saturation with 5% BSA, specimen were incubated overnight with PBC sera diluted to a final concentration of 1:500.

Thereafter, preparations were washed with PBS and incubated with secondary antibodies coupled to Cy2. Immunostained preparations were inspected under fluorescence microscopy using an FITC filter. Sera were considered positive for ANA at a dilution of $\geq$1:40. A fluorescein-conjugated antibody directed against human immunoglobulins (Ig) A, G, and M (Sigma ImmunoChemicals, St. Louis, MO) diluted to a final concentrations of 1:100 were used as secondary antibody. Positivity and patterns were evaluated using fluorescence microscopy (Orthoplan, Leitz, Wetzlar, Germany).

Cloning of Human p62 Nucleoporin

Human p62 cDNA generated and sequenced from a HeLa cDNA library in Dr. Hurt's lab [Carmo-Fonseca et al., 1991] was cloned into a baculovirus recombination vector (pBlueBacHis2B; Invitrogen Life Technologies, San Diego, CA). A poly-His-tag sequence was introduced in the NH_2-terminus of human recombinant p62 protein to facilitate its one-step purification by Ni-affinity chromatography. To ensure that newly generated human recombinant p62 protein possesses a proper primary structure, the p62 nucleporin was sequenced.

Transfection of Insect Cells

For infection, medium containing the virus harboring the pBlueBacHis2B vector with a p62 insert was used. After 3 days the baculovirus started to lyse the *Spodoptera frugiperda* (Sf9) insect cells and 1 week after transfection the supernatant including the newly formed virus particles could be saved for subsequent infection of cells [Wesierska-Gadek et al., 2003, 2005]. The optimal time for maximal expression of the recombinant p62 protein was determined. For selection, serial dilutions of the virus suspension (e.g., 1,000–100,000-fold) were put into petri dishes (10 cm diameter) containing 10^7 logarithmically growing Sf9 cells. After 1 h the medium was removed and the cells were covered with 6 ml of low melting agarose containing X-Gal. Starting 3 days later, the petri dishes were inspected for the appearance of blue plaques, generated by X-Gal metabolized by the protein expressed by the reconstituted *lacZ* gene. Blue plaques containing the recombinant virus were cut out and put into insect cell medium to elute the virus particles. This was then used for subsequent infections of Sf9 insect cells. The human p62 protein was in vivo labeled after exposure of insect cells for 15 h in methionine-deprived medium supplemented with ^{35}S-methionine.

In Vitro Transcription/Translation Reaction

To generate ^{35}S-labeled p62 and gp210 recombinant proteins, an in vitro transcription/translation reaction was performed using the rabbit reticulocyte Kit (Promega Corporation, Madison, WI), a cDNA coding for gp210 and p62 as well as ^{35}S-labeled methionine (New England Nuclear, Perkin-Elmer, Waltham, MA) [Wesierska-Gadek et al., 2007b]. The identity of the radioactively labeled recombinant p62 proteins was checked by immunoprecipitation using monoclonal anti-p62 antibodies (Transduction Laboratories) and by sequential analysis of the immune complexes.

Immunoprecipitation

^{35}S-labeled recombinant p62 protein was used for immuno-precipitation using human sera. For control, monoclonal anti-p62 antibodies (clone 53) from Transduction Laboratories were used. Immunoprecipitation was performed according to the previously described stringent protocol [Wesierska-Gadek et al., 1995, 1996a, 2007a]. The immune complexes were isolated by affinity chromatography on Protein G-Sepharose beads (Amersham Biosciences, Little Chalfont, UK). Protein-G-Sepharose bound immune complexes were eluted twice with 40 μl of non-reduced SDS sample buffer and analyzed on 10% SDS-gels. Proteins separated on gels were transferred electrophoretically onto the membranes. The amount of precipitated ^{35}S-labeled p62 protein was determined by liquid scintillation counting of the aliquots (3 μl) of both eluates and additionally by autoradiography after exposure of the dried membranes to BMR film for the appropriate time (Eastman Kodak Co., Rochester, NY). As a control, ^{35}S-labeled unrelated human recombinant proteins (e.g., p53 protein) were used for immunoprecipitation.

Preparation of Cell Extracts

PBS-washed cells were lysed in RIPA buffer [50 mM Tris–HCl (pH 7.4), 500 mM NaCl, 1% Nonidet-P40, 0.5% Na-deoxycholate, 0.1% SDS, 0.05% NaN_3, 1 mM phenylmethylsulfonylfluoride (PMSF)] for 20 min at +4°C [Wesierska-Gadek et al., 2005, 2007b]. For affinity purification of baculovirally expressed

proteins, insect cells were lysed directly in native binding buffer provided in the X-press purification Kit (Invitrogen Life Technologies), and cell structures were destroyed by repeated freeze/thaw procedure and sonication [Wesierska-Gadek et al., 2003, 2005]. The cell suspension was spun off. Clear supernatant was used for further analysis. The protein concentration of cell extracts was determined by the DC assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as standard.

Purification of His-Tagged Recombinant p62 Protein by Affinity Chromatography on Ni-Agarose

Cell lysates prepared in native binding buffer (pH 7.8) were loaded on pre-equilibrated Ni-agarose beads ProBond (Invitrogen Life Technologies) and purification of p62 recombinant protein was performed according to the manufacturer's protocol [Wesierska-Gadek et al., 2003, 2005]. Recombinant p62 protein was eluted from the Ni-beads with the native elution buffer at pH 4.0 and immediately neutralized.

Isolation of Nuclear Pore Complexes (NPCs) From Human $HeLaS_3$ Cells

The nuclear pore complex-lamina fraction (NPC-LF) was isolated from cultivated human $HeLaS_3$ cells according to the method of Dwyer and Blobel [1976]. Cells arrested in a stationary phase were used for isolation of nuclei. Their cell cycle status was determined by measurement of the propidium iodide stained cells in FACS. $HeLaS_3$ cells were harvested and washed in PBS as previously described in detail [Wesierska-Gadek et al., 1995, 1996a]. To avoid a proteolytic degradation of antigens during all isolation steps PMSF and Pefabloc were added to a final concentration of 1 mM and 50 μM, respectively. Briefly, PBS washed cells were suspended in ice-cold low salt buffer (RSB buffer), swollen and after stepwise addition of detergents (NP-40 and freshly prepared sodium deoxycholate) they were homogenized. After centrifugation through sucrose cushion nuclei were pelleted. Then isolated nuclei were digested with DNase I and then chromatin was extracted sequentially with the non-ionic detergent Triton X-100 and high salt buffer. The remaining insoluble structure pelleted after the last centrifugation step represents the NPC-LF.

Electrophoretic Separation of Proteins and Immunoblotting

Proteins of the NPC-LF fraction and proteins isolated by affinity chromatography dissolved in SDS-sample buffer were separated by SDS–PAGE on 10% slab gels and then they were electrophoretically transferred onto a PVDF membrane (Amersham International, Little Chalfont, UK) [Wesierska-Gadek et al., 1995, 1996a, 2007a,b]. The efficiency of the transfer as well as protein loading of proteins was confirmed by Ponceau S staining. Blots were incubated with human sera or with anti-p62 specific antibodies (clone 53) and the immune complexes were detected autoradiographically using appropriate peroxidase-conjugated secondary antibodies and the enhanced chemiluminescent detection reagent ECL+ (Amersham International). The radioactively labeled proteins were detected by autoradiography after exposure of the blots to BMR film (Eastman Kodak Co.).

Preadsorption Experiments

To examine the specificity of anti-p62 antibodies, an excess of cold p62 recombinant protein (free or immobilized on Ni-beads) was incubated with human PBC sera prior to the addition of ^{35}S-recombinant p62 protein or prior to the immunoblotting. After incubation for 1 h at RT, the immune complexes as well as free recombinant p62 protein were removed by Ni-agarose. The supernatant samples representing preadsorbed sera were used for immunoprecipitation and for immunoblotting. The effect of the excess of cold recombinant p62 on the amount of precipitated ^{35}S-p62 protein was evaluated [Wesierska-Gadek et al., 1995, 1996a, 2006; Invernizzi et al., 2001].

RESULTS

PBC Sera React Primarily With Two Components of NPC

Incubation of human PBC sera with HeLa preparations generated different staining pattern (Table I). Some PBC sera displayed homogenous nuclear (Fig. 1A) or nuclear dot (not shown) staining pattern by means of IIF. Moreover, four PBC sera stained the nuclear periphery of $HeLaS_3$ cells indicating that the antibody in PBC sera targets the components of the nuclear envelope (Fig. 1A). As depicted in

TABLE I. Reactivity of Human Sera With The Recombinant p62 Nucleoporin

		IIF		WB			IP		
				NPCs					
Patients	No. of sera	AMA	ANA	p200	p60	Human p62	Rat gp210	Rat p62	Human p62
PBC	20	19	7	5	7	8	2	11	14
AIH	10	0	n.d.	n.d.	n.d.	0	0	0	0
Healthy controls	10	0	0	0	0	0	0	0	0

n.d., not determined.

Figure 1A, two different PBC sera generated a rim-like staining pattern. Unfortunately, most PBC sera are heterogeneous and have antibodies with different specificities, for example, AMAs. For these reasons, overlapping staining patterns may occur and it is difficult to exactly distinguish which subcellular structures are stained by the reactive autoantibodies [Invernizzi et al., 2005]. Testing of PBC sera with proteins of isolated NPCs revealed a strong reactivity with two major autoantigens: with a protein at 200 kDa and with a protein at approximately 60 kDa (Fig. 1B, Table I). Some PBC sera additionally reacted with a few minor bands. However, the intensity of the minor bands was weak and it cannot be excluded that

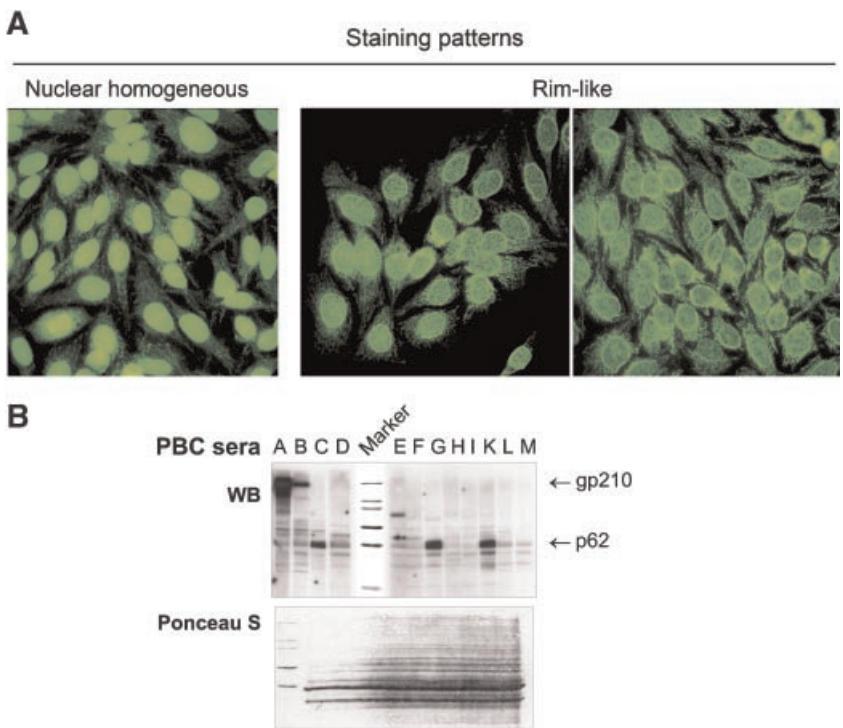

Fig. 1. Reactivity of PBC sera with NPCs. **A**: Immunostaining of $HeLaS_3$ cells by ANA-positive PBC sera. Fixed preparations of $HeLaS_3$ cells were incubated overnight with PBC sera diluted to a final concentration of 1:500. Thereafter, they were washed with PBS and incubated with secondary antibodies coupled to Cy2. Immunostained preparations were inspected under fluorescence microscopy using a FITC filter. The reactivity of a serum generating homogeneous nuclear staining (serum H) and two sera displaying a rim-like nuclear pattern (serum A and G) was shown. **B**: Detection of reactivity of PBC sera with proteins of NPCs using Western blotting. Proteins of isolated NPCs were resolved on 8% SDS-gel and electrophoretically transferred onto PVDF membrane. For control of equal protein loading and of proper electrotransfer, proteins immobilized on the membrane were detected by Ponceau S staining (**lower panel**). Blot strips were incubated with distinct PBC sera (A–D and E–M) at a final dilution of 1:500. After incubation with secondary antibodies linked to HRP, immune complexes were detected by chemoluminescence using ECL+ reagent. Marker proteins were loaded between **lanes D** and **E**. Arrows indicate the position of the major autoantigens. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the observed weak reactivity is attributable to unspecific cross-reactivity. Serum E was reactive with a protein at about 70 kDa, a position characteristic for lamin A.

The 200 kDa component of NPCs reacting with PBC sera (Fig. 1B, lane A,B) was identified a couple of years ago. The glycoprotein gp210, an integral component of NPCs was evidenced as the PBC-specific autoantigen. Unlike the 200 kDa protein, the 60 kDa antigen recognized by a number of PBC patients shares some features, for example, modification by N-acetylglucosamine (GlcNAc) residues with other related constituents of NPC and its identity assumed so far by different biochemical approaches has to be proved by the reactivity with the recombinant antigen.

Characterization of the Human Recombinant p62 Protein

In the first step we verified the overexpression of poly-His tagged human p62 recombinant protein. Proteins solubilized during lysis of control and p62 baculovirus infected Sf9 insect cells were separated on SDS slab gels. Proteins were Coomassie blue stained or were immobilized on a membrane. The comparison of the staining pattern revealed the presence of a prominent protein band at approximately 60 kDa solely in samples obtained from insect cells infected with baculovirus encoding p62, but not from cell lysate obtained from control cells (Fig. 2A, left panel). The identity of the overexpressed recombinant human protein was examined by immunoblotting using specific monoclonal anti-p62 nucleoporin antibodies and antibodies directed against the poly-His-tag sequence. As shown in Figure 2A (right panel), monoclonal anti-p62 reacted strongly with a 62 kDa protein only in a sample obtained from Sf9 cells infected with baculovirus. Antibodies targeting poly-His sequence generated a similar staining pattern (not shown). Monoclonal anti-p62 antibodies additionally stained the nucleoporin band in the nuclei sample isolated from human $HeLaS_3$ cells (Fig. 2A, right panel). Moreover, the human recombinant His-tagged p62 was purified by one-step Ni-affinity chromatography under native conditions. Sf9 cell extract loaded on Ni-Sepharose at slightly basic pH was eluted from the beads at acidic pH and immediately neutralized (not shown). Finally, the glycosylation status of the human recombinant p62 nucleoporin was proved by two independent methods, that is, WGA-affinity chromatography (Fig. 2B) and overlay with WGA-lectin (not shown); both methods evidenced that human recombinant p62 nucleoporin generated in insect cells was glycosylated by GlcNAc residues. Considering the fact that under physiological conditions p62 nucleoporin is modified by GlcNAc residues, the modification could be of importance for its reactivity.

Characterization of the Recombinant Rat p62 Protein Generated by In Vitro Transcription/Translation Reaction

To assess the glycosylation status of the recombinant rat p62 nucleoporin generated by in vitro transcription/translation reaction (Fig. 2C, upper panel), ^{35}S-labeled protein was purified on WGA-affinity chromatography. The analysis of the fractions stepwise eluted from WGA-Sepharose (eluates 1–4) (Fig. 2C, lower panel) revealed that also recombinant antigen in vitro synthesized in the rabbit reticulocyte system was post-translationally modified.

PBC Sera Precipitate ^{35}S-Labeled p62 Recombinant Protein

In the next step we performed immunoprecipitation assays using ^{35}S-labeled p62 recombinant protein. We used rat p62 protein radioactively labeled during an in vitro transcription/translation reaction and human p62 protein labeled in vivo in Sf9 cells. We tested 20 non-selected PBC sera. As shown in Figure 3 and Table I, more than 50% of non-selected PBC sera strongly precipitated ^{35}S-labeled rat p62 nucleoporin. Noteworthy, a few sera that did not recognize rat p62 protein precipitated human antigen (Table I). For control, ^{35}S-labeled p62 nucleoporin was precipitated by monoclonal anti-p62 antibodies (Fig. 3). The amount of ^{35}S-labeled p62 protein measured in both eluates was summed up and compared with the amount of radioactivity labeled protein precipitated by monoclonal anti-p62 antibodies. Moreover, to exclude the possibility that the p62 seroreactivity was overestimated by immunoprecipitation, rat gp210 protein ^{35}S-methionine labeled with comparable specific activity was used in parallel assays. Only 2 out of 20 PBC sera precipitated recombinant gp210 protein (Table I) [Wesierska-Gadek et al., 2007a]. These data are in concordance with our recent observations [Wesierska-Gadek et al., 2007a]. Finally, the

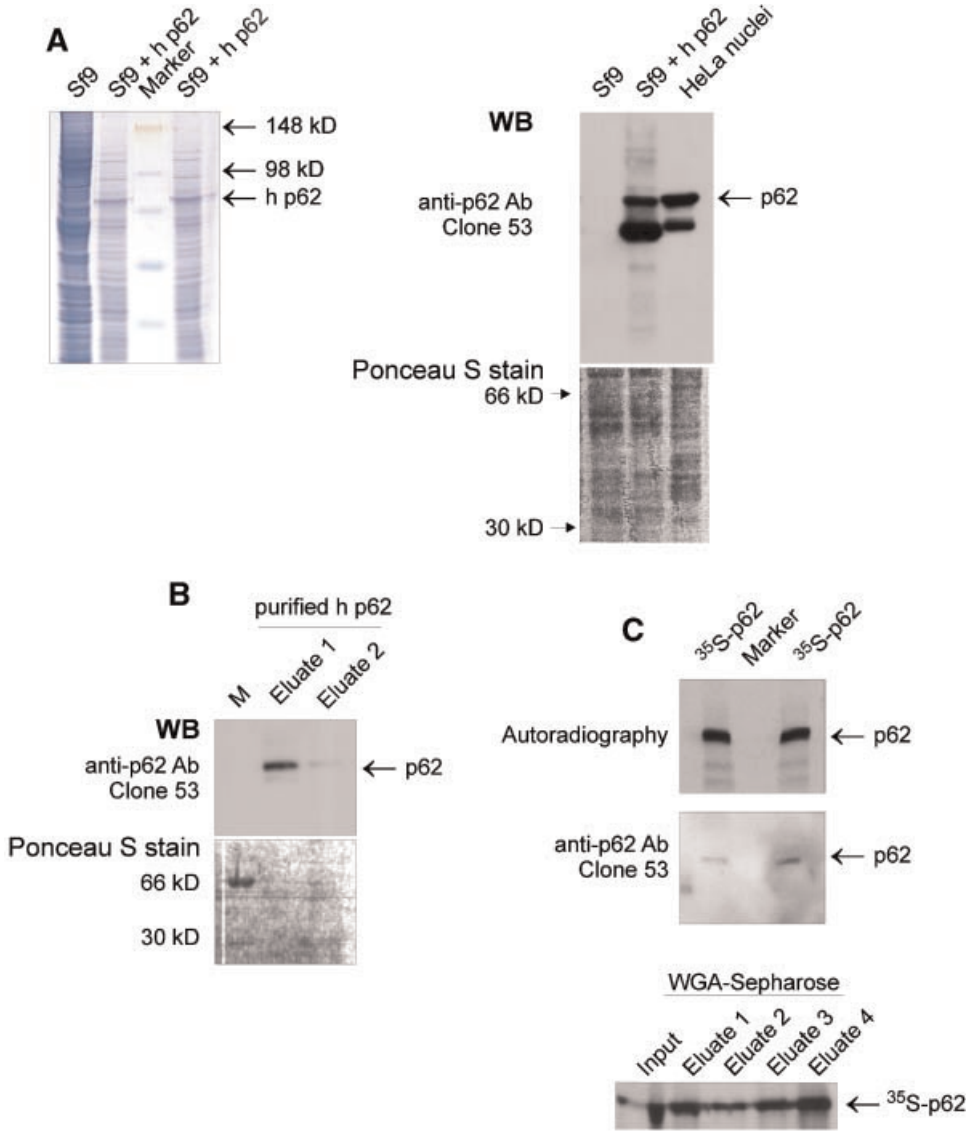

Fig. 2. Characterization of human recombinant p62 protein. **A**: Cell lysates obtained from control Sf9 cells and cells infected with a baculovirus encoding human p62 nucleoporin (Sf9 + h p62) were separated on 10% SDS slab gels. One gel was Coomassie blue stained (**left panel**) and the second was blotted onto the PVDF membrane (**right panel**). p62 protein was detected by immunoblotting using anti-p62 monoclonal antibody (clone 53) at a final dilution of 1:2,000. Position of molecular weight protein markers (M) is indicated by arrows. Sample of nuclei proteins isolated from $HeLaS_3$ cells was additionally loaded. To avoid overstaining, lysate from Sf9 cells expressing lower h p62 levels was loaded for immunoblotting. **B**: Human recombinant p62 nucleoporin was affinity purified on WGA-Sepharose. p62 nucleoporin bound to WGA-Sepharose was eluted with puffer containing N-acetylglucosamine. The majority of the recombinant protein was detected by immunoblotting in the first eluate. **C**: ^{35}S-labeled rat p62 protein generated by in vitro transcription/translation reaction was precipitated by a monoclonal anti-p62 (**upper panel**) or purified by affinity chromatography on WGA-Sepharose (**lower panel**) antibody and analyzed on 10% SDS-gels. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

human recombinant ^{35}S-methionine labeled p62 nucleoporin that was precipitated by PBC sera (Fig. 3C, upper panel) was additionally detected by immunoblotting using anti-p62-antibodies (Fig. 3C, lower panel) and anti-His antibodies (not shown). As depicted in Figure 3C, p62 nucleoporin positive signals were very strong thereby evidencing that distinct PBC sera precipitated high amounts of the recombinant antigen. Interstingly, two PBC sera (serum No. 4 and 13) that precipitated rat p62 displayed very weak, if any, reactivity with the ^{35}S-labeled human p62 nucleoporin and were evaluated as human p62 negative.

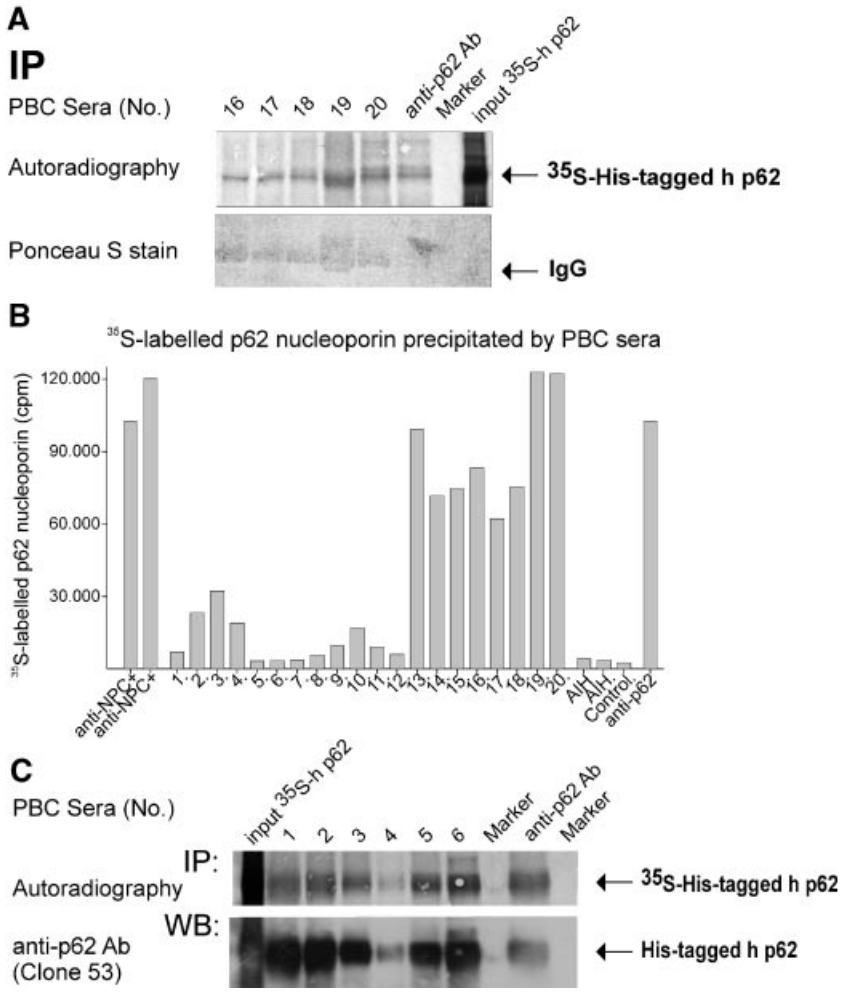

Fig. 3. PBC sera precipitate ^{35}S-labeled recombinant p62 protein. **A**: Analysis of the precipitated ^{35}S-labeled human p62 protein by autoradiography. An aliquot of the sample used for immunoprecipitation (input) was loaded. **B**: Semi-quantitative evaluation of the amount of precipitated ^{35}S-labeled rat p62 protein. The amount of precipitated ^{35}S-labeled p62 protein was determined by liquid scintillation counting of the aliquots (3 µl) of both eluates. Total activity of the eluate 1 and eluate 2 was summed up. As a positive control ^{35}S-labeled p62 protein precipitated by a monoclonal anti-p62 antibody was included. Two PBC sera previously tested as reactive with the WGA-purified 60 kDa component of NPC (designated anti-NPC+), two AIH sera and one serum from normal healthy control were additionally included. **C**: Human recombinant p62 nucleoporin was expressed and labeled with ^{35}S-methionine in Sf9 cells. Radioactively labeled p62 protein precipitated by human sera or by monoclonal anti-p62 antibody was detected by autoradiography (**upper panel**) and by immunoblotting (**lower panel**).

PBC Sera Recognize Human Recombinant p62 Protein by Immunoblotting

PBC sera reactive with ^{35}S-labeled nucleoporin p62 were further tested by immunoblotting. A lysate of Sf9 cells generating human His-tagged p62 nucleoporin and proteins of NPCs isolated from $HeLaS_3$ cells separated on 10% SDS-slab gels were immobilized on the membrane. As negative control a Sf9 cell lysate obtained from non-infected cells was loaded. As shown in Figure 4, PBC sera reacted with human recombinant p62 protein and reacted with the antigen in the NPC samples isolated from $HeLaS_3$ cells. However, the results of the immunoblotting experiments require an additional explanation. Serum No. 15 that precipitated huge amounts of ^{35}S-labeled p62 nucleoporin, heavily stained the His-tagged p62 band. It also reacted with the samples isolated from $HeLaS_3$ cells. However, to resolve the stained p62 band, this blot was exposed very shortly. Similarly, the blot probed with monoclonal anti-nucleoporin antibodies was also exposed very shortly and the p62 band in the sample of NPCs was not visible. In contrast, serum No. 20 displayed a moderate reactivity with the denaturated

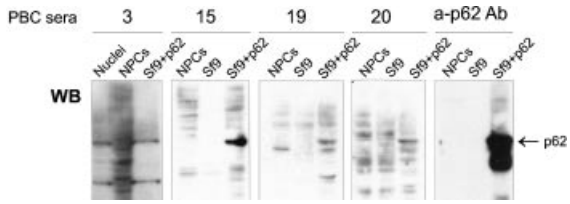

Fig. 4. Reactivity of human PBC sera with p62 nucleoporin. Proteins of nuclei and of NPCs isolated from $HeLaS_3$ cells as well as lysate of control and p62 baculovirus infected Sf9 cells were loaded on 10% SDS-gels and blotted onto membrane. Blots were incubated with distinct PBC sera at a final dilution of 1:500 or with monoclonal antibodies against p62 at a final dilution of 1:2,000. Due to extremely strong reactivity of the monoclonal antibody with the sample of Sf9 cells overexpressing p62, the blot was exposed only 1 s. After the short exposure p62 signal in NPCs was nicely detectable (without a strong background).

antigen. It generated a positive signal with the recombinant p62 protein but not with the sample of NPCs. The frequency of p62 positive PBC sera estimated by Western blotting is shown in Table I. It becomes obvious that much more PBC sera react with the native human recombinant p62 nucleoporin by immunoprecipitation than by immunoblotting.

Preadsorption of p62 Reactive PBC Sera With an Excess of Recombinant p62 Protein Abolishes Their Reactivity

To prove the specificity of the anti-p62 positive PBC sera, preadsorption experiments were performed. As shown in Figure 5, the preadsorption of PBC sera with human recombinant p62 protein abolished their reactivity with ^{35}S-labeled antigen. However, preadsorption with an excess of BSA or human normal serum did not affect the amounts of the precipitated radioactive antigen.

DISCUSSION

Approximately 30% of patients with PBC develop autoantibodies against proteins of the nuclear envelope [Invernizzi et al., 2005]. These sera frequently display a rim-like nuclear staining pattern. These autoantibodies are directed against different nuclear proteins such as lamin B receptor (lamin binding receptor, LBR) [Courvalin et al., 1990b; Lin et al., 1996], nuclear lamins [Wesierska-Gadek et al., 1988], translocated promoter region (Tpr) [Ou et al., 2004], LAP1 and two proteins [Miyachi et al., 2003; Invernizzi et al., 2005] and components of NPCs [Lassoued et al., 1990; Courvalin et al.,

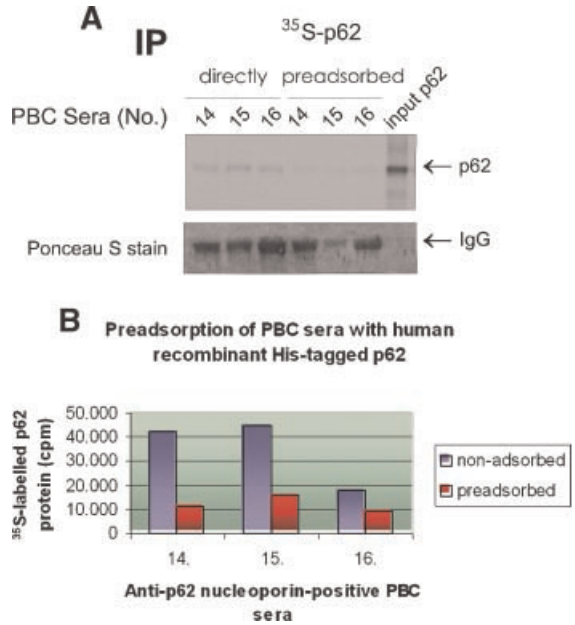

Fig. 5. Preadsorption of PBC sera abolished their reactivity with ^{35}S-labeled p62 recombinant protein. **A**: Detection of ^{35}S-labeled recombinant p62 protein precipitated directly by indicated PBC sera or after sera preadsorption with p62 recombinant protein by autoradiography. ^{35}S-labeled recombinant p62 protein used for immunoprecipitation (designated as input p62) was loaded as a positive control. **B**: Quantification of ^{35}S-labeled recombinant p62 protein precipitated by non- and preadsorbed PBC sera. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

1990a; Wesierska-Gadek et al., 1995, 1996a,b; Miyachi et al., 2003]. The incidence of autoantibodies directed against proteins of the NPC is relatively high and these antibodies seem to be specific for this entity. In particular, the gp210 glycoprotein [Lassoued et al., 1990; Courvalin et al., 1990a; Wesierska-Gadek et al., 1995, 1996b], the integral protein of the nuclear pores [Wozniak et al., 1989; Greber et al., 1990], and a protein of approximately 60 kDa represent the major autoantigens [Wesierska-Gadek et al., 1996a]. The autoantibodies directed against gp210 occur in about 30% of PBC sera [Lassoued et al., 1990; Courvalin et al., 1990a; Wesierska-Gadek et al., 1995, 1996a,b] and the carbohydrate moieties are an essential part of their antigenicity [Wesierska-Gadek et al., 1995, 1996a,b]. The antibodies against the 60 kDa component of NPC were detected in approximately 14–32% of PBC patients. Since the reactive autoantigen at 60 kDa was isolated from human cells by affinity chromatography on WGA, it has been assumed that p62 nucleoporin represents the autoantigen [Wesierska-Gadek

et al., 1996a]. Moreover, by immunoblotting after two-dimensional gel electrophoresis PBC sera reacted with 2–3 spots exhibiting pI values similar to that of p62 nucleoporin [Wesierska-Gadek et al., 1996a].

p62 nucleoporin belongs to a distinct subset of NPC proteins substituted by O-linked GlcNAc [Davis and Blobel, 1987]. This heterogeneous group of proteins, ranging in molecular weight from 45 to 200 kDa is glycosylated at serine and threonine with 10–15 GlcNAc residues per molecule [Davis and Blobel, 1987; Starr and Hanover, 1990; Starr et al., 1990]. The GlcNAc residues covalently linked to nucleoporins play an essential role in the regulation of transport through NPCs. However, it should be stressed that by WGA lectin binding not only the GlcNAc modified p62 was separated from its unmodified counterparts, but also other GlcNAc-linked NPC components of this protein family were isolated. Since nucleoporins possess a similar molecular mass, the testing of PBC sera with a recombinant p62 protein became indispensable. Insect cells and lytic baculoviruses provide a well-established method for high-level expression of functional full-length mammalian proteins. In addition, recombinant proteins expressed in insect cells are post-translationally modified in a similar manner to that catalyzed in mammalian cells.

Using the recombinant p62 nucleoporin, we determined the specificity of the autoantibodies by two independent approaches: immunoprecipitation and immunoblotting. PBC sera previously tested as reactive with the affinity purified NPCs 62 kDa protein precipitated recombinant p62 protein and recognized it by immunoblotting, thereby evidencing that these sera are directed against p62 nucleoporin [Wesierska-Gadek et al., 1996a]. More than 50% of 20 tested PBC sera were reactive with the recombinant p62 nucleoporin. Considering the fact that some previously published studies on the reactivity of PBC sera with p62 nucleoporin were performed with the antigen purified from rat liver [Miyachi et al., 1996], we decided to examine the reactivity of our PBC sera with both recombinant p62 proteins: human and rat. The analysis of precipitated immune complexes revealed higher incidence of anti-p62 reactivity using human antigen. The incidence of anti-p62 reactivity increased by 15% as compared with the rat counterpart.

Preadsorption of anti-p62 positive PBC sera with an excess of recombinant human p62 protein abolished their reactivity with ^{35}S-labeled p62 protein and with the p62 protein band in the sample of NPCs, thereby substantiating the high specificity of the autoantibodies. The incidence of anti-p62 nucleoporin positive sera assessed by immunoblotting is much lower (approximately 40%) and is in concordance with our previous results [Wesierska-Gadek et al., 1996a]. The nucleoporin p62 antibodies seem to be characteristic for PBC, although they were also reported in 13% of Sjögrens's syndrome [Miyachi et al., 2003; Enarson et al., 2004], and in rare cases of mixed connective tissue disease [Kraemer et al., 2003; Enarson et al., 2004]. The observed divergence in the number of anti-p62-positive PBC sera determined by immunoprecipitation and by immunoblotting may reflect the difference in their immunoreactivity depending on the status of the autoantigen. Whereas during immunoprecipitation autoantibodies interact with a native antigen, for Western blotting denaturated antigen immobilized on the membrane reacts with antibodies.

Evaluation of the clinical significance of ANA in PBC markedly progressed in recent years indicating [Invernizzi et al., 2001, 2004; Miyachi et al., 2003; Wesierska-Gadek et al., 2006] that, in contrast to AMA, ANA in general, and PBC-specific anti-NPC in particular, correlate with the severity of disease and may be of important prognostic value [Invernizzi et al., 2001, 2004; Wesierska-Gadek et al., 2006]. We generated in the present study a recombinant p62 nucleoporin antigen source that will be useful in future serum testing of patients with PBC. Based on this data further multi-center studies based on a large collection of PBC sera have to be performed to elucidate the clinical significance of the anti-p62 specific reactivities.

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