

Autoantibodies to the Ribosomal P Proteins React with a Plasma Membrane-related Target on Human Cells

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Abstract

Autoantibodies to ribosomal P-proteins are present in 12–16% of patients with systemic lupus erythematosus and are associated with neuropsychiatric disease. As the ribosomal P proteins are located in the cytoplasm, the pathogenic effects of their cognate autoantibodies are unclear. In this study affinity-purified anti-P autoantibodies were used to explore the cell surface of several types of human and animal cells. Immunofluorescence as well as EM immunogold analysis demonstrated, on the surface of human hepatoma cells, the presence of an epitope that is antigenically related to the immunodominant carboxy terminus of P-proteins. The presence of this epitope was also demonstrated on the surface of human neuroblastoma cells and, to a lesser extent, on human fibroblasts. Furthermore, the Western blot technique revealed in purified human and animal plasma membranes a 38-kD protein that is closely related or identical with ribosomal P₀ protein. The availability of reactive P peptide on the surface of cells makes possible the direct effect of autoantibodies on the function and viability of cells that express this antigenic target. This delineates one of the possible impacts of anti-P antibodies in disease expression. (*J. Clin. Invest.* 1992. 89:1236–1241.) **Keywords:** ribosomal P • autoantibodies • neuropsychiatric disease • neuroblastoma cells • hepatoma cells

Introduction

The sera of patients with systemic lupus erythematosus (SLE) contain autoantibodies to ribosomal P-proteins in 12–16% of cases (1, 2). These highly conserved proteins comprise P₀, P₁ and P₂ molecules corresponding to sizes of 38, 19, and 17 kD, respectively (3–7). P-proteins are acidic phosphoproteins primarily associated with 60S ribosomal subunit in eukaryotic cells although their presence in ribosome free cytoplasm has also been reported (5, 8). Most of SLE anti-P autoantibodies cross-react with P₀, P₁ and P₂ due to a common determinant present at the carboxy terminus of all three proteins. This epitope is confined within the 22 carboxy-terminal amino acids, the sequence of which is identical in P₀, P₁, and P₂ proteins as well as in eL12 and eL12' *Artemia salina* proteins commonly

considered crustacean counterparts of mammalian P₁ and P₂ (8). In addition, recent studies revealed that the ribosomal protein L₁₂ is also a target of autoimmunity in SLE. This protein of molecular weight of 20 kD is distinctive from the ribosomal P proteins (9).

SLE sera containing anti-P autoantibodies invariably show a strong cytoplasmic immunofluorescence pattern on fixed HeLa and mouse kidney cells due to the ribosomal, cytoplasmic distribution of P proteins. In this study, we demonstrate by immunostaining techniques the presence of an epitope on the surface of several types of human cells that is antigenically related to the immunodominant carboxy terminus of P-proteins. Furthermore, we demonstrate by Western blot the presence of a 38-kD protein, which is closely related or identical with ribosomal P₀ protein, in plasma membranes isolated from human and animal cells.

Pathogenic effects of autoantibodies specific for intracellular components require cell destruction, release of antigen, and formation of phlogistic immune complexes. Such autoantibodies could also cause damage after penetration into live cells, a phenomenon that has been reported but remains controversial (10, 11). However, the presence of P₀ protein on the cell surface could trigger direct pathogenic mechanisms in the presence of anti-P autoantibodies.

Methods

Human anti-P sera. Human sera from patients with SLE were defined as having anti-P antibodies if they met the following three criteria: (a) precipitated purified ribosomes in a reaction of identity in gel diffusion with a monospecific prototype anti-P serum; (b) reacted with a 38-kD band in Western blot with purified rat ribosomes; and (c) reacted in ELISA with plates coated with purified rat ribosomes and synthetic "P-peptide"-BSA conjugate.

Synthesis of "P-peptide". The carboxy-terminal 22-amino acid peptide of the ribosome P protein has the following sequence: Lys-Lys-Glu-Glu-Lys-Lys-Glu-Glu-Ser-Glu-Glu-Glu-Asp-Glu-Asp-Met-Gly-Phe-Gly-Phe-Leu-Phe-Asp-OH (8). It was synthesized by a solid phase method, purified by HPLC and its composition confirmed by amino acid analysis (12, 13).

Synthetic "P-peptide"-BSA conjugate. 20 mg of peptide and 40 mg BSA were dissolved in 4 ml 0.1 M PO₄ buffer, pH 7.0. 2.0 ml of 0.25 M glutaraldehyde were added dropwise with constant stirring. Blocking with lysine and dialysis were carried out as described previously (14).

Preparation of rat ribosomes. Ribosomes were prepared by a published method from a freshly prepared saline-perfused rat liver (15). The rat ribosome preparation precipitated strongly with sera with anti-ribosomal P₀-positive sera by gel diffusion.

Ribosome-Sepharose column. Rat ribosome solution (14.0 mg protein/ml) was coupled to cyanogen bromide activated Sepharose according to the manufacturers' instructions (Pharmacia, Uppsala, Sweden). 11 g of Sepharose in a slurry was mixed with 175 mg of ribosomal protein in 12.5 µl coupling buffer. 68% of the added ribosomal protein was coupled to sepharose.

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Synthetic "P-peptide"-sepharose column. 20 mg of synthetic "P-peptide" was dissolved in 5 ml of coupling buffer and added to 10 ml of cyanogen bromide-activated Sepharose slurry. The coupling and blocking was carried out according to the manufacturers' instructions (Pharmacia).

Affinity purification of anti-P antibodies. Serum from SLE patient S.S. was selected and used in all the studies. This serum gave a single precipitin line and a reaction of identity in gel diffusion when reacted with purified rat ribosomes and calf thymus extract. It also precipitated strongly with synthetic "P-peptide"-BSA conjugate but not with free BSA. 1 ml of S.S. serum was passed over the ribosome-Sepharose column or the synthetic "P-peptide"-Sepharose column. After washing, columns were eluted with 3 M MgCl₂. Eluates containing purified antibodies were dialyzed versus PBS, and reconcentrated to the original volume of serum. The unretained fractions from such columns were further absorbed by adding incremental amounts of purified rat ribosomes until no further precipitate formed. This was designated "depleted" serum. Such a depleted serum at a 1/100 dilution developed a net OD of 0.2 in a micro-ELISA using ribosome-coated plates whereas unabsorbed serum developed an OD of 0.2 at a dilution of 10⁻⁵ suggesting the removal of 99.9% of antibody activity. Specifically purified antibodies from either the rat ribosome column or the synthetic "P-peptide" column were highly active in precipitin reactions with rat ribosomes or in ELISA with rat ribosome-coated plates.

Cell culture. Cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD) and cultured according to ATCC instructions. These include human hepatoma cells HepG2 (ATCC-B8065) grown in Eagle's MEM supplemented with 10% FBS and human neuroblastoma cells SK-N-MC (ATCC-HTB10) grown in MEM supplemented with 10% FBS. Human fibroblasts were isolated from skin biopsy material of an asymptomatic male donor according to the method of Vijg et al. (16) and grown in MEM supplemented with 10% FBS.

Immunofluorescent staining. Cell monolayers grown in T-75 flasks were washed in sterile PBS two times and incubated with trypsin-EDTA solution (0.25% trypsin, 0.02% EDTA in PBS) for 5 min at 37°C. Trypsinization was stopped by addition of 5 ml FBS per flask. This was followed by vigorous pipetting to detach the cells. After 10 min of centrifugation at 400 g supernates were discarded and pellets were resuspended in respective media. Cell suspensions were adjusted to the concentration of 5 × 10⁴ cells/μl and seeded into eight-chamber cell culture slides (Labtech, Naperville, IL). After 2–4 d of growth (depending on the cell line) cells reached semiconfluency and were used for immunofluorescent staining. The staining was carried out on fixed cells at 25°C and on native cells at 0–4°C. Cells were washed three times with PBS for staining at 25°C and fixed (1 h) with 0.5% paraformaldehyde. Fixation was followed by a 10-min incubation with 0.2% Triton X-100 (Sigma Chemical Co., St. Louis, MO) and a triple washing with PBS. After this treatment cells were incubated with whole serum, antibodies purified by the use of ribosome column, antibodies eluted from an immunoaffinity column containing synthetic "P-peptide" and with serum adsorbed with rat liver ribosomes. Incubation was carried out at 25°C for 3 h. After triple washing with PBS, cells were incubated with FITC-labeled antibodies to human immunoglobulins for 2 h at 25°C. After three washes with PBS, plastic chambers and gaskets were removed from slides. A few drops of 50% glycerol diluted with PBS were added to each slide. Coverslips were carefully placed on each slide which made them ready for microscopic analysis. For the long-term storage slides were kept at -20°C, in dark, up to several months with no appreciable loss in fluorescence or change in cell morphology. Whole serum from an asymptomatic, healthy male donor was used as a negative control.

To stain native cells, slides were placed on ice for 10 min and washed with ice-cold PBS three times. Immediately after washing, cells were incubated with antibodies identical to those used for staining of fixed cells. The incubation lasted for 3 h at 0–4°C and was followed with triple washing with ice-cold PBS and a 2-h incubation with FITC-labeled antibodies to human immunoglobulins at 0–4°C. After a final

washing with ice-cold 3× PBS cells were fixed with ice-cold 0.5% paraformaldehyde for 1 h at 0–4°C. Glycerol and coverslips were placed on each slide as described above.

Fluorescence microscopy. Slides were analyzed for immunofluorescence by the use of an epifluorescent/phase contrast microscope (Optiphot; Nikon, Garden City, NJ) equipped with a photometer (Nikon, UFX) and an automated camera (Nikon, FX-35A). A 20× objective with high numerical aperture (NA 0.75) was routinely used.

Confocal microscopy. To accurately analyze immunofluorescence pattern of native cells stained at 0–4°C, a laser scanning confocal microscope (Zeiss, Jena, Germany) was used. Cells were excited at 488 nm with 2.5-W argon ion laser and subjected to optical sectioning going stepwise from the cell surface adhering to the slide towards the apical cell surface and using a 1.0-μm step size.

Immunogold staining and electron microscopy. A monolayer of HepG2 cells grown in T-75 flask was washed with PBS three times and fixed with 0.5% paraformaldehyde for 1 h. After fixation and additional washing, cells were scraped with a rubber policeman in PBS and centrifuged for 10 min at 400 g. The pellet was embedded with an epoxy resin followed by preparation of ultrathin (1 μm) sections according the method described by Hayat (17). After mounting to copper grids, sections were incubated at 25°C with anti-P autoantibody purified over a column with synthetic "P-peptide". Incubation was carried out for 2 h with copper grids floating on top of 10 μl antibody containing droplets. After five washes with PBS, grids were incubated for 2 h with goat anti-human IgG conjugated to 5 nm gold particles (Sigma Chemical Co.). This was followed by washing and analysis under JEOL's JEM-1200 EX electron microscope (JEOL, Tokyo, Japan). Grids incubated with serum from an asymptomatic male donor followed by immunogold conjugate served as controls.

Isolation of plasma membranes. Cells were grown in T-150 flasks until confluent. 18–20 h before membrane isolation, fresh medium was added. 10–15 flasks were routinely used per experiment. The isolation procedure started with washing of cell monolayers in PBS (3×) followed by a 15-min incubation in ice-cold PBS (20 ml/flask). From this point on the entire procedure was carried out at 0–4°C. Cold PBS was discarded and 10 ml of lysis medium (1 mM NaHCO₃, pH 8.3) was added to each flask. After a 2-min rotation on an orbital shaker the lysis medium was discarded. This step was repeated one more time and the cells were harvested in 10 ml of lysis medium containing protease inhibitors (5 mM PMSF and 5 mM EDTA). Scraped cells were homogenized in a glass homogenizer. The homogenate was centrifuged for 20 min at 28,000 g. The resulting pellet was resuspended in 8 ml of 10% sucrose. Discontinuous gradient gel consisting of 8 ml of 50% sucrose at the bottom, 8 ml of 38% sucrose in the middle and 8 ml of 27% sucrose on the top were prepared in 35-ml polycarbonate tubes. After layering 8 ml of resuspended cell pellet on top of the gradient, tubes were centrifuged in a "swing-out" rotor (model SW-27; Beckman Instruments, Palo Alto, CA) for 2 h at 76,000 g in an L3-50 ultracentrifuge (Beckman Instruments). The plasma membrane band, usually positioned 1.5 cm from the top of the gradient, was recovered with a transfer pipette and diluted in 5 vol of PBS. This was followed by a 25-min centrifugation at 70,000 g. The final pellet was resuspended in PBS and kept at -80°C. As revealed by EM, the described preparation consisted of bilayer vesicles of variable sizes with no detectable ribosomes or other subcellular organelles even at the magnification of 100,000. The lack of ribosomal contamination in membrane samples was confirmed by determination of the ribose content using conventional Orcinol reaction (18). The average concentration of ribose in isolated membranes was below 0.1 μmol/mg (n = 3) protein. By the same analysis ribose concentration was 5.4±0.7 (n = 3) μmol/mg of protein in isolated rat ribosomes. To prepare plasma membranes from animal kidneys, fresh organs from sheep, dog, and rat were minced and separately treated with collagenase for 30 min at 37°C with occasional gentle shaking. After filtration through a 200-μm nylon mesh, fibrous residue and large cell aggregates were discarded. Homogenous cell suspensions were washed with PBS three times and processed for membrane isolation as described for cultured cells.

Immunoblotting. Electrophoresis of rat ribosomal and various membrane preparations on SDS-polyacrylamide gels, electrotransfer to nitrocellulose membranes as well as incubation with anti-P antibodies were carried out by a method described previously (19).

Results

Immunofluorescence analysis. All analyzed cells showed strong cytoplasmic immunofluorescence after fixation, treatment with Triton X-100, and staining with anti-P antibodies at 25°C. This applies to anti-P whole serum, ribosome specific as well as synthetic "P-peptide" specific antibodies. Whole serum

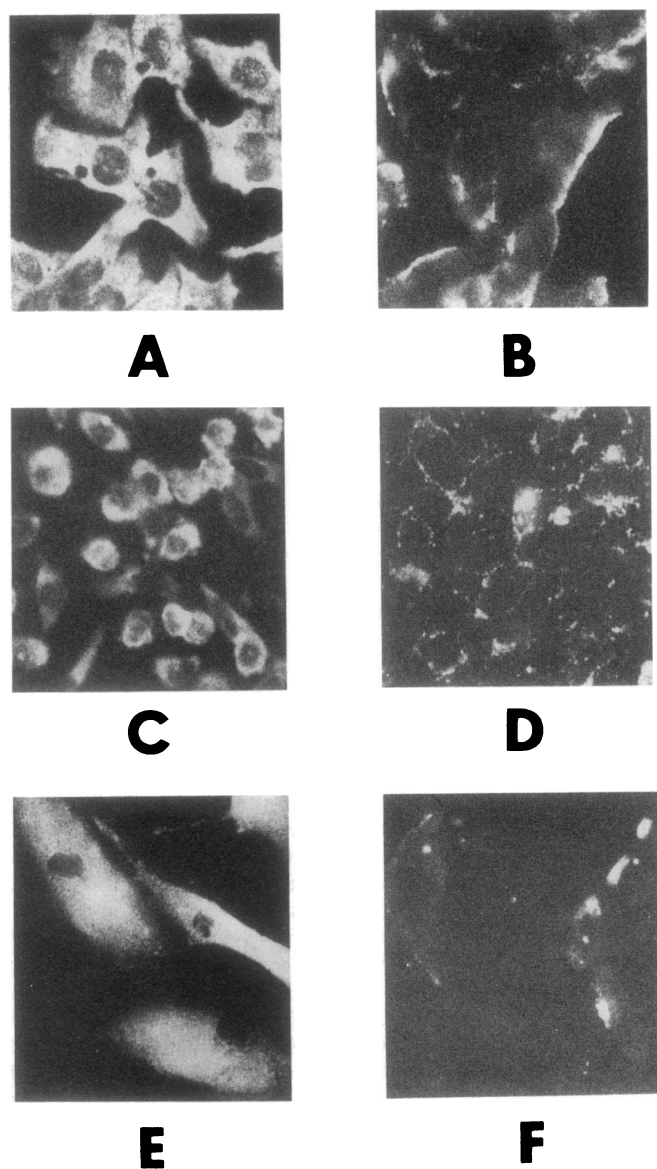


Figure 1. Indirect immunofluorescence analysis of human hepatoma cells (*HepG2*), human neuroblastoma cells (*SK-N-Mc*), and human fibroblasts stained with an affinity-purified anti-"P-peptide" autoantibody. Fixed cells show strong cytoplasmic fluorescence with dark nuclei in case of all three types of cells, i.e., (A) *HepG2*, (C) *SK-N-Mc*, and (E) fibroblasts. Staining of native cells at 0–4°C resulted in strong surface fluorescence in the case of (B) *HepG2* cells, moderate surface fluorescence in the case of (D) *SK-N-Mc* cells, and weak surface fluorescence in the case of (F) fibroblasts.

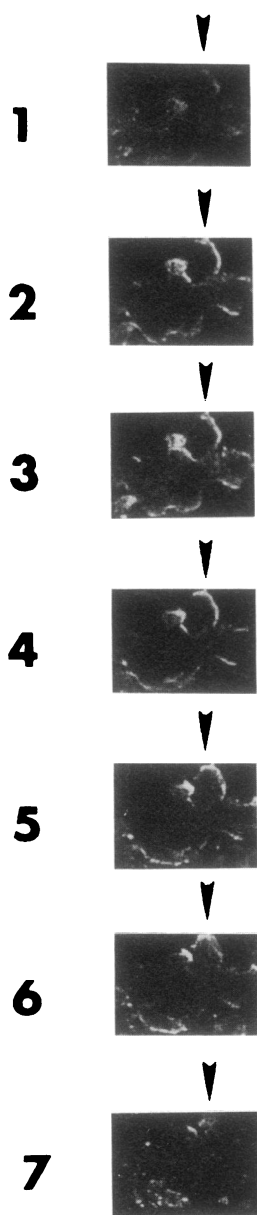


Figure 2. Confocal microscopy of native *HepG2* cells stained with immunofluorescence purified "anti-peptide" antibody at 0–4°C. Optical sections (1–7) were taken in sequence from the surface adhering to the slide (1) to the apical surface of the cell (7) using 1.0 μm step size. The cell marked with the arrow exemplifies an immunofluorescence pattern typical of a surface staining. The bottom (frames 1 and 2) as well as midsection (frames 3, 4, and 5) clearly demonstrate ring-shaped fluorescence, whereas the top of the cell (frames 6 and 7) shows rather "granular" fluorescence covering a circular area. A three-dimensional model of described fluorescence pattern is consistent with a "dome-like" structure. Obviously, the bottom surface of the cell adhering to the slide was not accessible to antibodies, whereas the sides and top were fully exposed. This observation applies to other cells shown on scans 1–7. However, they do not appear to be laid in a perfectly even monolayer.

depleted of anti-P antibodies showed no fluorescence with any of the cells. Staining of native cells at 0–4°C clearly showed typical surface immunofluorescence pattern in the case of hepatoma cells (*HepG2*) and neuroblastoma cells. Fibroblasts showed negligible surface immunofluorescence irrespective of the type of anti-P antibody used (Fig. 1). Surface fluorescence of native *HepG2* cells stained with antibodies purified over synthetic "P-peptide" column was confirmed by the use of confocal microscopy (Fig. 2).

Therefore, cytoplasmic expression of P antigen appears to be comparable in all analyzed cells. The surface expression of this antigen, however, is not equal in all cells. Hepatoma cells are the most abundant in the surface P antigen followed by neuroblastoma cells. Fibroblasts appear to be poor in membrane-associated P antigen.

EM analysis. Since hepatoma cells expressed highest surface fluorescence with anti-P antibodies they were further analyzed by electron microscopy. Immunogold staining with antibodies purified over synthetic "P-peptide" column revealed

two patterns of binding of these antibodies to the outer surface of cell membrane. Gold particles were distributed more or less evenly over "flat" and relatively long sections of cell surface whereas in coated pits they appeared localized in clusters. In addition to the surface distribution, numerous gold particles were also localized inside cells due to exposure of the cytoplasmic pool of antigen in sectioned cells (Fig. 3).

Western blotting. To explore the molecular nature of the cell surface antigen reacting with anti-P antibodies, plasma membranes were purified from cultured human hepatoma cells and from tissues of freshly isolated sheep, dog, and rat kidneys. SDS-PAGE under reducing conditions followed by immunoblotting clearly showed in all plasma membranes the presence of a protein band strongly reactive with anti-P antibodies. This applies to the whole anti-P antiserum, purified antibody from ribosome column, and to the antibody eluted from synthetic "P-peptide" column. No reaction was observed with depleted anti-P antiserum or normal human serum. Electrophoretic mobility of this plasma membrane protein was identical to the mobility of 38-kD P_0 protein present in rat liver ribosomes. Interestingly, plasma membranes showed a single 38-kD band with anti-P antibodies whereas rat liver ribosomes gave positive bands corresponding to 38-k (P_0), 19- (P_1), 17- (P_2), and 50-kD proteins (Fig. 4). Four separate Western blot analysis of the HepG2 cell culture supernates showed no detectable P proteins demonstrating the lack of "shedding" or leakage of these antigens from the cells. These data strongly support the presence in the plasma membrane of a 38-kD polypeptide

antigenically related to or identical with the ribosomal 38-kD P_0 protein.

Discussion

Data are presented which show that a polypeptide antigenically related to the 38-kD ribosomal P_0 protein is present on the surface of several types of human and animal cells as revealed by their reactivity with purified anti-P autoantibodies. Of interest is the apparent differential membrane expression of this protein in that the surface of hepatoma cells and neuroblastoma cells react strongly with the autoantibodies, whereas fibroblasts react weakly with the same autoantibody preparations. Immunogold staining indicates an even as well as clustered distribution of this antigen on the surface of hepatoma cells. It remains to be seen whether or not other cells have similar pattern(s). In addition to immunofluorescence and immunogold analyses, Western blotting of highly purified plasma membranes show strong and highly selective reactivity with the autoantibodies as they carry a 38-kD polypeptide antigenically related if not identical to the ribosomal P_0 protein. Interestingly the membrane preparation did not possess detectable quantities of the other two ribosomal P proteins, i.e., the 19-kD P_1 and 17-kD P_2 .

According to the currently assumed model, P_0 is attached to the large ribosomal subunit and serves as an anchor for the pentameric complex consisting of one P_0 molecule combined with two P_1 and P_2 homodimers (20–23). This complex of P

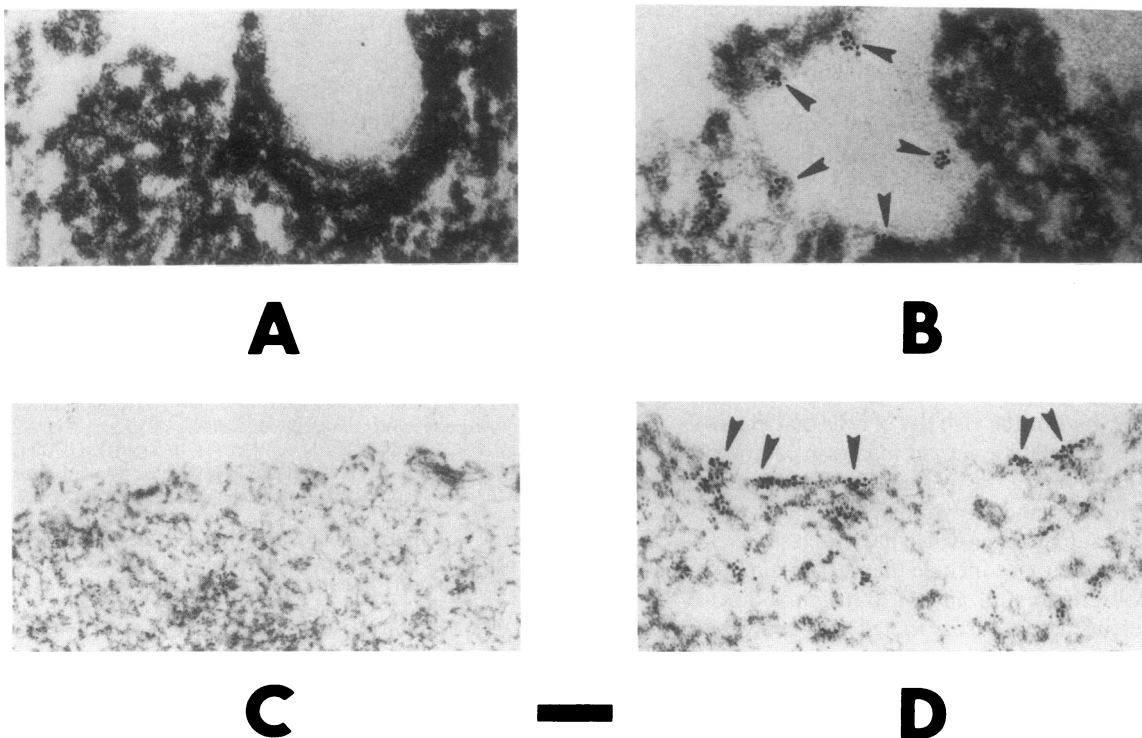


Figure 3. Immunogold electron microscopic analysis of human hepatoma cells (*HepG2*) stained with an affinity-purified anti-"P-peptide" autoantibody. Clusters of gold particles can be seen on the outer surface of a coated pit (*B*, arrows). On the surface of a "flat" section of the cell gold particles are more evenly distributed (*D*, arrows). Considerable number of gold particles can be seen inside cell membrane in both cases. *A* and *C* represent respective controls stained with serum from an asymptomatic healthy male. Both of these controls were virtually free of gold particles confirming specificity of the staining with anti-"P-peptide" autoantibody. Bar, 100 nm.

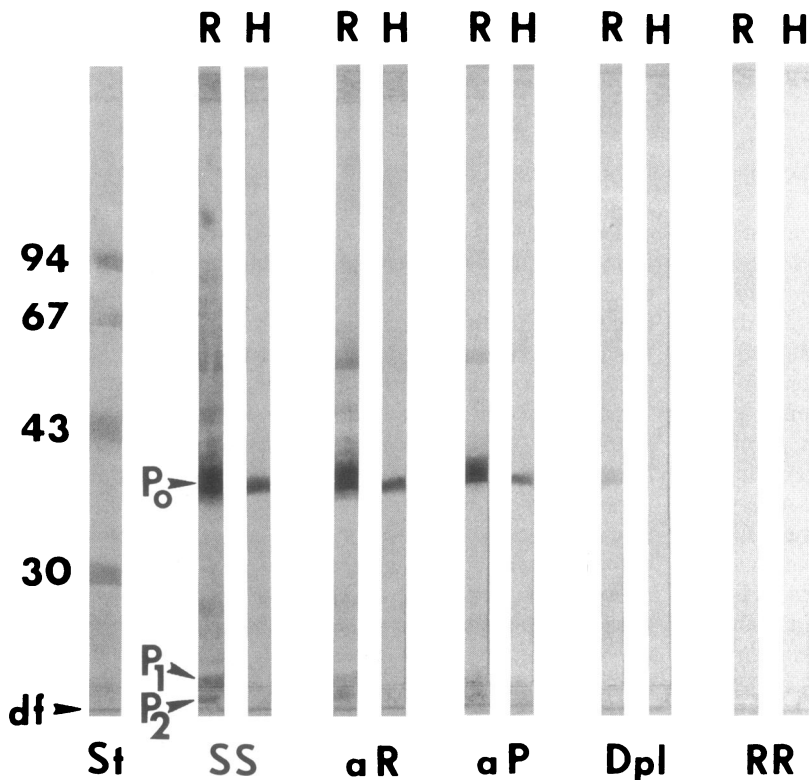


Figure 4. Western blot analysis of rat ribosomes (*R*) and human hepatoma cell (*HepG2*) plasma membrane (*H*). Electrophoretic separation was carried out on SDS polyacrylamide gel under reducing conditions. After transfer to the nitrocellulose the blots were stained with SLE serum containing anti-P autoantibodies (*SS*); anti-P autoantibodies purified over a rat ribosome affinity column (*aR*); anti-P autoantibodies purified over a synthetic "P-peptide" affinity column (*aP*); serum depleted of anti-P autoantibodies (*Dpl*) and with serum from asymptomatic healthy male donor (*RR*). *St* = molecular weight standards representing 94 kD (94), 67 kD (67), 43 kD (43) and 30 kD (30) proteins. *df*, dye front. The blots of rat ribosomes (*R*) clearly show all three ribosomal P proteins, i.e., 38-kD P_0 protein (P_0); 19-kD P_1 protein (P_1) and 17-kD P_2 protein (P_2) visualized with all three types of anti-P autoantibodies. The blots of human hepatoma cell plasma membrane show only the P_0 band. Depleted SLE serum visualized trace of P_0 on the rat ribosome blot whereas no staining is visible on the hepatoma membrane blot. Plasma membranes isolated from sheep, dog, and rat kidneys stained with anti-P antibodies revealed P_0 band identical to that of human hepatoma cell plasma membrane although somewhat variable and less intense. No bands are visible on blots stained with control serum from a healthy donor.

proteins is involved in the interaction of EF-1 α and EF-2 with ribosomes since mAbs to P proteins inhibit binding of these elongation factors to ribosomes (24). P proteins have also been found in the cytosolic fraction of HeLa cells (8) as well as in the cytoplasmic pool of *Artemia* (6). These data, however, offer no clues to the possible mechanism by which P_0 could find its way to the cell surface and make its COOH-terminal domain accessible to the anti-P autoantibodies. Particularly abundant expression of P_0 on the surface of HepG2 cells could be related to their metabolic activity. These cells are known for highly active synthesis of many proteins (25–29) and have also been used as a good model for the phosphorylation of ribosomal proteins (30).

The clinical significance of our findings lies in the expansion of the possible participation of anti-P autoantibodies in disease expression. The availability of the reactive peptide on the surface of cells makes possible the direct effect of autoantibodies on the function and/or the viability of cells that express this antigenic target. A role of these autoantibodies in pathogenesis and disease expression awaits experiments demonstrating such functional modulation and/or cytotoxic effects as well as in vivo binding. Other autoantigens which have been shown to be represented as the cell surface include the nuclear antigens chromatin (31), DNA receptor (32), and Ku (33). Antibodies to the ribosomal P proteins have been associated with neuropsychiatric disease in lupus patients (34). Most recently, specific depletion of anti-P autoantibodies in cerebrospinal fluid per milligram IgG relative to the serum concentration of anti-P per milligram IgG has been reported (2). This finding is consistent with specific uptake and/or binding of anti-P antibodies to the cells of the central nervous system. The presence of P antigen on the surface of cells of neural origin described in this report

provides a realistic setting for such possibilities. The presence of P antigen on the surface of other cells (e.g., hepatocyte, fibroblast, kidney cell) also enlarges the spectrum of potential targets in pathogenesis of SLE patients.

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