Integral Membrane Proteins Specific to the Inner Nuclear Membrane and Associated with the Nuclear Lamina

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Abstract. We obtained a monoclonal antibody (RL13) that identifies three integral membrane proteins specific to the nuclear envelope of rat liver, a major 75-kD polypeptide and two more minor components of 68 and 55 kD. Immunogold labeling of isolated nuclear envelopes demonstrates that these antigens are localized specifically to the inner nuclear membrane, and that the RL13 epitope occurs on the inner membrane's nucleoplasmic surface where the nuclear lamina is found. When nuclear envelopes are extracted with solutions containing nonionic detergent and high salt to solubilize nuclear membranes and pore complexes, most of these integral proteins remain associated with the insoluble lamina. Since the polypeptides recognized by RL13 are relatively abundant, they may function as lamina attachment sites in the inner nuclear membrane. Major cross-reacting antigens are found by immunoblotting and immunofluorescence microscopy in all rat cells examined. Therefore, these integral proteins are biochemical markers for the inner nuclear membrane and will be useful models for studying nuclear membrane biogenesis.

The nuclear envelope forms the boundary of the nuclear compartment in eukaryotes (reviewed in references 10, 13, 25). Its primary functions involve selective transport of macromolecules between the nucleus and the cytoplasm and organization of higher level nuclear architecture. The major components of the nuclear envelope are inner and outer membranes, nuclear pore complexes and the nuclear lamina. Nuclear pore complexes, which occur at regions where the inner and outer membranes are joined to form pores, are large supramolecular assemblies that provide channels for molecular movement across the nuclear envelope (30). The outer nuclear membrane is morphologically continuous with the rough and smooth endoplasmic reticulum (ER) and has functional properties of ER, while the inner nuclear membrane is lined by a filamentous meshwork called the nuclear lamina. In higher eukaryotic cells, the lamina contains mainly a polymer of one to four related proteins (nuclear lamins) which are biochemically and structurally very similar to cytoplasmic intermediate filament proteins (9, 11). The lamina is thought to provide a framework for organizing nuclear envelope structure and an anchoring site at the nuclear periphery for interphase chromosomes (11). During mitosis in higher eukaryotic cells the lamina is reversibly depolymerized by a process that involves lamin hyperphosphorylation, and lamina dynamics are suggested to control disassembly and reformation of the nuclear envelope that takes place during this period (13).

Associations of the lamina with the inner nuclear membrane and chromosomes are likely to be fundamental to its involvement in organization of nuclear architecture during interphase and in restructuring of the nuclear envelope during mitosis. The molecular basis for these interactions is not understood. Association of the lamina with chromatin could involve nonhistone proteins (20), DNA (18), or a nucleoprotein structure such as the nucleosome. By analogy with other membrane systems such as the red cell membrane (4) and the envelope of animal viruses (19), membrane attachment of the lamina may involve integral protein(s) specific to the inner nuclear membrane (12). A putative lipid modification recently described for mammalian lamin B also could promote the lamina-membrane interaction (3, 34).

The two nuclear membranes are in part functionally distinct since the lamina is associated only with the inner nuclear membrane and polysomes are restricted to the outer membrane. However, differences between the complement of integral membrane proteins in the inner vs. outer nuclear membrane are poorly defined biochemically, in part because convincing procedures to separate inner and outer nuclear membranes have not been developed. Immunocytochemical localization indicates that cytochrome P450 is restricted to the outer nuclear membrane in rat liver (22), but cellular integral membrane proteins restricted to the inner nuclear membrane have not been described.

Abbreviations used in this paper: ER, endoplasmic reticulum; NRK, normal rabbit kidney.

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Previously, a major integral membrane glycoprotein specific to the nuclear envelope (gp90) was identified and shown to be localized in the nuclear pore complex (15). In this paper, we describe other integral membrane proteins that occur only in the nuclear envelope. In contrast to gp90, these proteins are localized exclusively in the inner nuclear membrane and appear to be associated with the lamina. Since they are relatively abundant nuclear envelope components, these proteins are attractive candidates for integral proteins that anchor the lamina to the inner nuclear membrane. In addition, these proteins will provide useful models for studying inner nuclear membrane biogenesis.

Materials and Methods

Isolation and Fractionation of Nuclear Envelopes

Nuclear envelopes and total microsomal membranes were isolated from rat liver as described (15). All fractionation procedures were conducted at 0°–4°C. "Salt-washed nuclear envelopes" were obtained by extraction of crude nuclear envelopes with a buffer containing 0.5 M NaCl (7). For isolation of peripheral and integral proteins of nuclear envelopes, pellets of salt-washed nuclear envelopes were resuspended at 2.0 mg/ml protein concentration in either (a) 0.1 M NaOH and 0.001 M dithiothreitol or (b) 4 M guanidine-HCl, 0.05 M triethanolamine-HCl pH 7.4, and 0.001 M dithiothreitol. They were then immediately centrifuged at 40,000 rpm for 60 min in a rotor (model 50T; Beckman Instruments, Inc., Palo Alto, CA) to yield supernatants and pellets. Before gel sample preparation, the guanidine-HCl supernatant was dialyzed against distilled water.

For extraction of nuclear envelopes with Triton and salt, salt-washed nuclear envelopes were resuspended at 1.5 mg/ml protein in 10% sucrose, 0.02 M triethanolamine-HCl, pH 7.4, and 0.001 M dithiothreitol. 1 vol of this suspension was mixed with an equal volume of 10% sucrose, 2% Triton X-100, 0.02 M triethanolamine-HCl, pH 7.4, and 0.001 M dithiothreitol containing either 0.1 M KCl or 10 M KCl. After an incubation of 15 min, samples were centrifuged at 12,000 g for 15 min yielding supernatants and pellets.

Monoclonal Antibodies

A pore complex–lamina fraction isolated from rat liver nuclear envelopes by sequential extraction with 1 M NaCl and 2% Triton X-100 (7) was used to immunize 4- to 8-month-old BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) as described (26). Spleen cells from immunized mice were fused with the mouse myeloma cell line P3-X63-Ag8.653 (17), and hybridomas were isolated by a limiting dilution procedure exactly as described previously (26). The RL13 antibody is of the IgGl subclass. RL12 is a mouse monoclonal IgM that is specific for lamins A and C (26) and RL16 is a mouse monoclonal IgG that is specific for gp90. Dr. Ann Hubbard (Johns Hopkins University School of Medicine, Baltimore, MD) generously provided HAA, a mouse monoclonal IgG that does not react with the nuclear envelope (16). Dr. Gert Kreibich (New York University School of Medicine, New York) generously provided the anti-ribophorin I monoclonal antibody (4HID-F12). Monoclonal IgGs were purified by chromatography on DEAE Affi-gel Blue (33), and the monoclonal IgM was purified by chromatography on Sephacryl S-400 (26). Purified antibodies were used for all immunoblotting, immunofluorescence microscopy, and immunogold electron microscopy.

SDS Gel Electrophoresis, Immunoblotting and Immunoadsorption

SDS-PAGE was carried out on 10% gels (21) using the sample preparation conditions described (15). Apparent molecular masses of the RL13 antigens were determined from their mobilities on a 7% SDS–polyacrylamide gel. A standard curve was determined with Sigma Chemical Co. (St. Louis, MO) high molecular mass protein standards (myosin, 205 kD; β-galactosidase, 116 kD; phosphorylase B, 97.4 kD; BSA, 66 kD; and egg albumin, 45 kD. Relative mass of protein bands on Coomassie Blue–stained SDS gels was determined by dye elution in 25% pyridine followed by spectrophotometric measurement at 605 nm (8).

Immunoblotting and immunoadsorption were carried out as described previously (26). We used 125I-protein A and autoradiographic detection for the immunoblot procedure. For analysis of RL13 antigens in various rat tissues (liver, brain, kidney, spleen), fresh tissues were homogenized at 4°C with a motorized glass-tenson homogenizer in 2 vol of 0.25 M sucrose, 0.05 M triethanolamine-HCl, pH 7.4, 0.025 M KCl, 0.005 M MgCl2, 0.0005 M phenylmethylsulfonyl fluoride. Next, 1 vol of homogenate was mixed with 1 vol of 2× concentrated SDS sample buffer (7) and samples were immediately boiled for 5 min before SDS gel electrophoresis and immunoblot analysis. For analysis of RL13 antigens in cultured cells, cells were harvested by scraping into PBS (0.05 M sodium phosphate, pH 7.4, 0.140 M NaCl), resuspended in 2 vol of PBS, and processed as described above for tissue homogenates.

Immunolocalization

Normal rat kidney (NRK) or HTC rat hepatoma cells were grown on glass coverslips as described (26), fixed for 5 min at room temperature in 4% paraformaldehyde in PBS, permeabilized for 5 min in PBS containing 0.2% Triton X-100, and processed for immunofluorescence microscopy as described (31). Isolated rat liver nuclei (7) were centrifuged onto coverslips and processed for immunofluorescence microscopy in a similar fashion (26). For immunogold electron microscopy, salt-washed rat liver nuclear envelopes were centrifuged onto plastic petri dishes, fixed, incubated with antibodies, and prepared for thin section electron microscopy as discussed previously (26). In this work we used indirect immunogold labeling involving goat anti-mouse IgG conjugated with 10-nm gold and goat anti-mouse IgM conjugated with 5-nm gold (Janssen Life Sciences Products, Piscataway, NJ).

Results

Novel Integral Membrane Proteins of the Nuclear Envelope Associated with a Lamina Fraction

In the course of preparing monoclonal antibodies against proteins of rat liver nuclear envelopes, we obtained an antibody that reacts with a novel set of integral membrane proteins. On immunoblots of rat liver nuclear envelopes (Fig. 1, lanes 1 and 7), this antibody (RL13) labels three bands: a major component that migrates at 75 kD (p75), and two more minor components that migrate at 68 (p68) and 55 kD (p55). These antigens are present in the nuclear envelope but absent from a total microsomal membrane fraction of rat liver which contains rough and smooth ER membrane, Golgi apparatus, and plasma membrane (Fig. 1, lanes 2 and 8). When nuclear envelopes are treated with either 0.1 M NaOH (Fig. 1, lanes 3 and 4 and lanes 9 and 10) or 4 M guanidine-HCl (Fig. 1, lanes 5 and 6 and lanes 11 and 12) and centrifuged to pellet membrane vesicles, all three of the antigens appear in the pellet fraction together with integral membrane proteins such as cytochrome P450 (Fig. 1, lane 1, asterisk). Peripheral membrane proteins such as lamins A, B, and C (the three major lamins of rat liver; 12) are extracted into the supernatant by these conditions (Fig. 1, lanes 3 and 5). Thus, by this operational definition (27) the RL13 antigens are integral membrane proteins. These proteins are not obviously related to lamins, since immunoadsorbed RL13 antigens (e.g., Fig. 1, lane 13) do not react with nuclear lamin–specific polyclonal antibodies (data not shown).

From quantitation of Coomassie Blue bound to proteins on SDS gels of immunoadsorbed samples (see Fig. 1, lane 13 and Materials and Methods), we determined that the p75 species accounts for ~75% of the total staining mass. Thus, p75 is substantially more abundant than the other two species in rat liver. It is unlikely that p68 and p55 arise from p75 by in vitro proteolysis during the course of nuclear envelope isola-
Identification of related integral membrane proteins of the nuclear envelope with monoclonal antibody RL13. Rat liver nuclear envelopes were extracted with 0.1 M NaOH or 4 M guanidine-HCl and centrifuged to yield supernatants (S) containing peripheral membrane proteins and pellets (P) containing integral membrane proteins. These samples, together with samples of unfractionated nuclear envelopes (NE) and total rat microsomal membrane (MM) were subjected to electrophoresis on an SDS gel and stained with Coomassie Blue (lanes 1-6) or analyzed by immunoblotting with RL13 (lanes 7-12). Also shown is a sample of RL13 antigens immunoadsorbed from solubilized nuclear envelopes, run on an SDS gel, and stained with Coomassie Blue (lane 13). Lane 13 contains the antigens immunoprecipitated by RL13 from approximately three times the amount of nuclear envelope material subjected to electrophoresis in lane 1 under conditions where almost all of the RL13 antigens were immunoadsorbed as shown by immunoblot analysis. Lamins A, B, and C are indicated (laA, B, C), as well as cytochrome P450 (asterisk to left of lane 1).

The 75-kD band seen in the integral membrane protein fractions of nuclear envelopes by Coomassie Blue staining of SDS gels (Fig. 1, lanes 4 and 6, arrowheads) most likely corresponds to the p75 RL13 antigen. We analyzed the fractionation behavior of RL13 antigens during extraction of nuclear envelopes with nonionic detergent and salts, to obtain insight into possible associations of these proteins with either pore complexes or lamina (Fig. 2). When nuclear envelopes are incubated in a buffer containing Triton X-100 and low salt (50 mM KCl), nuclear membranes are solubilized while pore complexes and lamina remain largely intact and occur in the "pore complex–lamina" pellet fraction (7, 14). As shown by SDS gel electrophoresis and immunoblotting, extraction of nuclear envelopes with this condition solubilizes major integral membrane proteins of the outer nuclear membrane, including most of cytochrome P450 (Fig. 2, lanes 1 and 2, asterisk) and a major fraction of ribophorin I (Fig. 2, lanes 9 and 10). Proteins that remain insoluble include lamins (Fig. 2, lanes 1 and 2), gp190 (an integral membrane protein associated with the pore complex; Fig. 2, lanes 13 and 14), and a group of O-linked peripheral membrane glycoproteins of the pore complex recognized by the antibody RL1 (reference 26; Fig. 2, lanes 17 and 18). In addition, all of p75 and p68 and most of p55 identified by RL13 are associated with the pore complex-lamina fraction.

When nuclear envelopes are incubated in a buffer containing Triton X-100 and 500 mM KCl, the pore complexes as well as nuclear membranes are solubilized, while a fraction highly enriched in the insoluble nuclear lamina occurs in the pellet (14). As shown by SDS gel analysis, this condition extracts most nuclear envelope polypeptides except lamins (Fig. 2, lanes 3 and 4), including gp190 (Fig. 2, lanes 15 and 16), O-linked glycoproteins of the pore complex (Fig. 2, lanes 19 and 20), and almost all of ribophorin I (Fig. 2, lanes 11 and 12). In contrast the majority of p75 and p68 recognized...
Figure 2. Association of RL13 antigens with a nuclear lamina fraction. Rat liver nuclear envelopes were incubated with a buffer containing either 2% Triton X-100 and 0.05 M KC1 (lanes 1 and 2, 5 and 6, 9 and 10, 13 and 14, 17 and 18) or 2% Triton X-100 and 0.5 M KC1 (remaining lanes) and were centrifuged to yield supernatants (odd-numbered lanes) and pellets (even-numbered lanes). Samples were then subjected to electrophoresis on an SDS gel and stained with Coomassie Blue (lanes 1-4) or processed for immunoblotting with the following antibodies: RL13 (lanes 5-8), monoclonal anti-ribophorin I (lanes 9-12), RL16 recognizing gpl90 (lanes 13-16), and RLI (lanes 17-20). Lamins A, B, and C and cytochrome P450 (asterisk to left of lane 1) are indicated.

by RL13 remain associated with the insoluble lamina fraction, while p55 is solubilized (Fig. 2, lanes 7 and 8). Since integral membrane proteins of the pore complex and outer nuclear membrane are almost completely extracted by this condition while the majority of p75 and p68 are insoluble, these data suggest that p75 and p68 have a direct or indirect physical association with the nuclear lamina, which is the only major structure remaining in this fraction. It is plausible that p55 also has a significant (albeit weaker) interaction with the lamina, consistent with its presence in a Triton and low salt pellet (Fig. 2, lanes 5 and 6).

RL13 Antigens Are Specifically Localized in the Inner Nuclear Membrane

We analyzed the localization of the RL13 proteins in NRK cells and in isolated rat liver nuclei by immunofluorescence microscopy (Fig. 3). In NRK cells, RL13 yields strong specific labeling of the nuclei of all interphase cells (Fig. 3, A–C) but no specific staining of the cytoplasm. When the microscope is focused to obtain an optical section through the center of NRK nuclei, pronounced "rim" labeling of the nuclear periphery is seen (Fig. 3, A and B). Similarly, strong rim staining is obtained with isolated rat liver nuclei, which are more round than the nuclei of NRK cells (Fig. 3 E). Distinct intranuclear structures are not labeled in either sample. These immunofluorescence patterns closely resemble those obtained when cultured cells and rat liver nuclei are stained with other antibodies specific for nuclear envelope antigens (e.g., reference 26). In conjunction with the biochemical data presented above (Fig. 1), these results suggest that the RL13 antigens are localized predominantly if not exclusively in the nuclear envelope.

When the microscope is focused on the nuclear surface of NRK cells, a largely uniform fluorescence staining pattern is obtained (Fig. 3 C). This pattern is similar to the distribution of lamins seen by immunofluorescence microscopy, and is inconsistent with localization in the nuclear pore complex which is associated with finely punctate surface labeling (6, 26). During mitosis in NRK cells, the RL13 antigens lose chromosome associations and become distributed throughout the cytoplasm by metaphase (Fig. 3 D) and subsequently reappear at the chromosome surfaces during telophase (data not shown). This closely resembles the mitotic behavior of other nuclear envelope proteins such as lamins (e.g., reference 12).

Indirect immunogold labeling was used to localize the RL13 antigens at the electron microscope level in isolated rat liver nuclear envelopes (Fig. 4). Inner and outer nuclear membranes can be readily distinguished in these preparations, since the outer nuclear membrane is more fragile than the inner nuclear membrane and consequently is disrupted or absent in many areas (7). With RL13 we obtain a substantial level of specific gold labeling on the nucleoplasmic surface of the inner nuclear membrane (Fig. 4, A and B). To a first approximation, the antigens appear to occur uniformly over the nucleoplasmic surface of the inner nuclear membrane, and show no evidence of localized high concentrations. No significant labeling of pore complexes or the outer...
nuclear membrane is seen with RL13. An irrelevant monoclonal IgG gives no significant gold labeling of any nuclear envelope structures (Fig. 4 D). The labeling obtained with RL13 coincides with the localization of lamins in nuclear envelopes visualized by immunogold labeling (Fig. 4 C). Thus, these data indicate that the epitope recognized by RL13 is restricted to the nucleoplasmic surface of the inner nuclear membrane where the nuclear lamina is found. These data closely support our biochemical studies involving extraction of nuclear envelopes with Triton and high salt that suggest that the p75 and p68 RL13 antigens are associated with the nuclear lamina.

Cross-reacting Antigens in Other Rat Cells

We performed immunoblot analysis with RL13 to determine the distribution of cross-reacting RL13 antigens in various rat tissues and cells (Fig. 5). In total homogenates of rat liver and spleen (Fig. 5) as well as in brain and kidney (data not shown), the only major antigens detected by RL13 migrate at 75, 68, and 55 kD as do the antigens of isolated nuclear envelopes. In contrast, a 55-kD band is the only major antigen found in a number of cultured rat cell lines, including YB2 cells (a myeloma line), HTC cells (a hepatoma line), NRK cells, and PC12 cells (a pheochromocytoma cell line). Since immunofluorescence microscopy indicates that the 55-kD antigen of NRK cells (Fig. 3) and of HTC cells (data not shown) is localized in the nuclear envelope, the immunoreactive proteins detected in these cells by immunoblotting probably are similar to the antigens characterized in rat liver nuclear envelopes, although they do not necessarily derive from identical genes. Except for rat, RL13 does not react strongly with antigens in other species that we have tested.

Discussion

We obtained a monoclonal antibody (RL13) that recognizes three novel proteins of the nuclear envelope of rat liver, including a major species (p75) and two relatively minor components (p68 and p55). These cross-reacting antigens are integral membrane proteins based on chemical extraction of nuclear envelopes with alkali and chaotropic conditions. The RL13 antigens are localized specifically to the inner nu-

Figure 3. Localization of RL13 antigens by immunofluorescence microscopy. NRK cells growing on coverslips (A–D) or rat liver nuclei that had been centrifuged onto a coverslip (E) were labeled for immunofluorescence microscopy with RL13. Shown are fluorescence (A and C–E) and phase-contrast (B) images. D shows cell in a metaphase, where the RL13 antigens are dispersed throughout the cell. Bar, 10 μm.
Figure 4. Immunogold localization of the RL13 antigens. Shown are thin section electron micrographs of isolated salt-washed nuclear envelopes that had been labeled for immunogold electron microscopy with RL13 (A and B); RL12, an antibody specific for lamins A and C (C); or HA4, an monoclonal IgG that does not react with nuclear envelope antigens (D). A 10-nm gold antibody conjugate was used to detect RL13 and HA4, while a 5-nm gold antibody conjugate was used to detect RL12. In A, inner (INM) and outer (ONM) nuclear membranes are indicated and examples of pore complexes are designated by arrowheads. Bar, 200 nm.
p55 of rat liver nuclear envelopes is unknown, most likely the proteins are functionally similar: they all are integral proteins of the nuclear envelope that share the RL13 epitope, and the polypeptides appear to be associated with the lamina by chemical fractionation. Further analysis will be required to determine whether they arise from different genes, or from the same gene by differential processing such as alternate mRNA splicing or posttranslational proteolysis. Major cross-reacting antigens that comigrate with the three liver polypeptides are found in many different rat cells. Interestingly, the predominant immunoreactive species of several cultured cells is a 55-kD band rather than the prevalent 75-kD antigen seen in liver, indicating developmentally regulated expression of members of this antigen set.

Since RL13 antigens are relatively major proteins of the inner nuclear membrane that appear to be associated with the lamina, they are attractive candidates for integral proteins that may anchor the lamina to the inner nuclear membrane. The intermediate filaments forming the lamina are constructed of 50-nm-long lamin dimers (1) and may contain 8–20 dimers per filament cross section in analogy with other intermediate filaments (32). Based on these figures, RL13 antigens (estimated to occur at ~10% of the molar level of lamins) are sufficiently abundant to be present at ~1.5–4 copies for every 50 nm of lamin filament length. Thus, the RL13 proteins could engage in frequent periodic associations with lamina filaments and thereby could plausibly contribute to the salt-resistant association of the lamina with the inner nuclear membrane (7). Association of the RL13 antigens with the lamina during the cell cycle could be dynamic, and modulation of these interactions could be important for nuclear envelope restructuring during interphase and mitosis (13). Elucidation of the membrane interaction of the nuclear lamina could provide useful information for understanding apparent membrane associations of cytoplasmic intermediate filaments (e.g., reference 24).

To our knowledge the RL13 antigens are the only cellular integral membrane proteins specific to the inner nuclear membrane that have been described, although integral proteins of viruses that bud from the nuclear envelope also may become highly concentrated in the inner nuclear membrane (e.g., reference 2). Thus, the RL13 proteins are biochemical markers for the inner nuclear membrane. Furthermore, they will represent useful models to study inner nuclear membrane biogenesis. Nuclear envelope growth and remodeling during interphase (23) presumably involves continuous insertion of integral proteins into the inner membrane. Continuity of the lipid bilayers of inner and outer nuclear membranes at nuclear pores presumably allows ready access of lipids to the inner membrane by lateral diffusion. However, mechanisms by which integral membrane proteins are assembled into the inner nuclear membrane are unknown. In virus-infected cells, viral integral membrane proteins can move from rough ER where they are synthesized to the nuclear envelope (5, 28) and in at least one case movement is bidirectional (29). While it is conceivable that movement of integral proteins between outer and inner nuclear membranes involves lateral diffusion around membranes of nuclear pores, the pore complex may provide a topological “roadblock” to movement of proteins, particular to polypeptides with a large cytoplasmic domain. In this case, entry of integral proteins into the inner membrane might necessitate

Figure 5. Antigens that cross react with RL13 in different rat cells. Samples of whole tissue homogenates or cultured cells were subjected to electrophoresis on an SDS gel and processed for immunoblot analysis with RLI3. Shown are samples of liver, spleen, YB2 cells (a myeloma line), HTC cells (a hepatoma line), NRK cells, and PC12 cells (a pheochromocytoma line). When the autoradiogram was exposed for a shorter time than in this figure, the 68-kD band was clearly evident in liver and spleen samples, in addition to the 75- and 55-kD bands that are apparent in this figure.
periodic fusion between inner and outer membranes, transport of integral proteins into the nucleus as soluble precur-
sors, or transient restructuring of the pore complex.

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