

A major glycoprotein of the nuclear pore complex is a membrane-spanning polypeptide with a large luminal domain and a small cytoplasmic tail

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One of a small number of polypeptides of the nuclear pore complex that have been identified is a major glycoprotein called gp210. Since it is very resistant to chemical extractions from membranes, gp210 was suggested to be integrated into nuclear membranes. In this study we have determined the membrane topology of this protein by biochemical and immunological approaches. We found that limited proteolysis of isolated nuclear envelopes with papain released a 200 kd water-soluble fragment of gp210 containing concanavalin A-reactive carbohydrate. Immunogold electron microscopy with a monoclonal antibody showed that this domain is localized on the luminal side of nuclear membranes at pore complexes. Anti-peptide antibodies against two sequences near the C-terminus of gp210 were used to map possible membrane spanning and cytoplasmically disposed regions of this protein. From analysis of the protease sensitivity of these epitopes in sealed membrane vesicles, we determined that gp210 contains a small cytoplasmic tail and only a single membrane-spanning region. Thus, gp210 is a trans-membrane protein with most of its mass, including the carbohydrate, located in the perinuclear space. This topology suggests that gp210 is involved primarily in structural organization of the pore complex, for which it may provide a membrane attachment site.

Key words: glycoprotein/immunolocalization/membrane topology/nuclear envelope/nuclear pore complex

Introduction

The nuclear envelope, which forms the boundary of the nucleus, contains inner and outer nuclear membranes joined at large supramolecular assemblies called nuclear pore complexes (Franke *et al.*, 1981). The inner membrane is lined on its nucleoplasmic surface by the nuclear lamina (Franke, 1974; Newport and Forbes, 1987; Gerace and Burke, 1988; Nigg, 1989), while the outer membrane is continuous with the rough and smooth endoplasmic reticulum (ER) (Franke, 1974; Unwin and Milligan, 1982). Thus, the perinuclear space between inner and outer membranes is continuous with the ER lumen.

Nuclear pore complexes provide aqueous channels for molecular exchange between the nucleus and the cytoplasm (for reviews, see, e.g. Maul, 1977; Gerace and Burke, 1988). The pore complex has a diameter of ~120 nm (Akey, 1989) and a mass of ~125 × 10⁶ daltons (Reichelt *et al.*,

1990). It contains a number of distinct substructures, including two 'rings' that frame the cytoplasmic and nuclear sides of the pore complex and an assembly of 'spokes' that project from the membrane walls toward the pore center between the rings. In addition, a central 'plug' or putative 'transporter' is located in the middle of the spoke assembly (Franke, 1974; Unwin and Milligan, 1982; Akey, 1989). Both the rings and the spoke assembly have 8-fold radial symmetry when viewed in a direction perpendicular to the nuclear surface. Due to the superposition of these structures, the pore complex itself shows strong 8-fold symmetry.

Although intact pore complexes have not been isolated from any source by biochemical fractionation, several proteins of the pore complex have been identified in higher eucaryotes by immunocytochemistry (for review, see Gerace and Burke, 1988). The first of these was a glycoprotein called gp190 that was identified in rat liver nuclear envelopes with polyclonal antibodies (Gerace *et al.*, 1982). This protein is present in a number of different vertebrate cells (Gerace *et al.*, 1982) as well as in *Drosophila* (Fisher *et al.*, 1982). This glycoprotein was renamed gp210 (Wozniak *et al.*, 1989) on the basis of its deduced amino acid sequence, which predicted a polypeptide mol. wt of ~201 600 daltons for the mature protein. Although the mol. wt of the carbohydrate is unknown, we will call the protein 'gp210' in this paper.

By operational criteria (Steck and Yu, 1973; Fujiki *et al.*, 1982), gp210 appears to be an integral membrane protein or a lipid-anchored protein, since it is not extracted from the membrane by alkaline pH or chaotropic agents (Gerace *et al.*, 1982). The protein is the predominant nuclear envelope polypeptide that binds concanavalin A (Con A) (Gerace *et al.*, 1982), a lectin specific for α -D-mannopyranose and sterically related sugars (Poretz and Goldstein, 1970). When treated with endo- β -N-acetylglucosaminidase H (Endo H), gp210 undergoes a small increase in mobility on SDS gels and loses the reactivity with Con A (Berrios *et al.*, 1983; Wozniak *et al.*, 1989), consistent with the notion that it contains N-linked high mannose-type oligosaccharide (Tarentino and Maley, 1974) like many proteins located in the lumen of the ER (Kornfeld and Kornfeld, 1985). Gp210 also binds lentil lectin, which reacts with mannose residues, but does not bind wheat germ agglutinin (WGA), which binds N-acetylglucosamine residues and sialic acids (for review, see Lis and Sharon, 1973). Sequencing of cDNA clones encoding this protein indicated that it consists of 1886 amino acids, including an N-terminal signal sequence that could target the nascent polypeptide to the ER (Wozniak *et al.*, 1989). The signal sequence is absent from the mature gp210, which contains two additional hydrophobic segments of ~20 amino acids each, one at position 1482 and the other near the C-terminus at position 1809. Calculations of hydrophathy suggested that both segments could function potentially as membrane-spanning domains.

In this report, we describe the *in situ* membrane topology of gp210 using structural and biochemical approaches. We

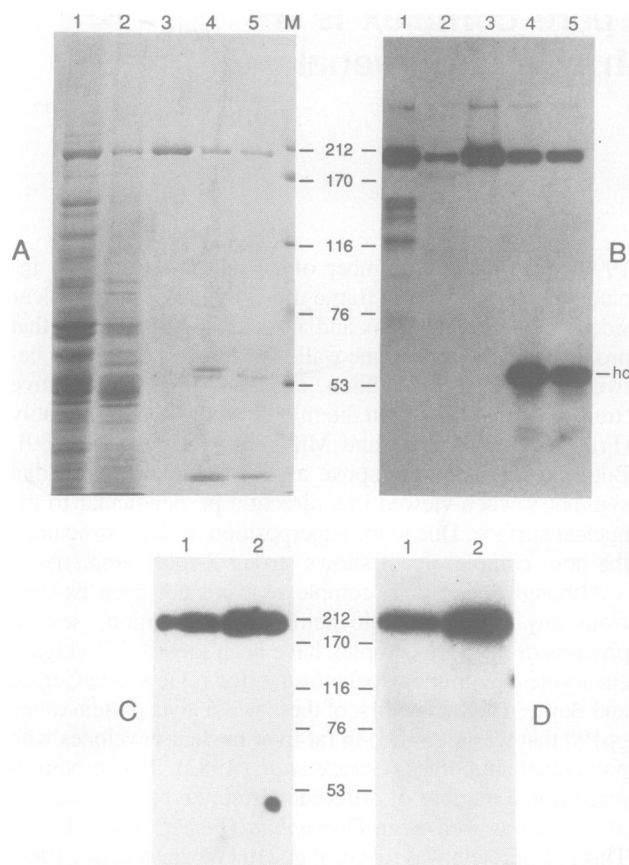


Fig. 1. Characterization of anti-gp210 antibodies by immunoblotting and immunoadsorption. Fractions of rat liver nuclear envelopes were resolved by SDS-PAGE on an 8% polyacrylamide gel and proteins were stained with Coomassie Blue R-250 (A) or processed for immunoblotting with RL16 (B), Rb-68 (C) and Rb-72 (D). In (A) and (B), lanes 1 display 4 OD units (see Materials and methods) of salt-washed nuclear envelopes, lanes 2 show 2 OD units of alkali-extracted nuclear envelopes, lanes 3 depict 8 OD units of purified gp210, and lanes 4 and 5 show protein immunoadsorbed by RL16 and RL20, respectively. The heavy chain of IgG in these samples reacts with protein A and is indicated (hc). In (C) and (D), lanes 1 show 4 OD units of salt-washed nuclear envelopes and lanes 2 depict 8 OD equivalents of purified gp210. Migration of molecular weight markers is indicated by numbers between (A)–(B) and (C)–(D).

demonstrate that gp210 is a transmembrane polypeptide with a single membrane-spanning segment located near its C-terminus. Interestingly, most of the mass of gp210 occurs in the perinuclear lumen, while only a short segment is found on the endoplasmic surface of nuclear membranes. (While this endoplasmic domain may occur in both the cytoplasmic and nuclear spaces, we will describe this segment as 'cytoplasmic' in this paper.) These data provide a framework for studying possible roles of gp210 in pore complex structure and assembly and for identification of accessory components involved in linking pore complexes to nuclear membranes.

Results

Characterization of antibodies specific for gp210

We used both monoclonal antibodies and polyclonal anti-peptide antibodies to map the membrane topology of gp210. Two hybridomas secreting monoclonal antibodies specific for gp210 (RL16 and RL20) were generated following

immunization of mice with purified rat liver gp210 or with pore complex–lamina fraction of rat liver nuclear envelopes. RL16 reacted strongly with the SDS-denatured protein and was used for detection of gp210 on immunoblots, while RL20 strongly recognized the native protein and was used for immunocytochemistry.

The specificities of these antibodies are shown in Figure 1. On immunoblots of total nuclear envelope proteins, RL16 reacted with one major protein with an apparent mol. wt of ~210 kd (Figure 1A and B, lanes 1), as well as some minor, faster-migrating bands that were probably proteolytic fragments of the 210 kd band. RL16 also reacted with a major 210 kd band in sodium carbonate-extracted nuclear membranes (Figure 1A and B, lanes 2), as well as with gp210 purified to near homogeneity by lentil lectin chromatography (Figure 1A and B, lanes 3). Furthermore, both RL16 and RL20 specifically bound a single, major 210 kd band corresponding to gp210 when used to immunoadsorb antigen from nuclear envelopes solubilized in Triton and high salt (Figure 1A and B, lanes 4 and 5).

Polyclonal antibodies were prepared by immunizing rabbits with two different synthetic peptides corresponding to regions of predicted amino acid sequence near potential membrane-spanning segments of gp210 (Wozniak *et al.*, 1989). Rb-68 was immunized with a peptide comprising residues 1868–1886, while Rb-72 was injected with a peptide corresponding to residues 1648–1664. By immunoblot analysis, both Rb-68 and Rb-72 reacted strongly with a single major band of 210 kd in salt-washed nuclear envelopes and also bound to purified gp210 (Figure 1, Panel C and D).

A 200 kd domain of gp210 is outside the lipid bilayer of nuclear membranes

To define regions of gp210 that were external to the membrane bilayer, we digested salt-washed nuclear envelopes with a number of different proteases. Since the outer nuclear membrane is physically ruptured in isolated rat liver nuclear envelopes (see Dwyer and Blobel, 1976), both luminal and cytoplasmic sides of the membranes are accessible to added proteases or other macromolecular probes. We obtained the most interesting results by digestion with papain (Figure 2).

When nuclear envelopes were incubated in the absence of papain and subsequently centrifuged at 100 000 g to pellet membranes, all of the gp210 detected by RL16 or Con A appeared in the pellet (Figure 2A). Incubation with 2 µg/ml papain released a water-soluble fragment of gp210 of ~200 kd into a supernatant. This fragment contained most or all of the Con A-reactive sites (Figure 2A). Most of the gp210 was released from the nuclear envelope as a 200 kd water-soluble fragment by incubation with 5 µg/ml papain. However, at 10–50 µg/ml, the soluble 200 kd fragment was extensively degraded to a smaller fragment of ~170 kd, which reacted with Con A but not with RL16 (Figure 2A). The 200 kd soluble fragment released by 5 µg/ml papain was immunoadsorbed by RL20, and also contained the epitopes recognized by RL16 and Rb-72 but not the epitopes seen by Rb-68 (Figure 2B). In contrast, the membrane-bound gp210 immunoprecipitated with RL20 reacted strongly with all three antibodies (Figure 2B) showing that it contained an intact carboxyl-terminus. Considered together, these data indicate that most of the mass of gp210 is external to the

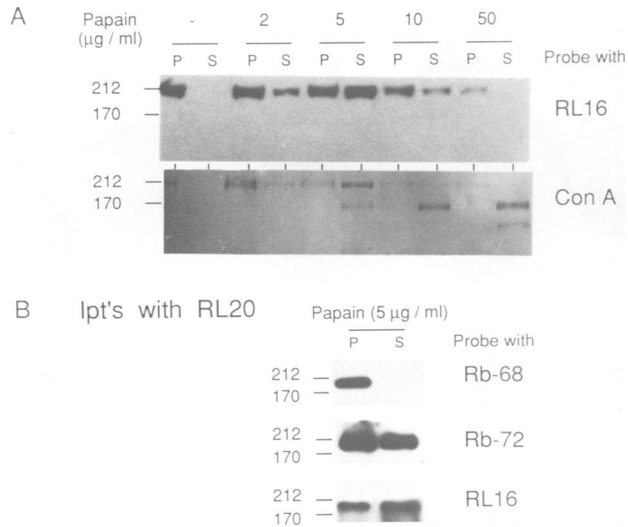


Fig. 2. Digestion of rat liver nuclear envelopes with papain. (A) Salt-washed nuclear envelopes were incubated with papain at the concentrations indicated for 30 min on ice, separated into a membrane pellet (P) and a soluble fraction (S) by centrifugation and analysed by SDS-PAGE on a 5% acrylamide gel. Immunoblot analysis was performed using RL16 antibodies or Con A-biotin as probes and visualized by autoradiography and development of color, respectively. (B) The membrane pellets and supernatants obtained with 5 µg/ml papain were immunoadsorbed with RL20 antibodies and subsequently analysed by immunoblotting using RL16, Rb-68 and Rb-72.

lipid bilayer of nuclear membranes as a single continuous domain uninterrupted by any transmembrane segment. This domain contains epitopes for RL16, RL20 and Rb-72 as well as most or all of the Con A-binding sites.

Immunolocalization of the 200 kd domain of gp210

We performed immunocytochemical labeling with RL20 to locate the 200 kd papain-released domain of gp210. When HTC cells (a rat hepatoma cell line) were labeled with RL20 for immunofluorescence microscopy, we observed prominent nuclear staining, with enhanced 'rim' labeling at the nuclear periphery (Figure 3) that is characteristic of nuclear envelope proteins (Gerace *et al.*, 1978; Snow *et al.*, 1987). A similar labeling pattern for gp210 was previously obtained with chicken polyclonal antibodies (Gerace *et al.*, 1982). In addition to this nuclear staining, RL20 also labeled small cytoplasmic bodies that were frequently near the nucleus and that varied in number from cell to cell (Figure 3A, arrows). This cytoplasmic labeling was observed also with a hamster cell line (CHO) and two other rat cell lines (NRK and BRL) using several different monoclonal antibodies specific for gp210 (data not shown). The significance of this staining is presently unknown, but it may reflect cytoplasmic pore complexes of annulate lamellae (Gerace and Burke, 1988) or some uncharacterized biosynthetic intermediates of pore complexes.

To locate the RL20 epitope with respect to the topology of nuclear membranes, we performed indirect immunogold electron microscopy on salt-washed rat liver nuclear envelopes that had not been treated with detergent (Figure 4). Strikingly, in samples incubated with RL20 (Figure 4A-C) most gold labeling occurred inside the lumen of nuclear membranes in close vicinity to pore complexes (typically, gold was located ~80–120 nm from the center of the pore complex). The relatively small amount of labeling

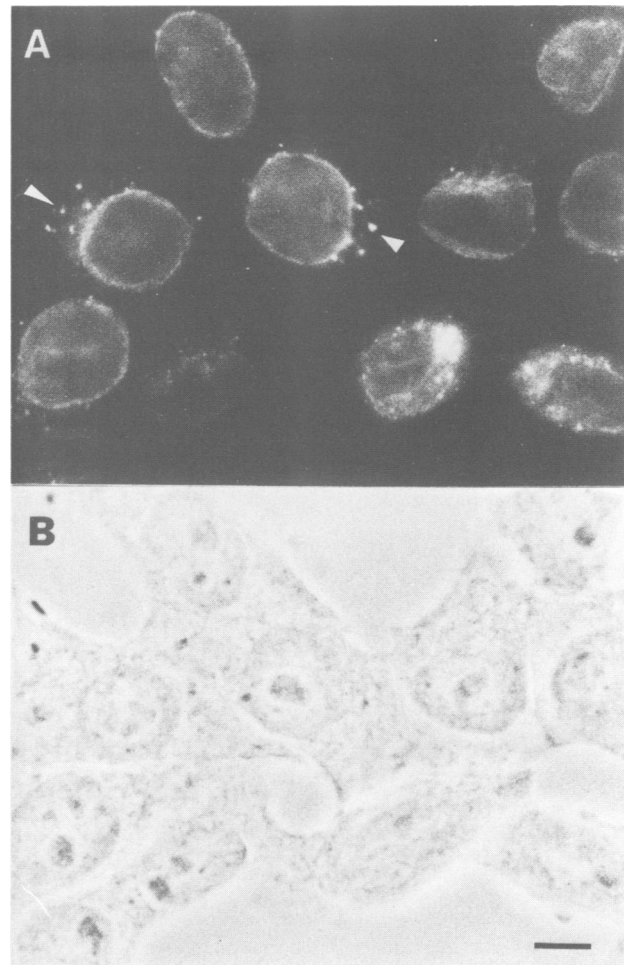


Fig. 3. Immunofluorescence localization of gp210 in cultured HTC cells with RL20. HTC cells grown on cover slips were labeled for indirect immunofluorescence microscopy with RL20. Shown are (A) a fluorescence micrograph of interphase cells and (B) the corresponding phase-contrast image. Arrowheads in (A) indicate punctate cytoplasmic staining seen to varying extents in different cells. Bar, 5 µm.

seen on the cytoplasmic side of nuclear membranes in these samples was similar to that obtained with a control antibody that did not react specifically with the nuclear envelope (Figure 4D). In sections tangential to the nuclear surface, roughly continuous labeling around the margins of the pore complex was observed with RL20 (Figure 4A and C, arrows), indicating that, to a first approximation, gp210 is distributed around the circumference of this structure. While most gold labeling occurred at recognizable pore complexes in structurally well-preserved areas of the nuclear envelopes, it is possible that a minor amount of gp210 in the nuclear envelope is not associated with the nuclear pore complex (e.g. in an unassembled pool) and could not be resolved by our labeling procedure.

Our results clearly demonstrate that the 200 kd domain of gp210 recognized by RL20 is located in the perinuclear space and is highly concentrated at the nuclear pore complex. They both confirm and extend the earlier immunoelectron microscopy studies on gp210, which were performed on Triton-treated nuclei (Gerace *et al.*, 1982), where gp210 remained attached to the pore complex after membranes were solubilized.

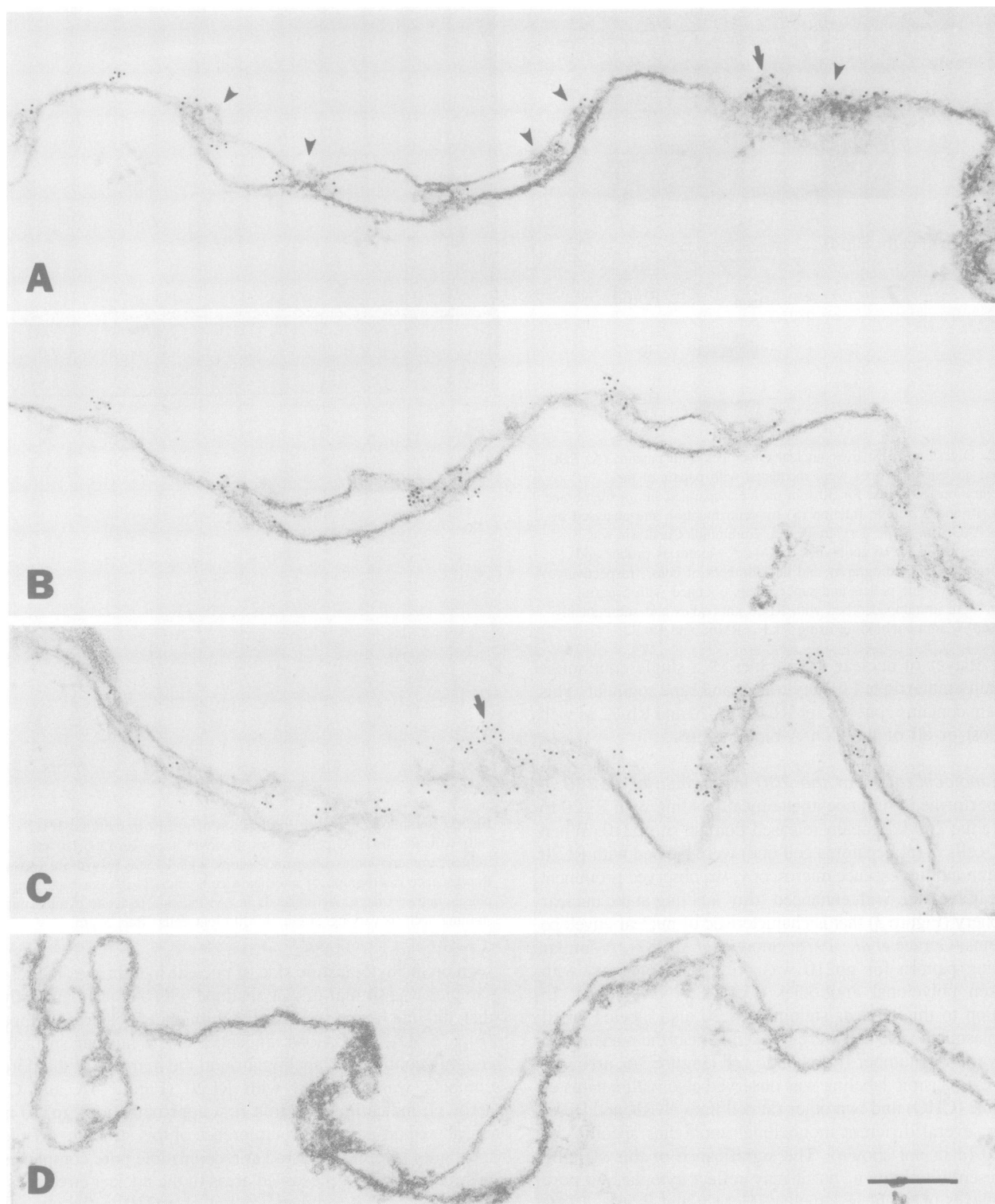


Fig. 4. Immunogold localization of the RL20 epitope in rat liver nuclear envelopes. Shown are thin section electron micrographs of salt-washed nuclear envelopes labeled with RL20 IgGs (A)–(C) or the control HA4 monoclonal (D) followed by rabbit anti-mouse IgG coupled to 5 nm gold particles. Examples of cross-sectioned pore complexes are indicated by arrowheads (A), while several tangentially cut pore complexes are indicated by arrows (A and C). Bar, 200 nm.

Gp210 has a transmembrane domain and a small cytoplasmic tail

Since a continuous 200 kd stretch of gp210 is external to the lipid bilayer, any stable insertions of gp210 into the membrane of nuclear envelopes must occur near the N- or C-terminus of the protein. An N-terminal membrane

insertion is inconsistent with the amino acid sequence of the mature protein, but a membrane insertion site at the C-terminus is possible, since gp210 contains a hydrophobic segment extending from amino acid 1809 to 1828.

To investigate whether gp210 has a transmembrane segment and cytoplasmic domain in this region, we attempted

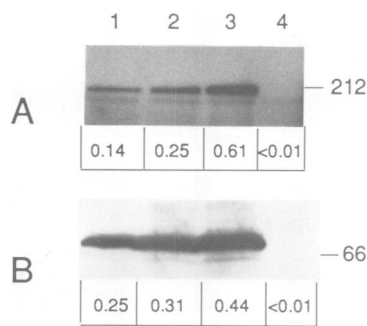


Fig. 5. Analysis of gp210 in mitotic cell homogenates by sucrose gradient centrifugation. Homogenates of mitotic HTC cells were adjusted to 1.65 M sucrose and overlaid with zones containing 1.4 M sucrose and 0.25 M sucrose. After centrifugation, the gradient was fractionated into pellet plus loading zone (lanes 1), the 1.65/1.4 M sucrose interface (lanes 2), the 1.4/0.25 M interface (lanes 3) and the 0.25 M sucrose zone (lanes 4). (A) 10^7 cell equivalents of each fraction were immunoadsorbed with RL20 and the bound material visualized with RL16. Immunoadsorptions were quantitative, as no additional gp210 protein was found by a second round of immunoadsorption. (B) 10^6 cell equivalents were analyzed by immunoblotting with a ribophorin I-specific antibody. Numbers at the bottom of (A) and (B) indicate the relative fraction of protein in each sample as determined by elution of silver grains from the autoradiogram (Suissa, 1983).

to prepare sealed membrane vesicles from rat liver nuclear envelopes to assess the protease sensitivity of C-terminal epitopes recognized by Rb-68 and Rb-72. Unfortunately, using either chemical or mechanical fragmentation approaches we were unsuccessful in preparing sealed vesicles in which the 200 kd luminal domain of gp210 was protected from added proteases.

As an alternative approach, we purified membranes from metaphase cells, where nuclear envelope and ER membranes are naturally fragmented into vesicles and small cisternae (for reviews, see Warren, 1985, 1989). Mitotic membranes were isolated from synchronized HTC cell populations containing 70–80% metaphase cells. After cell homogenization, membranes were floated from a loading zone into a sucrose step gradient and the fractions were analysed for the presence of gp210 by immunoblotting. This analysis showed that ~60% of the mitotic gp210 was recovered at the 1.4/0.25 M sucrose interface (Figure 5A, lane 3), the fraction where smooth ER membranes and most membranes of the Golgi apparatus of interphase cells would band (Kreibich *et al.*, 1978; Howell and Palade, 1982). Approximately 25% was at the 1.65/1.4 M sucrose interface, where most rough microsomal membranes of interphase cells would be found (Blobel and Sabatini, 1970; Kreibich *et al.*, 1978; Hortsch and Meyer, 1985). Roughly 14% of gp210 remained in the loading zone plus pellet fraction of the gradient, where interphase nuclei and membrane–cytoskeleton aggregates would appear. In contrast to mitotic cell homogenates, when an interphase cell homogenate was fractionated by this procedure, all of the detectable gp210 occurred in the pellet plus loading zone fraction (not shown).

The fractionation pattern of gp210 in mitotic cell homogenates was very similar to that of ribophorin I (Figure 5B), an integral membrane protein of the ER (Kreibich *et al.*, 1978; Marcantonio *et al.*, 1982). Approximately 44% of the total ribophorin I was recovered in the 1.4/0.25 M sucrose fraction, 30% was in the 1.65/1.4 M sucrose interphase and 25% in the loading zone. Thus, gp210

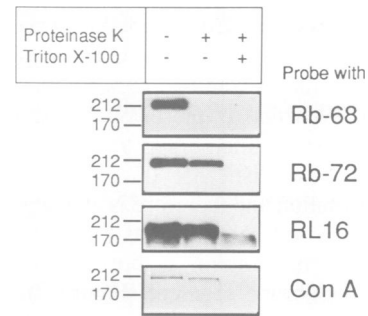


Fig. 6. Protease digestion of gp210 in mitotic membranes. HTC mitotic membranes collected from the 1.4/0.25 M sucrose interface (Figure 5; Materials and methods) were incubated with or without proteinase K (0.05 mg/ml) for 30 min on ice in the presence or absence of 0.5% Triton X-100. The reactions were stopped by adding protease inhibitors and samples were immunoadsorbed with RL20 antibodies, electrophoresed on an SDS gel (8% polyacrylamide) and analysed by immunoblotting with Rb-68, Rb-72 or RL16 or for glycoprotein detection with Con A.

fractionated with mitotic membranes as expected for an integral membrane protein.

To evaluate the protease sensitivity of gp210 in isolated mitotic membranes, we incubated the membrane fraction banding at the 1.4/0.25 M sucrose interface with proteinase K in the presence or absence of non-ionic detergent. After protease treatment, gp210 was enriched by immunoprecipitation with RL20 antibodies and then analyzed by immunoblotting using domain-specific gp210 antibodies. When membranes were proteolyzed in the absence of detergent, a fragment of slightly smaller than 210 kDa was protected from degradation (Figure 6). This fragment included the epitopes for Rb-72, located 222 amino acids from the C-terminus of the protein, as well as the epitopes for RL16 and RL20 and the binding sites for Con A (Figure 6). In contrast, the epitopes for Rb-68 located in the C-terminal 19 amino acids were completely degraded by this treatment. When the non-ionic detergent Triton X-100 was added to permeabilize membranes prior to protease digestion, no detectable fragment of gp210 was immunoadsorbed (Figure 6).

These data demonstrate that a major population of gp210 in mitotic membranes occurs in a sealed membrane compartment in which gp210 maintains the overall membrane orientation seen in interphase nuclear envelopes, i.e. it has a luminal domain >200 kd. Most importantly, these results show that the C-terminus of gp210 is on the cytoplasmic side of mitotic vesicles, as shown by the protease accessibility of the Rb-68 epitope. Thus, gp210 must have a membrane-spanning segment near the C-terminus in mitotic membranes. It is very likely that gp210 retains the same transmembrane topology in interphase nuclear envelopes.

Discussion

In this study we investigated the membrane orientation of gp210, a glycoprotein tightly associated with nuclear membranes that we previously found to be located at the nuclear pore complex (Gerace *et al.*, 1982). Since this is a particularly abundant component (present at ~25 copies per pore complex), it could be important for pore complex structure. Many different topological arrangements of gp210

are theoretically possible from its deduced sequence, since mature gp210 contains two segments that could potentially serve as membrane-spanning regions based on their hydrophobicity (Wozniak *et al.*, 1989). Using antibodies and proteases to probe the membrane topology of gp210, our studies directly establish that this protein has a transmembrane orientation and therefore is an integral membrane protein. As discussed below, it has an exceptionally large glycosylated luminal domain, a short cytoplasmic tail and a single transmembrane segment (Figure 7).

Most of the mass of gp210 can be released from membranes as a water-soluble fragment of >200 kd by controlled digestion of isolated nuclear envelopes with papain under conditions of approximately physiological ionic strength and pH. This soluble 200 kd fragment contains the Con A-reactive carbohydrate and epitopes present on a peptide encompassing amino acids 1648–1664 recognized by Rb-72. Therefore, it contains one of the two major hydrophobic regions of gp210 at amino acids 1482–1504, termed HS1 (Figure 7). While HS1 comprises a 23 residue stretch of predominantly hydrophobic amino acids that could potentially span a lipid bilayer (Wozniak *et al.*, 1989), our data indicate that this region has no stable hydrophobic interaction with membranes of rat liver nuclear envelopes. However, a transient or peripheral association of this region with membrane lipids could possibly occur during pore complex biogenesis (see below).

Indirect immunogold labeling of nuclear envelopes that were not treated with detergents directly demonstrated that the 200 kd papain-releasable domain of gp210 is located on the luminal side of nuclear membranes. Most of the detectable gp210 occurs at or very near pore complexes, confirming our previous immunoelectron microscopy studies performed with detergent-treated nuclei (Gerace *et al.*, 1982). While our analysis did not determine the location of gp210 with respect to different substructures of the pore complex, we often obtained approximately uniform gold labeling around the circumference of pore complexes viewed in tangential sections, suggesting that gp210 may be periodically associated with one of the major 8-fold symmetrical substructures of the pore complex (i.e. the peripheral rings or central spokes; Unwin and Milligan, 1982). Interestingly, recent studies involving image analysis of pore complexes visualized in vitreous ice revealed eight 'radial arms' extending outward an additional 12–14 nm beyond the 60 nm outer radius of the peripheral rings (Akey, 1989). Since these radial arms are the most peripheral structures of the pore complex, they could in part or entirely contain the luminal domain of gp210, based on our immunolocalization results.

To resolve definitively the question of whether gp210 is a transmembrane protein, we examined the protease accessibility of different regions of this protein in isolated mitotic membranes, where most of the gp210 was in a sealed membrane compartment. Proteinase K digestions of these sealed mitotic membranes directly demonstrated that gp210 has a short cytoplasmically exposed region at its C-terminus. Epitopes present in 19 C-terminal amino acids were degraded by digestion with proteinase K, while epitopes present on the luminal 200 kd domain were protected. Since the region of gp210 up to at least residues 1648–1664 is lumenally disposed, a transmembrane sequence must occur between this domain and the C-terminus. This transmembrane stretch

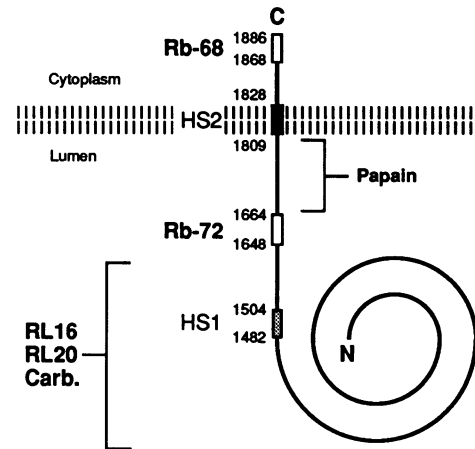


Fig. 7. Model describing the membrane topology of gp210. The schematic drawing shows that gp210 is a transmembrane glycoprotein with a large luminal domain and a small cytoplasmic tail. The luminal domain, which has a region of high papain sensitivity close to the membrane, contains the Con A-reactive sites (Carb.), the RL16 and RL20 epitopes and also the epitopes for the anti-peptide antibody Rb-72 (displayed as an open box). The cytoplasmic tail, bearing the epitopes for the anti-peptide antibody Rb-68 (indicated by an open box), is separated from the luminal domain by the transmembrane segment HS2 (displayed as a closed box). The other hydrophobic segment, HS1 (displayed by a hatched box), is not stably associated with the lipid bilayer. Numbers designate the amino acid residues from the amino terminus of the full-length protein to the C-terminus (1886), as derived from the cDNA sequence (Wozniak *et al.*, 1989).

very likely occurs at the single large hydrophobic segment of gp210, at residues 1809–1828, termed HS2 (Figure 7). Thus, it is likely that gp210 has a cytoplasmic tail composed of 58 amino acids and a mature luminal domain of 1783 amino acids including Con A-reactive carbohydrates and a region of high papain sensitivity close to the membrane-spanning segment (Figure 7).

Potential role of gp210 in pore complex structure and assembly

Most of the mass of the pore complex is found on the cytoplasmic surface of nuclear membranes (Franke, 1974), where all detectable events of nucleocytoplasmic transport take place (Akey and Goldfarb, 1989). In contrast, only a small region of gp210 is found in the cytoplasm. This argues that gp210 is not in direct contact with the putative 'transporter' of the pore complex. Rather, the results in this paper combined with previous studies (Gerace *et al.*, 1982) suggest that gp210 is involved primarily in organizing pore complex structure.

Gp210 could be important for pore complex architecture in several ways. First, it could provide a membrane anchoring site for certain substructures of the pore complex (Unwin and Milligan, 1982; Akey, 1989). Both the cytoplasmic and luminal domains of gp210 could be involved in membrane attachment, through interactions with other peripheral or integral proteins located lumenally or cytoplasmically. Because of its large size, the luminal domain of gp210 in principle could engage in multiple different protein–protein interactions, both homotypic and heterotypic. Second, gp210 may be an integral component of certain substructures of the pore complex such as the rings or spoke assembly, acting as a 'linchpin' for these structures in addition to attaching them to the membrane. However,

since the pore complex contains a number of different substructures that are closely associated with the membrane, it is reasonable to expect that other integral proteins in addition to gp210 are involved in attaching the pore complex to membranes.

Interestingly, the transmembrane topology of gp210 closely resembles the topology of envelope glycoproteins of many animal viruses, e.g. the vesicular stomatitis virus (VSV) G glycoprotein (Rose *et al.*, 1980), the Semliki forest virus (SFV) E2 glycoprotein (Garoff and Simons, 1974) and influenza virus hemagglutinins (for review, see Nayak and Jabbar, 1989). These viral glycoproteins have a large luminal domain, a single transmembrane segment and a short C-terminal cytoplasmic tail (typically 10–40 residues). Gp210 may resemble in function as well as in structure these viral proteins, whose cytoplasmic tails are thought to mediate attachment of a supramolecular complex (the viral nucleocapsid) to the membrane, as demonstrated for the SFV E2 glycoprotein (Vaux *et al.*, 1988).

Assuming that gp210 is involved in the structure of the pore complex, then it also must have a role in pore complex assembly, which occurs throughout interphase in cycling cells as well as at the end of mitosis (Gerace and Burke, 1988; Lohka, 1988). Gp210 could direct assembly of the pore complex at membrane surfaces by providing a membrane anchor and/or an essential component for formation of pore complex substructures.

In addition, gp210 conceivably could have a role in fusion of inner and outer membranes (Wozniak *et al.*, 1989), a process required for pore complex assembly and probably coordinated with the latter. The hydrophobic segment of gp210 that is not stably integrated into the lipid bilayer of nuclear membranes (HS1) could be involved in such a fusion process, if initiation of pore complex assembly alters the conformation of the luminal domain of gp210 such that HS1 becomes exposed and favorably interacts with membrane lipids. In this respect HS1 could be analogous to apolar segments present in the luminal domains of many viral fusion proteins such as influenza hemagglutinins (see, e.g. Daniels *et al.*, 1985) and the simian virus 5 (SV5) envelope glycoprotein F (Paterson and Lamb, 1987). These viral sequences are thought to interact with lipids only after a conformational change in the protein is induced, often upon proteolysis and/or acid pH (for review, see White, 1990). Since HS1 of gp210 does not stably interact with the membrane bilayer in assembled pore complexes, it would be necessary for gp210 to undergo further conformational changes during ensuing steps of pore complex assembly to release HS1 from a lipid environment and fold it back into a protein interior.

Materials and methods

Isolation and fractionation of rat liver nuclear envelopes

Rat liver nuclei were isolated as described (Gerace *et al.*, 1978). Nuclear envelopes were prepared from rat liver nuclei (Dwyer and Blobel, 1976) and salt-washed (Snow *et al.*, 1987) according to previous procedures, except that the solutions were buffered with HEPES–KOH, pH 7.35, instead of TEA–HCl, pH 7.4, and did not contain protease inhibitors.

For alkali extraction of salt-washed nuclear envelopes, membrane pellets were resuspended at 0.6 mg/ml protein in ice-cold 0.1 M sodium carbonate containing 5 mM DTT, incubated for 15 min and pelleted at 100 000 g for 30 min at 4°C (Fujiki *et al.*, 1982).

Gp210 was purified from salt-washed nuclear envelopes by a modification of a previous procedure (Gerace *et al.*, 1982). Briefly, membranes were

solubilized at ~2 mg/ml protein in high salt buffer (0.5 M NaCl, 0.02 M HEPES–NaOH, pH 7.35, 1 mM MgCl₂, 1 mM DTT, 1 mM PMSF) containing 2% Triton X-100 for 30 min at 4°C. The extract was then centrifuged at 50 000 g for 30 min and the supernatant was passed over a column of *Lens culinaris* agglutinin (lentil lectin, Sigma) conjugated to agarose (3000 OD units of nuclear envelopes/5 ml agarose) under continuous recycling for 14 h at 4°C. [One unit of nuclear envelopes is the amount derived from 1 A₂₆₀ unit of isolated nuclei (Dwyer and Blobel, 1976).] The column was washed with 20 volumes of high salt buffer containing 0.1% Triton X-100 and gp210 was eluted with high salt buffer containing 0.5 M α-methylmannoside and 0.1% Triton X-100.

Isolation of mitotic membranes

Two liters of rat hepatoma cells (HTC) growing exponentially in suspension culture at 3 × 10⁵ cells/ml were arrested in S phase by adding 2 mM thymidine the growth medium [Joklik's spinner medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Suprynowicz and Gerace, 1986)] and incubating for 12 h at 37°C. The cells were then washed once in growth medium, resuspended in 2 l of fresh medium and incubated for a further 12 h in medium containing 0.1 µg/ml nocodazole to arrest cells in metaphase (Zieve *et al.*, 1980). This yielded a cell population of 70–80% metaphase-arrested cells. Cells were then centrifuged at 500 g and incubated in 50 ml of medium containing 0.02 mM cytochalasin B and 0.1 µg/ml nocodazole for a further 30 min at 37°C prior to homogenization.

After treatment with cytochalasin B populations enriched in metaphase cells were washed twice in PBS and once in H buffer (0.05 M HEPES–KOH, pH 7.35, 0.09 M KOAc, 2 mM MgCl₂, 1 mM DDT). Cell pellets were then resuspended in 1 volume of H buffer and homogenized with 20 strokes of a tight fitting dounce homogenizer. The homogenate was adjusted to 1.65 M sucrose with H buffer containing 2.3 M sucrose, overlaid with 5 ml of H buffer containing 1.4 M sucrose, 5 ml of H buffer containing 0.25 M sucrose and 1 ml H buffer, and centrifuged at 120 000 g for 8 h in an SW28 rotor.

Protease treatment of membranes

Salt-washed nuclear envelopes (60 OD) were resuspended at 0.7 mg/ml protein in 0.25 M sucrose, 0.02 M HEPES–KOH, pH 7.35, 0.05 M KOAc, 0.001 M EDTA, 1 mM DDT and incubated with various concentrations of papain (Boehringer) for 30 min on ice. The reactions were stopped by sequentially adding *N*-ethylmaleimide (5 mM), PMSF (1 mM), leupeptin and pepstatin (1 µM each) and samples were centrifuged at 100 000 g for 1 h to yield pellets and supernatants.

For protease treatment of mitotic membranes, material from the 1.4/0.25 M sucrose interface was removed from the gradient (see above) and diluted with H buffer to a protein concentration of 1.5 mg/ml. As required, 1 mg/ml proteinase K (freshly prepared in H buffer containing 0.1 mM CaCl₂) and 10% Triton X-100 (Pierce) were added to final concentrations of 0.05 mg/ml and 0.5%, respectively. The samples were incubated on ice for 30 min and the reactions were stopped by sequentially adding protease inhibitors described for the papain digestions, except that *N*-ethylmaleimide was omitted.

Preparation of monoclonal and polyclonal antibodies and immunolocalization of gp210

The monoclonal antibody RL16 (an IgG1) was obtained after immunizing mice with a pore complex lamina fraction of rat liver nuclear envelopes (Snow *et al.*, 1987), while RL20 (IgG1) was isolated after immunization of mice with purified gp210. Production of ascites fluid and purification of monoclonal IgGs over DEAE–Sephacel was done as described (Kiehart *et al.*, 1984; Williams and Chase, 1967). Polyclonal gp210-specific anti-peptide antisera were obtained by immunizing rabbits with peptide–keyhole limpet hemocyanin (KLH) conjugates. The peptides used for immunization (Multiple Peptide Systems, La Jolla) corresponded to amino acids 1868–1886 (Rb-68) and 1648–1664 (Rb-72) of the deduced amino acid sequence of gp210 (Wozniak *et al.*, 1989). Each contained an additional amino terminal cysteine residue for coupling to KLH using *m*-maleimido-benzoyl-*N*-hydroxysuccinimide ester (MBS) (Lui *et al.*, 1979) at a peptide to carrier ratio of 1/0.8 (w/w). The following immunization schedule was used: day 1, subcutaneous injection of the peptide–KLH conjugate (150 µg) emulsified in complete Freund's adjuvant (1:1); days 14 and 23, subcutaneous injections of 150 µg in incomplete Freund's adjuvant (1:1). Serum used in this study was taken on day 26.

For immunofluorescence localization of gp210, HTC cells were grown on glass coverslips, fixed by immersion in 3% *p*-formaldehyde for 4 min and permeabilized in 0.2% Triton X-100 as described (Warren *et al.*, 1984). The samples were then incubated with RL20 monoclonal antibodies (2 µg/ml)

followed by rhodamine conjugated rabbit anti-mouse IgG (2 µg/ml, Cappel) that had been preadsorbed against *p*-formaldehyde fixed rat liver homogenate.

Indirect immunogold electronmicroscopy of RL20 was performed on salt-washed rat liver nuclear envelopes exactly as described (Snow *et al.*, 1987) using rabbit anti-mouse IgG conjugated to 5 nm gold (Jannsen Life Sciences Products) to detect the mouse antibodies. A mouse monoclonal IgG HA4 provided by Dr Ann Hubbard was used as an irrelevant control antibody for these labeling studies.

Immunoadsorption, gel electrophoresis and immunoblotting

For immunoadsorption of gp210, proteins were precipitated with 15% TCA and washed in acetone/1 M HCl (9:1) on ice. They were then resuspended in 0.5 ml 1% SDS containing 0.02 M Tris-HCl, pH 7.4, and samples were adjusted to contain 1% Triton X-100, 0.1% SDS, 0.4 M NaCl, 0.02 M HEPES-NaOH, pH 7.4 and 2 mM EDTA. After addition of PMSF, leupeptin, pepstatin and aprotinin (see above), samples were incubated for 14 h at 4°C with purified monoclonal antibodies conjugated to CNBr-Sephacrose 4B (Pharmacia) at 3 mg/ml antibody/Sephacrose. The immuno-beads were then washed 4 times in immunoadsorption buffer and once in the same buffer containing 0.1 M NaCl and lacking detergents and protease inhibitors. Adsorbed proteins were finally eluted and prepared for SDS gel-analysis as described (Gerace and Blobel, 1980).

SDS-PAGE was performed in either 5% or 8% polyacrylamide gels (Laemmli, 1970) as indicated. Protein samples were prepared for SDS gels as described (Gerace and Blobel, 1980) after precipitation with TCA and extraction with acetone/HCl (see above). Apparent molecular weights of proteins were determined from their mobilities compared to commercial standards (Pharmacia): rabbit muscle myosin (212 kd), bovine plasma α₂-macroglobulin (170 kd), *Escherichia coli* β-galactosidase, (116 kd), human transferrin (76 kd) and bovine liver glutamic dehydrogenase (53 kd).

For immunoblotting, proteins were transferred from SDS-polyacrylamide gels to nitrocellulose filters (0.2 µm, Schleicher and Schuell) as described (Gerace *et al.*, 1982) and processed for antigen detection using [¹²⁵I]protein A (Snow *et al.*, 1987). Glycosylated gp210 was visualized by incubating nitrocellulose filters with biotinylated Con A (10 µg/ml, United States Biochemicals), followed by avidin-peroxidase (5 µg/ml, ICN Biochemicals) in a buffer containing 0.25 M NaCl, 0.02 M Tris-HCl, pH 7.4, 2 mM MgCl₂, 2 mM CaCl₂ and 1% BSA as a blocking agent. Diaminobenzidine was used as a peroxidase substrate.

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