The Single Transmembrane Segment of gp210 Is Sufficient for Sorting to the Pore Membrane Domain of the Nuclear Envelope

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Abstract. The glycoprotein gp210 is located in the "pore membrane," a specialized domain of the nuclear envelope to which the nuclear pore complex (NPC) is anchored. gp210 contains a large cisternal domain, a single transmembrane segment (TM), and a COOH-terminal, 58-amino acid residue cytoplasmic tail (CT) (Wozniak, R. W., E. Bartnik, and G. Blobel. 1989. J. Cell Biol. 108:2083-2092; Greber, U. E, A. Senior, and L. Gerace. 1990. EMBO (Eur. Mol. Biol. Organ.) J. 9:1495-1502). To locate determinants for sorting of gp210 to the pore membrane, we constructed various cDNAs coding for wild-type, mutant, and chimeric gp210, and monitored localization of the expressed protein in 3T3 cells by immunofluorescence microscopy using appropriate antibodies. The large cisternal domain of gp210 (95% of its mass) did not reveal any sorting determinants. Surprisingly, the TM of gp210 is sufficient for sorting to the pore membrane. The CT also contains a sorting determinant, but it is weaker than that of the TM. We propose specific lateral association of the transmembrane helices of two proteins to yield either a gp210 homodimer or a heterodimer of gp210 and another protein. The cytoplasmically oriented tails of these dimers may bind cooperatively to the adjacent NPCs. In addition, we demonstrate that gp210 co-localizes with cytoplasmically dispersed nucleoporins, suggesting a cytoplasmic association of these components.

The nuclear envelope (NE) consists of three morphologically and biochemically distinct domains (for review see 9, 12). The outer nuclear membrane domain contains bound ribosomes and is continuous with and biochemically indistinguishable from the RER. The inner nuclear membrane domain is adjacent to the nuclear lamina and the underlying chromatin, and contains a distinct set of integral membrane proteins (1, 29, 36). Finally, the outer and inner membranes of the NE are connected with each other at numerous sites forming circular openings in the NE (nuclear pores) of ~100 nm diam. The connecting membrane is sharply bent (180°), and is referred to as the "pore membrane" domain of the NE. The nuclear pore is occupied by the nuclear pore complex (NPC) and the pore membrane is adjacent to the NPC (for review see 9, 21). The pore membrane contains at least one distinct glycoprotein, referred to as gp210 (10, 37). This, and perhaps other yet to be identified pore membrane–specific integral proteins, may function in nuclear pore formation and in the assembly and attachment of the NPC to the pore membrane.

Gp210 has been estimated to be present in 16-24 copies per nuclear pore (9). Its primary structure has been deduced from overlapping cDNA clones (37), and its topology has been determined using proteolytic enzymes and domain-specific antibodies (11; E. Bartnik, R. W. Wozniak, and G. Blobel, unpublished results). gp210 has an estimated mass of ~210,000 D, 5% of which consists of N-linked, high mannose-type oligosaccharides (2, 37). The deduced primary structure reveals an NH2-terminal, cleaved signal sequence of 1,861 residues of the mature gp210. The NH2-terminal 1,783 residues of mature gp210 are exposed on the intracisternal side of the pore membrane. This is followed by a transmembrane segment (TM) of 21 residues and a COOH-terminal tail of 58 residues. The tail is exposed at the nuclear pore and is therefore a potential anchor for components of the NPC.

How is gp210 sorted to the pore membrane after its signal- and stop-transfer sequence–mediated integration into the RER and the outer nuclear membrane? Like resident integral proteins of the RER and the NE, gp210 is designed to resist bulk flow from the RER into downstream membranes of the exocytotic pathway. In addition, it is sorted specifically to the pore membrane of the NE. Retention in the ER of a number of resident integral proteins, both endogenous and viral, has been shown to be mediated by sequences in the cytoplasmically exposed domains of these proteins (13, 24, 26). Because of its likely interaction with the NPC, the cytoplasmically exposed tail of gp210 seemed a likely candidate to be the determinant for sorting to the pore membrane. However, using various mutant forms of gp210, we found that its TM is sufficient for sorting to the pore membrane. The cytoplasmic tail (CT) serves as an additional, but weaker, sorting determinant.

1. Abbreviations used in this paper: CT, cytoplasmic tail; HA, hemagglutinin; NE, nuclear envelope; NPC, nuclear pore complex; PCR, polymerase chain reaction; TM, transmembrane segment.
Expression of Recombinant cDNAs in Mouse Balb/c 3T3 Fibroblasts

The expression of chimeric proteins in mouse Balb/c 3T3 cells was accomplished by direct microinjection of plasmid DNA into nuclei using a procedure described by Capecchi (5). Subconfluent cultures of Balb/c 3T3 cells were grown on 12-mm-diam. glass coverslips in DME medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Gibco Laboratories), 20 mM Hepes, pH 7.4, and 50 μg/ml Gentamycin (Gibco Laboratories). Nuclei were injected with plasmid DNA purified using a plasmid isolation kit (Qiagen Inc., Chatsworth, CA) at 1 μg/ml in PBS containing 1 mM MgCl2. The microinjection equipment consisted of an Eppendorf injector 5.242 and a Zeiss micromanipulator MR mounted on a Zeiss Axiosvert 10 microscope (Zeiss, Oberkochen, Germany). After injection, the cells were returned to a 5% CO2 incubator at 37°C for 4–5 h.

Immunofluorescence Microscopy

Coverslips containing injected 3T3 cells were processed for immunofluorescence microscopy. Cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After blocking with 2% normal horse serum, coverslips were incubated with primary antibodies at 4°C overnight. Secondary antibodies conjugated to Alexa Fluor 488 or 594 (Invitrogen) were added for 1 h. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole). Images were captured using a Zeiss Axiosvert 10 microscope equipped with an AxioCam MRm camera and analyzed with ImageJ software.

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Results

After its integration into the ER membrane, gp210 is sorted to the pore membrane, most likely by lateral diffusion in the plane of the membrane. To locate the determinant(s) for sorting, we constructed various cDNAs coding for wild-type, mutant, and chimeric gp210 (Fig. 1); subcloned them into a transient eukaryotic expression vector (pSVL); injected the DNA directly into the nuclei of subconfluent, unsynchronized 3T3 cells; and monitored localization of the expressed protein by immunofluorescence microscopy using the appropriate antibodies. Typically, 100–200 cells were injected for each experiment and the location of expressed protein was assessed 4–5 h after injection. This time period was experimentally determined to be optimal as it yielded levels of expressed protein that were sufficient for immunofluorescence detection but avoided overexpression and concomitant mislocalization.

Epitope-tagged gp210:HA Is Sorted to the Pore Membrane

To distinguish the expression of recombinant from endogenous gp210, we inserted an epitope tag encoding 12-amino acid residues of HA (8, 34) after the second residue of the mature form of gp210 (see Fig. 1). Using an mAb against the tag (anti-HA antibody), the expressed gp210:HA showed the punctate distribution at the nuclear surface (Fig. 2 A) that was characteristic for endogenous gp210 in the pore membrane (6). To further substantiate the localization of gp210:HA in

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**Figure 1.** Schematic representations of cDNA constructs. The terms used for each construct are shown on the left. The amino terminus (N) and the carboxy terminus (C) are indicated. Segments positioned to the left of the schematic lipid bilayer lie within the luminal or extracellular compartments and those to the right extend into the cytosol. Amino acid residues within regions of interest are listed below each schematic diagram. Those residues that span the membrane are underlined. Regions of the constructs derived from gp210 or CD8 are distinguished graphically and by print type (gp210 === or CD8 ——). Amino acid residues are numbered in accordance with publications describing their positions within the wild-type proteins (18, 37). The arrowheads in gp210:HA and gp210-(TM + CT):HA point to the approximate position of the HA epitope tag.

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Wozniak and Blobel Sorting of gp210 to the Nuclear Pore Membrane
gp210: HA

Figure 2. gp210:HA is targeted to the pore membrane domain. Cells expressing the epitope-tagged gp210:HA protein (see Fig. 1) were fixed, permeabilized, and probed with an mAb (12CA5) directed against the HA epitope tag. Binding to gp210:HA was visualized with Texas red-labeled goat anti-mouse IgG (mAb anti-HA, A). The locations of pore complexes within the same cell were determined by probing with a FITC-labeled mAb specific for a subset of the nucleoporins (mAb 414, B). The focal plane shown in A and B is tangential to the nuclear surface. gp210:HA is visible at densely packed points along the surface of the nucleus and, to a much lesser extent, in a dispersed pattern in the cytoplasm. Note the extensive coincidence between this pattern and the NPC pattern shown in B. Bar, 10 μm.

the pore membrane, we carried out double immunofluorescence with mAb 414 (Fig. 2 B). This mAb reacts with several nucleoporins (7) (all proteins of the NPC, excluding integral proteins of the pore membrane). A comparison of the two staining patterns (Fig. 2, A and B) showed considerable congruity, not only between the densely punctate pattern at the nuclear surface, but also between the more dispersed punctate pattern observed in the cytoplasm. The matching patterns in the cytoplasm suggest that gp210:HA and at least some of the nucleoporins are present as assembled structures in the ER. These structures might be similar to annulate lamellae (15). Together these data suggest that epitope tagging of gp210 does not interfere with sorting.

Gp210:HA Lacking the TM and the CT Is Not Sorted to the Pore Membrane

Having shown that gp210:HA is correctly sorted, we constructed a mutant cDNA coding for a protein that lacked both the TM and the CT, referred to as gp210-(TM + CT):HA (see Fig. 1). This protein showed a distribution characteristic of ER proteins (Fig. 3 B) and was no longer visible in the pore membrane, either when focusing on the nuclear surface (not shown), or when focusing on the nuclear rim (Fig. 3 B). Double immunofluorescence with mAb 414 did show the characteristic punctate rim pattern of the nucleoporins (Fig. 3 A). Likewise, a punctate nuclear rim staining pattern was seen with the anti-HA antibody in cells expressing gp210:HA (Fig. 3 C). We conclude that the NH2-terminal ectoplasmic domain preceding the TM and comprising ~95% of gp210 lacks topogenic determinants for sorting to the pore membrane.

The TM of gp210 Is Sufficient for Sorting to the Pore Membrane

As the NH2-terminal ectoplasmic domain of gp210 is not localized to the pore membrane, the topogenic determinants for pore membrane localization of gp210 must reside either in its TM, or in its CT, or in both. To distinguish between these possibilities, we constructed a cDNA coding for a chimeric protein, referred to as CD8/gp(TM + CT) (see Fig. 1) that consists of the ectoplasmic domain of CD8, used as a reporter, and the TM plus the CT of gp210. CD8 is an integral protein of the plasma membrane present in a subset of human T lymphocytes (27). Like gp210, CD8 is a bitopic integral membrane protein. Its NH2-terminal ectoplasmic domain (161 residues) is followed by a TM and a CT (28 residues) (18, 31). After signal- and stop-transfer sequence-mediated integration into the RER, CD8 ends up, via the exocytic pathway, in the plasma membrane. If the TM and the CT (TM + CT) portions of gp210 contain the determinants for sorting to the pore membrane, the chimera CD8/gp(TM + CT) should be localized to the pore membrane. Indeed, staining of cells expressing this fusion protein with an anti-CD8 antibody showed a characteristic punctate pore
Figure 3. The targeting of gp210:HA to the pore membrane is dependent on the presence of the TM and the CT. Cells expressing the gp210-(TM + CT):HA mutant (see Fig. 1) were examined using the double-immunofluorescence procedure described in Fig. 2 to compare the localization of this chimeric protein (mAb anti-HA, B) to the position of pore complexes (mAb 414, A). For comparison, a cell expressing gp210:HA is shown in C. In each case, the focal plane passes through the center of the nucleus and reveals the nuclear rim. In this focal plane, pore complexes are visible as a punctate ring around the nucleus (A). Note the presence of the gp210-(TM + CT):HA protein in the ER (B) and compared this with the positions of pore complexes (A) and the contrasting pattern seen for gp210:HA (C). Bar, 10 μm.

The CT of gp210 Is a Sufficient, but Weak, Determinant for Sorting to the Pore Membrane

To examine whether the CT of gp210 by itself contains topogenic determinants for pore membrane localization, we prepared a chimeric construct referred to as CD8/gpCT, where the CT of CD8 was replaced with that of gp210 (see Fig. 1). Immunofluorescence with anti-CD8 antibody (Fig. 6 B) detected CD8/gpCT at the plasma membrane, in a densely stained region near the nucleus (presumably representing the Golgi complex), and in a punctate pattern along the nuclear rim. This punctate nuclear rim pattern of CD8/gpCT matched the nucleoporin pattern when cells were double stained with mAb 414 (Fig. 6 A). However, the match was less than perfect, apparently obscured by the Golgi and plasma membrane localization of CD8/gpCT. Additional support for a pore membrane localization of CD8/gpCT was provided by comparing its staining pattern with that of CD8/E19, a resident integral membrane protein of the ER (13, 24, and Fig. 6 C). CD8/E19 is a chimeric protein where the CT of CD8 has been replaced by the CT of E19, a viral protein that is retained in the ER (30). Although both chimeric proteins showed nuclear rim staining, only CD8/E19 additionally showed the typical ER staining pattern, indicating that the nuclear rim staining of CD8/gpCT is likely the result of localization to the pore membrane, rather than retention in the ER. Moreover, a deletion mutant of CD8/gpCT lacking 20 COOH-terminal residues from the CT (CD8/gpCT-20, see Fig. 1) is no longer localized to the pore membrane, but rather is present exclusively at the plasma membrane (Fig. 7, compare A and B). Taken together, these data suggest that the CT of gp210 contains a determinant for sorting to the pore membrane and that this determinant might reside in its COOH-terminal 20 residues.

Discussion

Most integral proteins of the membranes in the exo- and endocytotic pathway are asymmetrically integrated into the ER
Figure 4. A reporter protein can be targeted to the pore membrane by the TM and the CT of gp210. 3T3 cells expressing the chimeric protein CD8/gp(TM + CT) (see Fig. 1), were examined by double immunofluorescence microscopy. Cells were fixed, permeabilized, and probed with an mAb, OKT8, directed against the extracellular domain of CD8 (mAb anti-CD8; A). Binding of anti-CD8 antibody was determined with Texas red-labeled goat anti-mouse IgG. Two cells expressing the CD8/gp(TM + CT) chimera are shown in A. The focal plane is positioned to show the nuclear surface of one cell and the nuclear rim of the other. The locations of pore complexes within the same cells were identified using FITC-labeled mAb 414 (C). Those cells not expressing the chimeric protein are recognized only by mAb 414. Note the extensive coincidental staining at the nuclear surface and in the cytoplasm when comparing A and C. For comparison, the cell-surface expression of wild-type CD8 in 3T3 cells is shown in B. Bar, 10 μm.
Targeting to the pore membrane of the CD8/gp(TM + CT) fusion protein is unaffected by deletions of the CT. 3T3 cells expressing the COOH-terminal deletion mutant CD8/gp(TM + CT)-20 or CD8/gp(TM + CT)-54 (see Fig. 1) were examined using the procedure described in Fig. 4 to visualize the chimeric proteins (mAb anti-CD8; A and B) and the pore complexes (mAb 414; C and D). All cells are recognized by mAb 414 (C and D), whereas only those expressing the chimeric proteins are detected with the anti-CD8 antibody (A and B). Bar, 10 μm.

It should be noted that at present little is known about the dynamics of pore formation and NPC assembly or the stability of the interacting components. Whether involved in de novo formation of pores or in lateral exchange, we suggest that gp210’s TM functions as the dominant sorting determinant because of the specific surface features of the α-helix that it forms. These features would allow specific lateral interactions with another transmembrane α-helix, either of gp210 or another integral membrane protein, to form a homodimer or heterodimer, respectively.

That lateral interactions between α-helices of TMs of integral membrane proteins can be specific was first demonstrated for the dimer of human erythrocyte glycophorin A (4, 16). Disruption of the dimer can be accomplished with a synthetic peptide representing the TM of glycophorin A but not with similar peptides representing the TMs of other integral membrane proteins (4, 16). Limited mutational analysis of this interaction suggests that close packing of side chains at the interface of adjacent helices may be required for dimerization (for a review of other examples of specific interactions via transmembrane helices see reference 16).

The TM of gp210 does not contain charged residues; thus, dimerization stabilized by charged ion pairs can be ruled out. It is noteworthy that the aromatic residues, two tyrosines and four phenylalanines, are arranged on one face of the membrane helix, with five of the six aromatic residues located on the NH₂-terminal half of the α-helix embedded into the cisternal leaflet of the pore membrane bilayer. Whether these or other features of the gp210 transmembrane α-helix allow specific lateral interactions with another transmembrane α-helix remains to be investigated.

There are precedents for TMs serving as topogenic determinants. Most resident integral proteins of the Golgi membrane that have so far been analyzed are retained there by sorting determinants in their TMs (20, 23, 25, 28, 33, 35, for review see 19).

The sorting determinants in gp210’s CT, albeit apparently weaker than those of the TM, are likely to be relevant. If gp210 were to form homodimers via the TM, this could spacer...
Figure 6. The CT of gp210 also contains a pore membrane targeting signal. The chimeric protein CD8/gpCT (see Fig. 1) was expressed in 3T3 cells and double immunofluorescence was performed, as described in Fig. 4, to visualize the chimeric protein (mAb anti-CD8, B) and pore complexes (mAb 414, A). Also shown is the distribution pattern of the CD8/E19 chimeric protein within a separate cell (C). In all cases, the focal plane shown passes through the center of the nucleus. In focus are the nuclear rim, adjacent regions of the cytoplasm, and portions of the plasma membrane. Note the presence of CD8/gpCT (B) at the cell surface as well as at the NE in a pattern similar to that revealed with mAb 414 (A). This distribution is compared to the CD8/E19 protein which is retained within the ER network and the NE (C). Bar, 10 μm.

Figure 7. A 20-amino acid residue deletion from the COOH-terminal tail of the CD8/gpCT chimera abolishes NE localization. 3T3 cells expressing CD8/gpCT (A) or the deletion mutant CD8/gpCT-20 (B) were fixed, permeabilized, and probed with anti-CD8 antibody. Binding was visualized with Texas red-labeled goat anti-mouse IgG. The focal plane shown reveals the cell surface adjacent to the coverslip. Note that both chimeric proteins are visible at the cell surface (A and B), but the punctate NE staining pattern seen with CD8/gpCT (A) is absent in the CD8/gpCT-20 expressing cell (B). Bar, 10 μm.
cytoplasmic structures as long as they are also sorted to the pore membrane domain of the NE (see Figs. 2, 4, and 5). It therefore seems likely that sorting of gp210 to a pore membrane domain of the NE (see Figs. 2, 4, and 5).

This paper is dedicated to George E. Palade.

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