Intracellular distribution of an integral nuclear pore membrane protein fused to green fluorescent protein

Localization of a targeting domain

Henrik SÖDERQVIST1, Gabriela IMREH1, Madeleine KIHLMARK1, Carolina LINNMAN2, Nils RINGERTZ2 and Einar HALLBERG1

1 Department of Biochemistry, Stockholm University, Sweden
2 Department of Cell and Molecular Biology (CMB), Karolinska Institutet, Stockholm, Sweden

(Received 4 July/9 September 1997) – EJB 97 0953/1

The 121-kDa pore membrane protein (POM121) is a bitopic integral membrane protein specifically located in the pore membrane domain of the nuclear envelope with its short N-terminal tail exposed on the luminal side and its major C-terminal portion adjoining the nuclear pore complex. In order to locate a signal for targeting of POM121 to the nuclear pores, we overexpressed selected regions of POM121 alone or fused to the green fluorescent protein (GFP) in transiently transfected COS-1 cells or in a stably transfected neuroblastoma cell line. Microscopic analysis of the GFP fluorescence or immunostaining was used to determine the intracellular distribution of the overexpressed proteins. The endofluorescent GFP tag had no effect on the distribution of POM121, since the chimerical POM121-GFP fusion protein was correctly targeted to the nuclear pores of both COS-1 cells and neuroblastoma cells. Based on the differentiated intracellular sorting of the POM121 variants, we conclude that the first 128 amino acids of POM121 contains signals for targeting to the continuous endoplasmic reticulum/nuclear envelope membrane system but not specifically to the nuclear pores and that a specific nuclear pore targeting signal is located between amino acids 129 and 618 in the endoplasmically exposed portion of POM121.

Keywords: nuclear pore; endoplasmic reticulum; protein trafficking; green fluorescent protein.

The eukaryotic cell nucleus is surrounded by the nuclear envelope, a double lipid membrane that separates the cytoplasm from the nucleoplasm (for reviews see Gerace and Burke, 1988; Miller et al., 1991). At numerous circumscribed points, referred to as the pore membrane domains of the nuclear envelope, the outer and inner nuclear membranes of the nuclear envelope are fused together to form nuclear pores. The nuclear pore complexes (NPCs) are multiprotein structures embedded in the nuclear pores and constitute the exclusive sites of nucleocytoplasmic transport (Panté and Aebi, 1996). The pore membrane proteins, i.e. integral membrane proteins of the nuclear pore complex, are believed to function in pore formation and assembly of the NPC (Hallberg et al., 1993; Wozniak et al., 1989, 1994). The cDNA of the 121-kDa pore membrane protein (POM121) was cloned and sequenced and its sequence was deduced (Hallberg et al., 1993). The protein has a short luminally exposed N-terminal tail, a single transmembrane domain and a large C-terminal portion adjoining the NPC (Söderqvist and Hallberg, 1994). The C-terminal third of POM121 contains several pentapeptide XFXFG repeats in common with a subfamily of peripheral nuclear pore complex proteins and are believed to function as docking sites in nucleocytoplasmic transport (reviewed by Görlich and Mattaj, 1996).

Our knowledge about the signals guiding proteins of the inner nuclear membrane and the nuclear pores to their proper location is very limited and only a few studies have been made. Studies of the polytopic integral inner membrane protein (the lamin B receptor) performed by different groups has identified targeting signals in the nucleoplasmically located N-terminal portion as well as the first transmembrane segment (Furukawa et al., 1995; Smith and Blobel, 1993; Soullam and Woman, 1993, 1995). Another integral protein of the inner nuclear membrane (LAP2) was found to have multiple targeting signals in its nucleoplasmically exposed N-terminal portion (Furukawa et al., 1995). In gp210, a bitopic integral pore membrane protein, a strong sorting determinant was located in its single transmembrane segment and a weaker one in its endoplasmically exposed C-terminal tail (Wozniak and Blobel, 1992). A targeting signal has also been identified in the 609 amino terminal residues of the peripheral NPC protein NUP153 (Bastos et al., 1996).

In a previous study we showed that rat POM121, or its C-terminal portion alone, displayed a punctate distribution in the nuclear envelope of COS-1 cells when overexpressed at low levels and formed intranuclear bodies when overexpressed at high levels (Söderqvist et al., 1996). In that study overexpressed rat POM121 was monitored by affinity purified species-specific antibodies raised against a synthetic peptide, P9107 (corresponding to amino acids 485–496 of POM121; Hallberg et al., 1993). In order to be able to follow the intracellular distribution of fragments of POM121, not containing the P9107 epitope, we constructed hybrid genes encoding various POM121 domains fused...
Fig. 1. POM121-GFP chimeras and deletion mutants. The terms used for each construct, POM121[x–y]-GFP, indicate the first (x) and last (y) residues of the POM121 peptide. POM121 is represented as a white box with the XFXFG repetitive domain indicated as a hatched box and the P9107 epitope (amino acids 485–496) as a black box. The positions of the x and y residues of the selected POM121 regions are indicated below the constructs. The GFP tag is represented by a gray box.

Fig. 2. Western blot analysis of overexpressed POM121-GFP fusion proteins. 200 μg total protein of COS-1 cells transfected with cDNA encoding POM121 or POM121-GFP were separated on an 8% SDS/PAGE, transferred to nitrocellulose membranes and probed with P9107 antibodies. The migration of molecular mass markers (in kDa) are indicated on the left.

MATERIALS AND METHODS

DNA constructs. In order to fuse the genes encoding rat POM121 and GFP, restriction sites were generated by PCR using modified primers. The clone c11 (Hallberg et al., 1993) was used as a template in a PCR reaction with a complementary to a green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. The GFP chromophore is spontaneously formed by an autocatalytic mechanism and fluoresces independently of any substrates or cofactors. The cDNA encoding GFP was recently cloned and sequenced (Prasher et al., 1992), opening the door for its growing popularity as a reporter protein (Chalfie et al., 1994).

In this paper we have studied the intracellular distribution of various deletion mutants of POM121 fused to GFP by fluorescence microscopy after overexpression in tissue culture cells. As will be discussed in the paper, we have demonstrated that the POM121-GFP chimera is correctly targeted to the nuclear pores. Furthermore, we show that whereas the N-terminal and transmembrane domains of POM121 can target GFP to the endoplasmic reticulum/nuclear envelope membrane, targeting to the nuclear pores required a discreet region in the endoplasmically exposed C-terminal portion.
Fig. 3. POM121-GFP is targeted to the nuclear pores. Transiently transfected COS-1 cells (a–f) or a cloned cell line, G197-1 (g–l) expressing POM121-GFP were examined by fluorescence microscopy. Equatorial (a–c, g–i) and grazing (d–f, j–l) sections of single nuclei are shown. The images show the fluorescence from POM121-GFP in the FITC channel (a, d, g, j) and the immunostaining using the mAb414 monoclonal antibody in the TRITC channel (c, f, i, l). The punctate distribution of the GFP fluorescence and the immunostaining almost perfectly matched when corresponding images from the FITC and the TRITC channels were superimposed (b, e, h, k). As a control for overbleaching, the superimposed images from the FITC and TRITC channels of parts of two adjacent nuclei in a sample were analyzed (m–o). The upper nucleus was from a COS cell expressing POM121-GFP and the lower nucleus from a non-expressing COS cell. Both nuclei displayed a punctate immunostaining using the mAb414 antibody, but only the nucleus from the expressing cell (upper) displayed a punctate GFP fluorescence matching that of the immunostaining. The relative pixel intensities from left to right of the FITC (green) and TRITC (red) channels along line A (n) and line B (o) are shown. Note that along line A the intensities in both channels matched (n). In contrast, the GFP fluorescence (green) was zero along line B except in one point representing autofluorescence from a contamination of the sample. Bars, 7.5 μm.

sense primer (positions 3533–3552) and an antisense primer (5'-AATCTAGAGCTAGCCTTCTTGGGGTGTGCT, complementary to nucleotides 3581–3597 of POM121 and containing 14 additional bases). A 79-bp DNA fragment was amplified with the stop codon replaced by an inner NheI site and an outer XbaI site. The fragment was cut by BamHI (position 3536 of the POM121 cDNA sequence) and XbaI producing a 69-bp fragment, which was used to replace a region in c11 (nucleotides 3536–3740), containing the stop codon of POM121) excised by BamHI and XbaI. The modified plasmid was cleaved using the XbaI site and the novel NheI site in order to incorporate GFP cDNA. A portion of the pGFP10.1 plasmid (positions 726–1718; Chalfie et al., 1994) containing the entire coding region of GFP was amplified using a sense primer (5'-ACAAGGCTAGCAAGGAGAAGAAC, complementary to nucleotides 726–750 of the pGFP10.1 sequence with base substitutions at positions 732–734 and 737) and an antisense primer (5'-TGGCGGCCGCTCTAGAACTAG, complementary to nucleotides 1698–1718) containing novel NheI and XbaI restriction sites to match those of the modified c11 plasmid. The resulting DNA encodes a fusion protein where the initiating Met of GFP is replaced with Ala which is fused to the C-terminal Lys-1199 of POM121. Unique Ncol and BsrBI restriction sites in the GFP gene were used to exchange amino acids 54–206 of the wild-
type GFP with the corresponding region of the mutated forms. We previously reported (Imreh et al., 1997) that, out of six different GFP mutants fused to the C-terminus of POM121, [Leu64, Thr65]GFP (Cormack et al., 1996) gave rise to the brightest fluorescence when overexpressed in COS-1 cells grown at 37°C and it was therefore used throughout this study. The fusion genes were cleaved by SalI and XbaI and incorporated in the eukaryotic expression vector pcDNA1Neo (Invitrogen), which was cut by XhoI (which is complementary to SalI) and XbaI. The gene for the fusion protein between POM121-(129–1199)-peptide and GFP was constructed in the same way starting with the c6 instead of the c11 plasmid. c6 lacks the initiation Met of POM121 (Hallberg et al., 1993), which results in translational initiation at Met129 downstream of the transmembrane domain. Plasmids for transfection were purified using a kit (Quiagen Inc.)

In order to fuse the POM121 1–129 and 803–1199 peptides to GFP the gene for [Leu64, Thr65]GFP was first inserted into the pcDNA1Neo expression vector. The GFP gene was amplified by PCR using the pGFP1.0.1 vector as template with a complementary sense primer containing a novel XhoI site (position 717, 5'-TAACTGAGACAAACCATGAGTAAGAGGAA) and an antisense primer containing a novel XbaI site (position 1698, 5'-TGCGGCACGCTCTAGAACTAG). The 982-bp fragment was cleaved using Xhol and XbaI and inserted into corresponding sites of the pcDNA1Neo expression vector.

To construct POM121-(1–129)-peptide–GFP, the c11 plasmid (Hallberg et al., 1993) was used as a template in a PCR reaction with a complementary sense primer (position –15, 5'-AAAAAAGCTTGGCGATGTCTCCGCGG) and an antisense primer (position 396, 5'-TTTCTGAGCTGTAAGAGTTTCCAGCGGG). POM121-(803–1199)–GFP was constructed in the same way except that the sense primer was (position 2388, 5'-AAAAAAGCTTGGCGATGTTTSGGCTTGGAGTGG) and the antisense primer (position 3606, 5'-AAAAACTGAGCTTGTTCGGGTTGTGCGT). The 411-bp and the 1219-bp fragments, respectively, were cut by HindIII and XhoI and ligated into pcDNA1Neo-GFP.

The POM121 1–618 and 129–618 peptides were constructed from the clones c11 and c6, respectively. Both plasmids were cleaved by Apol at position 1848 and 3454 to eliminate amino acids 619–1199 of POM121 (containing the XFXFG repeat domain). Religation of the linearized plasmids produced nucleotide sequences encoding the selected regions of POM121 with 24 additional amino acids originating from a different reading frame. The coding sequences were cut by SpeI and SalI and inserted into the pcDNA1Neo plasmid linearized by XbaI and XhoI.

Cell culture and transfection. COS-1 cells (African green monkey kidney cells) were cultured at 37°C on 13-mm glass coverslips as described (Soderqvist et al., 1996); 75% confluent monolayer cultures were transfected using the polycationic reagent N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methysulfate (DOTAP liposomal transfection regent, Boehringer Mannheim) according to the manufacturer’s manual; 5 h after transfection in serum-free medium the cells were allowed to incubate for an additional 19 h or 43 h at 37°C in normal culture medium. The human neuroblastoma SH-SY5Y cells were grown in modified Eagle medium containing 2 mM glutamine, 1% non-essential amino acids, 15% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin. Then 75% confluent neuroblastoma cells were transfected with POM121-GFP cDNA as described above; 48 h post-transfection the cells were grown for 15 days in the same medium containing 0.4 mg/ml G418 (Gibco Laboratories) after which time the G418 concentration was reduced to 0.25 mg/ml.

After 4 weeks G418-resistant colonies were subcloned and passaged two or three times in 100-mm petri dishes. One of the POM121-GFP expressing clones, denoted GI97-1, was used throughout this study.

Western blotting and immunostaining. For immunoblots, proteins of whole cell lysates were separated on 8% SDS/PAGE and electrotransferred to nitrocellulose filters as described (Hallberg et al., 1993). The immunoblots were probed using affinity purified P9107 antibodies, which are specific for rat

{{# beiden

**Fig. 4. Intracellular distribution POM121-GFP chimeras and deletion mutants.** Equatorial sections of nuclei of COS-1 cells studied by GFP fluorescence (a, g, i) or immunofluorescence using the P9107 antibody (c, e) are shown together with the corresponding DNA staining using Hoechst 33258 (b, d, f, j). Overexpressed POM121-(129–1199)-peptide–GFP (a), POM121-(1–618)-peptide (c) and POM121-(129–618)-peptide (e) were distributed as a punctate pattern in the nuclear rim. Overexpressed POM121-(1–128)-peptide–GFP (g) was evenly distributed in the nuclear rim and in the endoplasmic reticulum, whereas POM121-(803–1199)-peptide–GFP (i) was distributed throughout the entire cell without any particular location. Bars 7.5 μm.


```
POM121 and do not crossreact with the endogenous COS cell homologue (Söderqvist et al., 1996). Transfected COS-1 cells grown on 13-mm coverslips were prepared for immunofluorescence as described (Söderqvist et al., 1996). Affinity purified rabbit P9107 antibodies (see above), mouse monoclonal mAb414 antibodies (Davis and Blobel, 1986) or rabbit polyclonal antibodies raised against a synthetic peptide corresponding to amino acids 556–573 of the endoplasmic reticulum protein calnexin (Wada et al., 1991) were used as primary antibodies. Fluorescein-isothiocyanate-labeled donkey anti-(rabbit IgG), Cy3-conjugated donkey anti-(mouse IgG) or Cy3-conjugated donkey anti-(rabbit IgG) were used as secondary antibodies (both from Jackson ImmunoResearch Laboratories). For visualization of DNA, Hoechst 33258 (0.5 μg/ml) was included in the second of the four washes prior to mounting.

Fluorescence microscopy. The GFP fluorescence was examined after fixation, permeabilization, immunostaining and mounting using a Zeiss Axioplan 2 microscope (Carl Zeiss) equipped with a cooled C4880 digital CCD camera (Hamamatsu Photonics). The fluorescein-isothiocyanate (FITC) and GFP fluorescence was analyzed using a BP 470/40 nm excitation and a LP 500-nm emission filter combination (Chroma Technology Corp.). The Cy3 fluorescence was analyzed using a BP 546/12-nm excitation and a LP 500-nm emission filter combination and the Hoechst 33258 staining was analyzed using a BP 365/12-nm excitation and a LP420-nm emission filter combination (Carl Zeiss). The images were processed using Adobe Photoshop 3.0 software. The pixel intensity analysis was performed using Delta Vision software (Applied Precision Inc.).

RESULTS AND DISCUSSION

After being synthesized and inserted into the endoplasmic reticulum membrane, the pore membrane proteins are believed to be sorted to their proper location by lateral diffusion. In order to locate the targeting signal(s), the intracellular distribution of POM121 deletion mutants and fusion chimeras (Fig. 1) was studied after overexpression in tissue culture cells. Selected portions of POM121 cDNA was fused to the 5’-end of a mutant cDNA encoding [Leu64, Thr65]GFP (Cormack et al., 1996) in order to generate various deleted POM121-GFP chimeras.

Western blots of SDS/PAGE-separated proteins from lysates of cells transfected with the various constructs (Fig. 2) were probed with affinity purified polyclonal antibodies against an internal peptide (P9107) of rat POM121 (Hallberg et al., 1993). The overexpressed POM121-GFP fusion protein migrated with an apparent molecular mass of 172 kDa (compared to 145 kDa for POM121). The electrophoretic mobility of POM121-GFP is consistent with the 27 kDa calculated mass contributed by GFP. The data suggest that the fluorescence emitted from the transfected cells came from undegraded correctly folded POM121 tagged with GFP.

The intracellular distribution of GFP fluorescence in transiently transfected COS-1 cells or an immortalized neuroblastoma cell line stably expressing POM121-GFP at low levels was studied by conventional and CCD-enhanced fluorescence microscopy (Fig. 3). In both cell types the GFP fluorescence in the FITC channel was distributed in a finely punctate pattern in grazing sections and in the nuclear rim in equatorial sections. Similar patterns were obtained in the tetramethylrhodamine isothiocyanate (TRITC) channel showing the corresponding immunostaining using the monoclonal antibody mAb414 which recognizes a family of nuclear pore proteins, but not POM121. In fact, the distribution of GFP fluorescence almost perfectly matched the immunostaining pattern when the images were superimposed. As a control, the fluorescence from two adjacent nuclei (one from a transfected COS cell and the other from an untransfected COS cell) were analyzed (Fig. 3 m–o). The results clearly rule out the possibility that the match between the corresponding images from the two channels was due to overbleeding. The results show that the POM121-GFP chimera was targeted to the nuclear pores when overexpressed at low levels either in transiently transfected COS-1 cells or stably transfected neuroblastoma cells. The present study demonstrates the successful use of GFP as a tag for a nuclear pore protein in vertebrate cells.

We previously reported that, in transiently transfected COS-1 cells, POM121 and its 129–1199 peptide distributed in a punctate pattern over the nuclear surface (partially overlapping with that of nucleolar pores) when overexpressed at low levels but formed intranuclear bodies when overexpressed at high levels (Söderqvist et al., 1996). Nuclear body formation also occurred at high expression levels in the present study, but will be the subject for another investigation. In this study we focused on capturing images of cells overexpressing the various POM121 variants at levels which were lower than the level where nuclear body formation starts.
The intracellular distribution of a series of deletion mutants of POM121 and POM121-GFP was studied in transiently transfected COS-1 cells by microscopic analysis of the GFP fluorescence or the immunostaining using the P9107 antibody. Fusion proteins between GFP and POM121 peptides 129–1199 (missing the N-terminal and transmembrane domains; Fig. 4a), 1–618 (missing the C-terminal half; Fig. 4c) and 129–618 (missing the N-terminal and transmembrane domain and the C-terminal half; Fig. 4e) displayed a punctate distribution over the nuclear surface consistent with a localization at the nuclear pores. The 1–129 fusion peptide displayed an even distribution in the endoplasmic reticulum and the nuclear periphery (Fig. 4g), consistent with a localization in the endoplasmic reticulum/nuclear envelope membrane, but not at the nuclear pores. The 803–1199 fusion protein was evenly distributed throughout the cell without any specific localization (Fig. 4i).

In order to define the intracellular localization of overexpressed POM121-(1–129)-peptide–GFP, transiently transfected COS cells were immunostained using a polyclonal antibody raised against the 18 most C-terminal amino acids of calnexin, which is located in the endoplasmic reticulum (Wada et al., 1991). The GFP fluorescence distributed around the nuclear rim and in reticular structures throughout the cytoplasm matched the immunostaining using anti-calnexin antibodies (Fig. 5). The results unequivocally demonstrate that this fusion protein is targeted to the endoplasmic reticulum/nuclear envelope membrane when overexpressed in COS cells.

We conclude that the first 129 amino acids of POM121 were able to target GFP to the endoplasmic reticulum/nuclear envelope membrane, suggesting that they contain the information necessary for synthesis and integration in the rough endoplasmic reticulum but not to the nuclear pores. In contrast, amino acids 129–618 were sorted to the nuclear pores, either alone or together with the N-terminus and the transmembrane domain (1–618). We therefore conclude that a domain between amino acids 129 and 618 contains at least one nuclear pore targeting signal. The results support a model where nascent POM121 is first inserted into the endoplasmic reticulum and then diffuses laterally in the membrane guided by a nuclear pore targeting signal located between amino acids 129–618 in the cytoplasmically exposed C-terminal portion of POM121. It appears likely that this domain carries signals for interaction with other NPC proteins for integration into the NPC. This domain in POM121 is presently subject to a detailed analysis.

The conclusion is consistent with previous studies of integral proteins of the inner nuclear membrane (Furukawa et al., 1995; Smith and Blobel, 1993; Soullam and Worman, 1993, 1995) or the pore membrane (Wozniak and Blobel, 1992), which show that these proteins are at least partially guided to their proper locations by endoplasmically located sorting determinants. However, in contrast to the other pore membrane protein gp210, the transmembrane domain of POM121 does not contain a nuclear pore targeting signal. Although the repetitive pentapeptide XFXFG domain is a common motif among several NPC proteins, it does not appear to mediate sorting to the nuclear pores in the case of neither POM121 nor NUP153 (Bastos et al., 1996).

We thank Drs B. Cormack and S. Falkow for the generous donation of the [Leu64,Thr65]GFP mutant and Drs A. Andersson and R. Petterson for generous donation of antibodies towards calnexin. This work was supported by grants to EH (from The Swedish National Science Research Council, The Swedish National Board for Laboratory Animals, Carl Tryggers Foundation, Åke Wibergs Foundation and Stiftelsen Lars Hiertas Minne) and grants to NR (from The Swedish Medical Research Council and The Knut and Alice Wallenberg Foundation).

REFERENCES


