Essential Role of the Prion Protein N Terminus in Subcellular Trafficking and Half-life of Cellular Prion Protein*

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Received for publication, June 25, 2002, and in revised form, October 21, 2002
Published, JBC Papers in Press, November 12, 2002, DOI 10.1074/jbc.M206313200

Transmissible spongiform encephalopathies in humans and animals can be manifested as sporadic, familial and acquired diseases and include Creutzfeldt-Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle. These neurodegenerative diseases are caused by the accumulation of a conformationally altered isoform of the cellular prion protein (PrPc), denoted PrPSc (1–3). During biogenesis, PrPSc is directed cotranslationally into the lumen of the endoplasmic reticulum by a 22-amino acid N-terminal signal peptide. This is then removed together with a 23-amino acid C-terminal signal sequence promoting attachment of a GPI anchor. The protein undergoes further post-translational modifications with the addition of two N-linked carbohydrate chains. Properly folded PrPSc transits through the Golgi compartment and the secretory pathway and is attached to the outer leaflet of the plasma membrane by its GPI anchor (4–6).

Conversion of PrPSc into PrPc has been reported to occur close to the plasma membrane along the endocytic pathway, probably in caveolae-like domains or in rafts, membranous domains or invaginations of the plasma membrane rich in cholesterol and glycosphingolipids (7). Here also the first steps in PrPSc degradation occur (8, 9) before reaching acidic compartments for final degradation. Cell surface localization of PrPSc is thought to be essential for subsequent conversion into PrPc (4–6), and studies in transgenic mice suggest a direct interaction between the two PrP isoforms, possibly in a complex with auxiliary factors (1). The cellular function of the prion protein is still unknown, although binding of copper to the octapeptide repeat sequence located at its N terminus suggests a role of PrP for the N-terminal part of PrPc in subcellular trafficking and to inhibit conversion into the scrapie isoform (7).

Studies performed on the subcellular trafficking of GPI-anchored proteins have revealed that their sorting is not a simple default process because specific signals are required for transport from the Golgi to the cell surface and for endocytosis. These targeting signals are still poorly characterized. Although sorting and endocytosis of most transmembrane proteins require recognition of specific motifs in their cytoplasmic domains by multimeric adaptor protein complexes, other targeting elements are obviously necessary for the trafficking of GPI proteins which lack such cytoplasmic extensions. Several elements have been shown to influence PrPc trafficking. Studies done with PrP constructs lacking the GPI anchor revealed slower transport through the secretory pathway compared with wild type PrPc (wtPrPc) (14), although conversion of these constructs into PrPSc was previously not found to be compromised significantly (15). Prevention of glycosylation affected the transport to the cell surface and changed the biochemical properties of PrPc (16, 17). Addition of a transmembrane moiety to the C terminus of PrPc has been shown to affect subcellular trafficking and to inhibit conversion into the scrapie isoform (7).

In this report we were interested in devising a physiological function for the N-terminal part of PrPc in subcellular trafficking. We therefore performed various metabolic labeling and surface biotinylation assays to follow the intracellular trafficking and turnover of the PrP. We expressed specific N-terminal PrP deletion constructs and one chimeric Xenopus laevis-mouse construct in murine neuroblastoma cells and compared their...
internalization from the exosomal plasma membrane, their transport along the secretory pathway, and half-life with those of transfected wtPrP. Our results showed significant differences in the behavior of these proteins, although all constructs effectively passed the cellular quality control in the endoplasmic reticulum/Golgi. On the other hand, the chimeric protein consisting of the short N-terminal segment of Xenopus (amino acids 23–69) fused to the truncated mouse PrP nearly restored wild type secretory and endocytotic kinetics. These data indicate that the N-proximal domain of the PrP function as a potential target for the chimeric protein and is essential for both transport of the plasma membrane and modulation of endocytosis. These targeting functions of the N terminus are also highly conserved in evolution.

EXPERIMENTAL PROCEDURES

Reagents—Monoclonal PrP-specific antibody 3F4 (Signet Pathology) recognizes the sequence encompassing amino acids 109 and 112 in hamster and human PrP and has been described before. Polyclonal anti-PrP-specific antibody A7 was obtained in our laboratories after immunization of rabbits with recombinant dimeric mouse PrP. Cell culture media and trypsin-EDTA were obtained from Invitrogen. [35S]Met/Cys (Promix; 1,000 Ci/mmol) for labeling of proteins, protein A-Sepharose, and an enhanced chemoluminescence blotting kit were from Amersham Biosciences. Endoglycosidase H (Endo-H), PNGase F, and Pefabloc protease inhibitor were all obtained from Roche Molecular Biochemicals. Biotin-sulfo-NHS and horseradish peroxidase-conjugated streptavidin were obtained from Pierce, and trypsin inhibitor from Sigma. Transient and stable transfections were carried out using FuGENE (Roche Molecular Biochemicals) or Effectene (Qiagen) transfection reagents.

Cell Culture and Transient Expression of wtPrP and N-terminally Truncated PrP Constructs—The mouse neuroblastoma cell line N2a (ATCC CCL-131) has been described before. Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, penicillin/streptomycin, and glutamine in a 5% CO₂ atmosphere. The medium was changed every 48 h. wtPrP and PrP chimeric constructs were all cloned into the pcDNA3.1Zeo expression vector (Invitrogen). Substitution of amino acids 109 and 112 (numbering referring to human PrP according to Ref. 19) from mouse prion protein (PrP c) for amino acids 69 of X. laevis was generated from an Image cDNA clone using site-directed mutagenesis allows recognition of transiently transfected mouse PrP by the monoclonal antibody 3F4 and discrimination from endogenous PrP because the antibody does not recognize murine wtPrP. Construction of PrP chimeric constructs was done by PCR-based standard techniques using 3F4-tagged mouse PrP as a template. Insertion of appropriate restriction sites allowed cloning of PCR fragments into the multiple cloning site of the vector pcDNA3.1 using standard cloning techniques. Briefly, PrPα (23–90) was obtained by deleting amino acids 23–90 of full-length PrP (numbering referring to human PrP); in PrPα (48–93) residues 48–93 were deleted; in PrPα (23–51) and PrPα (68–91) amino acids 23–51 and 68–91 were absent, respectively. For cloning of PrP(Xen/23–69) a PCR fragment encompassing residues 23–69 of X. laevis was generated from an Image cDNA clone using primers allowing insertion of the fragment between amino acids 22 and 92 of murine PrP. Constructs were always confirmed by nucleic acid sequencing. Plasmid pEGFP (Clontech) was used as a control for specificity of results. All cloned constructs were transiently transfected into N2a cells by lipofection according to manufacturer’s directions, and cells were lysed for experiments 72 h post-transfection. For selected constructs, stable transfections were performed in addition, using Zeocin as a selection marker.

Detergent Solubility Assay and Western Blot Analysis—Confluent transfected cells were lysed in cold lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate). Postnuclear cell lysates were supplemented with 0.5 mM Pefabloc protease inhibitor and N-lauryl sarcosine to 1% and centrifuged for 1 h at 100,000 × g at 4 °C in a Beckman TL-100 centrifuge. Soluble fractions (supernatants) were precipitated with ethanol. Insoluble fractions (pellets) were resuspended in 50 μl of TNE (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) and ethanol-precipitated samples were resuspended for 30 min at 2,500 × g, and the pellets were redissolved in TNE buffer with the addition of gel loading buffer. Samples were then boiled for 10 min, and an aliquot was analyzed on 12.5% SDS-PAGE. Proteins were electrotransferred to a polyvinylidene difluoride membrane. This was blocked with non-fat dry milk (5%) in TBST (0.05% Tween 20, 100 mM NaCl, 10 mM Tris-Cl, pH 7.8), incubated overnight with antibody 3F4 at 4 °C, and stained using an enhanced chemoluminescence blotting kit from Amersham Biosciences.

Proteinase K (PK) Digestion—Aliquots of postnuclear lysates were incubated for 30 min at 37 °C with 20 μg/ml PK; the digestion was stopped by the addition of Pefabloc. Samples were precipitated with ethanol and analyzed in an immunoblot assay.

Metabolic Labeling and Immunoprecipitation Assay—Confluent transfected N2a cells were washed twice with phosphate-buffered saline (PBS) and starved for 1 h in RPMI medium without methionine/ cysteine containing 1% fetal calf serum. Labeling was carried out by adding 400 μCi/ml [35S]Met/Cys to the medium for 5 min or, for half-life studies, for 1 h. After incubation, cells were washed twice in cold PBS and then incubated with lysis buffer containing 100 μg/ml Pefabloc for different lengths of time in complete culture medium to allow transport to the cell surface. After appropriate chase times, cells were washed with PBS and either harvested directly or 1 ml of trypsin-EDTA was added on the dishes for 10 min on ice. Trypsinized cells were transferred to polypropylene tubes, and the reaction was stopped by centrifugation of the cells twice at 960 × g in PBS containing 20% fetal calf serum and 100 μl of trypsin inhibitor. Cells were then lysed in cold lysis buffer and cell debris removed by centrifugation for 40 s at 18,000 × g. After addition of 1% N-lauryl sarcosine postnuclear lysates were boiled at 95 °C for 10 min. Samples were placed on ice, and Pefabloc protease inhibitor was added, lysates were incubated with antibody A7 or 3F4, as indicated, overnight at 4 °C (dilution 1:300). Protein A-Sepharose beads were added to the protein-antibody complex for 90 min at 4 °C. The beads were centrifuged at 18,000 × g for 1 min and washed in radioimmune precipitation assay buffer (0.5% Triton X-100, 0.5% deoxycholate in PBS) supplemented with 1% SDS at 4 °C. All samples were treated with 0.1 unit/μl PNGase F at 37 °C to remove N-linked oligosaccharides and analyzed by 12.5% SDS-PAGE. Gels were exposed to an x-ray film (Kodak), and the autoradiographic signals were quantified by PhosphorImage analysis of the gel (Molecular Dynamics). The amount of total or intracellular (after surface trypsin digestion) PrP present at each time point after the chase was expressed as a percentage of nascent PrP rescued from the cell lysate directly at the end of the labeling period.

Internalization Assay Using Surface Biotinylation—Endocytosis of wtPrP and PrP mutants located on the cell surface was assessed by surface biotinylation. Briefly, confluent transfected N2a cells were washed twice with cold PBS and incubated on ice for 15 min with 1 ml of PBS containing 250 μg of biotin sulfo-NHS. Cells were washed again three times with cold PBS to remove unbound biotin and were either harvested directly with cold lysis buffer on ice for 10 min, or culture medium was added for appropriate chase times as indicated in the respective experiments at 37 °C to allow internalization. Cells were washed with PBS and either lysed directly or treated with 1 ml of trypsin-EDTA on ice for 10 min before lysing and immunoprecipitation as described above, using monoclonal antibody 3F4 for detection of transfected constructs. Immunoadsorbed proteins were subjected to 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Biosciences). Blots were developed with horseradish peroxidase-conjugated streptavidin and visualized with enhanced chemoluminescence. Films were digitized using an APB Image Scanner and images quantified using Image Master 1D software (both from Amersham Biosciences).

Treatment with Endo-H, PNGase F, and Immunoblot Analysis—For Endo-H digestion, aliquots of protein lysates were incubated after immunoprecipitation with 0.1 m β-mercaptoethanol and 0.1% SDS and with Endo-H of human placental origin for 30 min at 37 °C. Endoglycosidase H (Endo-H) buffer (0.1 m sodium citrate, pH 5.5, 0.5% phenylmethylsulfonyl fluoride, 6 milliliters of Endo-H) and incubated overnight at 37 °C. All samples were then subjected to SDS-PAGE. PNGase F treatment was carried out by mixing 100 μl of postnuclear lysate or immunoprecipitated proteins with 20 μl of mercaptoethanol-containing EDTA and incubated at 95 °C. Controls from immunoprecipitation were incubated with lysis buffer, and all samples were supplemented with protease inhibitor and 0.1 unit/μl PNGase F overnight at 37 °C before analysis on SDS-PAGE.

RESULTS

Internalization of Mouse PrP is Impaired by Progressive N-terminal Deletions—To assess the relevance of the N-terminal part of PrP in its intracellular trafficking, we transiently transfected murine neuroblastoma cells with a series of PrP constructs in which amino acids within the N terminus had
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Because the partial or complete removal of PrP on the plasma membrane we wondered whether altered internalization also interfered with the degradation and cellular stability of the proteins. In pulse-chase experiments we compared the half-lives of wtPrP, PrPΔ(23–90), and PrPΔ(48–93). N2a cells expressing these constructs were metabolically labeled with [35S]methionine/cysteine for 1 h and either had been progressively deleted (Fig. 1A). All experiments were performed in parallel with and compared with transfected wtPrP, to rule out possible effects on the kinetics caused by overexpression or metabolic stress upon transfection. The constructs were first characterized biochemically in a solubility assay. Postnuclear lysates were ultracentrifuged in a buffer containing 1% sarcosyl for separation of soluble from insoluble proteins. Aliquots of the lysates were subjected to a solubility assay (Fig. 1B, lanes 2, 5, 8, 11, and 14), and the PK-treated fractions (lanes 3, 6, 9, 12, and 15) were all analyzed by immunoblotting using the monoclonal antibody 3F4. Molecular mass markers are indicated in kDa on the left. C, 200-μl fractions of postnuclear lysates described in B were treated overnight with PNGase F for deglycosylation of the proteins and then subjected to immunoblotting with the antibody 3F4. The molecular masses are shown in kDa on the left.

Deletion of N-proximal Sequences Significantly Prolongs the Half-life of PrP—Because the partial or complete removal of the N-terminal part of PrP significantly prolonged the presence of wtPrP in the plasma membrane we wondered whether altered internalization also interfered with the degradation and cellular stability of the proteins. In pulse-chase experiments we compared the half-lives of wtPrP, PrPΔ(23–90), and PrPΔ(48–93). N2a cells expressing these constructs were metabolically labeled with [35S]methionine/cysteine for 1 h and either had...
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Fig. 2. Comparison of internalization of wtPrP\(^\text{+}\) and deletion mutants after 45 and 60 min of chase. A, N2a cells transfected transiently with wtPrP\(^\text{+}\) or PrP deletion mutants were surface biotinylated on ice for 15 min and then incubated for 0, 45, or 60 min at 37 °C. Cells where lysed immediately (−) or treated with trypsin (+) for 10 min on ice before harvesting and were immunoprecipitated with antibody 3F4 (to detect only transfected proteins). Lane 7 describes N2a cells transfected with the unrelated plasmid pEGFP and precipitated with antibody 3F4. Samples were subjected to SDS-PAGE, and signals were detected by streptavidin. Molecular mass markers are depicted in kDa on the left. B, quantification of the signals shows the amount of internalized protein after 45 and 60 min calculated as a percentage of protein without treatment with trypsin at the same time point (each bar represents the mean values from two independent experiments). The blot was digitized using an APB Image Scanner, and specific bands were quantified with Master 1D analysis software.

Fig. 3. Internalization of PrPΔ(23–90) and PrPΔ(48–93). A, transiently transfected N2a cells expressing PrP deletion mutants were surface biotinylated on ice and then incubated at 37 °C for 0, 3, 6, and 10 h, respectively. Cells were harvested directly or treated with trypsin for 10 min on ice and lysed. PrPs were immunoprecipitated with antibody 3F4. The blot shows samples treated (+) and untreated (−) with trypsin. Numbers on the top indicate the chase times (in hours) after the pulse. Molecular mass markers are indicated in kDa on the left. B, mean values from two independent experiments represent the amount of internalized protein expressed as a percentage of total labeled protein without trypsin digestion (at same time points) and plotted as a function of different time points.

vested directly or chased for different intervals of time in \(^{35}\text{S}\)-free culture medium before lysis. PrP present in the lysates was immunopurified with the anti-PrP antibody A7, deglycosylated with PNGase F, and analyzed by SDS-PAGE (Fig. 4A). The use of the polyclonal antibody A7 in this and in the following experiments did not alter the results due to cross-reaction with endogenous PrP\(^\text{+}\) because differences in molecular mass allowed discrimination between deletion mutants and endogenous wtPrP\(^\text{+}\). The autoradiogram was evaluated by densitometric analysis and the specific bands quantified as fractions of the signal observed in the absence of chase. Time points of 2, 3, 6, 8, and 22 h show that for all constructs the signal declined as a function of the chase. Curves in Fig. 4B show the mean values from three independent experiments: wtPrP\(^\text{+}\) revealed a half-life of ~2.6 h. On the other hand, the turnover of our PrP mutants proved to be significantly prolonged. Our measurements indicated a half-life of ~4.8 h for PrPΔ(23–90) and ~4.2 h for PrPΔ(48–93). These data corroborate the idea that prolonged accumulation of N-terminal deleted mutants on the plasma membrane indeed affects the turnover of these proteins.

N-proximal Region of PrP Modulates Transport to the Plasma Membrane—Having found that the N-terminal part of PrP\(^\text{+}\) plays an essential role in modulation of endocytosis and stability, we next focused on the secretory pathway and on the transport of PrP to the cell surface to identify a possible function of the N-proximal segment in this arm of the PrP life cycle. Using pulse-chase experiments we evaluated the time required by wtPrP\(^\text{+}\) and PrP deletion constructs to reach the plasma membrane. After transfection, N2a cells were metabolically labeled with a short pulse of 5 min, to have a more homogeneous population, and then harvested immediately or incubated in \(^{35}\text{S}\)-Met-free medium at 37 °C for different time periods to allow transport to the cell surface. Molecules that had reached the plasma membrane were separated from those still undergoing synthesis or transport to the cell surface by trypsin treatment on ice for 10 min and were then immunoprecipitated with an anti-PrP antibody. All samples were deglycosylated with PNGase F for simplifying quantitative comparison of the constructs. Immediately after the pulse, wtPrP\(^\text{+}\) and the PrP constructs were still protected from extracellular trypsin digestion (Fig. 5, lanes 2, 8, and 14), consistent with their presence in the endoplasmic reticulum and/or Golgi apparatus. Our assay revealed that most of the wtPrP\(^\text{+}\) molecules reach the cell surface within 1 h after synthesis (Fig. 5A). In Fig. 5B the mean data from three independent experiments show that although after 45 min 50% of total wtPrP can still be detected after trypsin treatment, the amount rapidly decreases to ~25% after 60 min. The transport of PrPΔ(23–90) and PrPΔ(48–93) to the cell surface showed important differences compared with wt-PrP. Even after 60 min of chase, both constructs were still detectable intracellularly in considerable amounts (Fig. 5A); PrPΔ(48–93) was still clearly detectable after 75 min of chase. Quantification of the signals verified that 50% of labeled proteins were not susceptible to trypsin treatment after 65 and 75 min, respectively, for PrPΔ(23–90) and PrPΔ(48–93). This analysis corroborated the idea that deletion of N-terminal sequences negatively affects transport of PrP\(^\text{+}\) even along the
secretory pathway. For further analysis of the domains involved in trafficking of PrP\(_c\), we compared the transport of PrP\(_c/H9004\) (23–51) with that of another construct where three of the five repeats (residues 68–91) had been removed, in a pulse-chase assay combined with trypsin treatment of transfected cells using the same time points for easier comparison (Fig. 6, A and B). Because in these constructs segments of similar lengths were deleted in distinct sections of the N terminus, different kinetics of transport should reveal the presence of a domain with a dominant role in the secretory pathway. The time for detection of 50% of intracellular PrP was 65 and 70 min for PrP\(_c/H9004\) (68–91) and PrP\(_c/H9004\) (23–51), respectively, which was longer than that measured for wtPrP\(_c\) (45 min). These data confirmed that truncation of even small segments within the N terminus encompassing or preceding the octapeptide repeat region negatively interferes with the efficiency of transport of PrP\(_c\) to the plasma membrane.

**X. laevis N Terminus Restores Wild Type Phenotype in Truncated Mouse PrP**—In light of the results described above, we studied the influence of the N-terminal part of a PrP belonging to a different species on the trafficking of the analyzed mouse PrP. We therefore expressed a chimeric PrP comprising the first 47 amino acids after the signal peptide (residues 23–69) of X. laevis PrP inserted between residues 22 and 92 of mouse PrP (Fig. 7A). This chimeric protein, (Xen(23–69)), was first characterized biochemically in a solubility assay and upon PK digestion. The construct appeared to be soluble and entirely PK-sensitive (Fig. 7B, lanes 2 and 3). After deglycosylation with PNGase F, the construct migrated as a single band of about 23 kDa, consistent with its shorter amino acid sequence.

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**Fig. 4.** Half-life times of wtPrP\(_c\) and deletion mutants PrP\(_c/(23-90)\) and PrP\(_c/(48-93)\). A, confluent N2a cells expressing wtPrP or deletion constructs were metabolically labeled with \[^{35}S\]Met/Cys for 1 h at 37 °C and were either lysed after the pulse or incubated in culture medium without \[^{35}S\] at 37 °C for 2, 3, 6, 8, and 22 h, respectively, before harvesting. Proteins were precipitated with polyclonal antibody A7 and deglycosylated with PNGase F to facilitate molecular mass comparison and quantification. Samples were subjected to SDS-PAGE and autoradiography. Molecular mass markers are designated in kDa on the left. On the right unglycosylated PrP-specific bands are indicated. B, evaluation of autoradiograms from three independent experiments. The amounts of protein are expressed as percentage of total protein rescued directly after the labeling period and plotted as a function of the chase time points. The data points were fitted to an exponential curve using nonlinear regression analysis.

**Fig. 5.** Kinetics of transport to the cell surface of wtPrP\(_c\), PrP\(_c/(23-90)\), and PrP\(_c/(48-93)\). A, wtPrP and N-terminal truncated constructs were transiently transfected into N2a cells, metabolically labeled with \[^{35}S\]Met/Cys for 5 min on ice, and then chased in \[^{35}S\]-free culture medium for 0, 45, 60, 75, 90 min, respectively. For each construct, one plate was harvested immediately after the pulse or treated with trypsin and then lysed. All other plates were subjected to digestion with trypsin before lysis. Proteins were immunoprecipitated with antibody A7, deglycosylated with PNGase F, and subjected to SDS-PAGE. The blot shows PrP signals before (−) and after (+) treatment with trypsin. Molecular mass markers in kDa are shown on the left. B, PhosphorImager evaluation of autoradiograms representing the amounts of protein detected after digestion with trypsin. Each point represents a mean value of three independent experiments. Quantities are calculated as a percentage of protein precipitated immediately after the pulse without trypsin treatment.
ysis of the transport of Xen(23–69) proteins were subjected to Endo-H treatment. As shown in Fig. 8, chased them for different time intervals before lysis. After metabolically labeling of proteins, we either immediately harvested or treatment of wtPrPc with Endo-H immediately after labeling. Immunoprecipitation led to similar results. After a short label of 5 min, 50% of Xen(23–69) moiety reached the cell surface after ~43 min (Fig. 7D), a value very similar to that of wild type mouse PrP. These data suggest that the N terminus of X. laevis, like the corresponding segment of the mouse PrP, has the same intrinsic sorting function and is able to restore the wild type trafficking kinetics.

N-terminal PrP Deletions Affect the Secretory Pathway after PrP Has Reached the Mid-Golgi Compartment—To identify a specific compartment along the secretory pathway where N-terminal truncation of PrP might result in a delayed transport to the cell surface, we subjected immunoprecipitated samples to Endo-H treatment, which removes high mannose glycans from glycoproteins before they reach the mid-Golgi. This assay therefore enables us to associate the localization of a protein with its glycosylation state. In pulse-chase experiments we measured at which time points wtPrP, PrPΔ(23–90), and PrPΔ(48–93) acquired Endo-H resistance (Fig. 8). After metabolic labeling of proteins, we either immediately harvested or chased them for different time intervals before lysis. After immunoprecipitation of proteins with antibody 3F4, all samples were subjected to Endo-H treatment. As shown in Fig. 8A, treatment of wtPrPc with Endo-H immediately after labeling resulted in a shift of the 33 and 28 kDa bands to a band of ~25 kDa (lane 2). We could detect initial signs of resistance after 30 min, when the monoglycosylated form appeared (data not shown). Complete Endo-H resistance was achieved between 40 and 50 min (lanes 4 and 6). Observing the other two constructs, we could detect the conversion to unglycosylated molecules of 19 and 22 kDa for PrPΔ(23–90) and PrPΔ(48–93), respectively, when treated with Endo-H immediately after the pulse (Fig. 8A, lane 12, and Fig. 8B, lane 2). Partial resistance was detected after 40 min for PrPΔ(23–90) (lane 14) and for PrPΔ(48–93) (Fig. 8B, lane 4), and both mutants became fully Endo-H-resistant after 50 min. We therefore conclude from these results that deletions within the N-terminal part of PrP do not significantly affect kinetics of glycosylation and transport of PrP to the Golgi.

**DISCUSSION**

The structure of the PrP displays enigmatic features because it seems to be composed of two highly different parts: the N-terminal part has a defined secondary structure consisting of three α-helices and two short β-strands. In contrast, the N-terminal region (i.e., amino acids 23–89) is devoid of any defined secondary structure and has the properties of a flexible random coiled polypeptide (20–22). After conversion of PrP into PrPSc, residues 23–89 of PrP remain entirely susceptible to PK digestion, whereas the rest of the protein becomes partly protease-resistant (1). Regions within the N-terminal part of PrP have been suggested to influence interactions required for PrPSc formation and to stabilize the conformation of PrPSc (23). The hypothesis for a biological function of the N-terminal part of PrP, besides the copper binding mentioned earlier, is also supported by the fact that this domain, despite its lack of ordered structure, is highly conserved in evolution. Interferences here mainly concern insertions and deletions (19). The current study aimed to assess a possible cellular function of the N-terminal part of PrPSc in the life cycle of PrP and therefore to characterize the subcellular trafficking of N-terminally deleted PrP constructs.

Progressive Deletions within the N Terminus of PrP Sc Result in Highly Reduced Endocytosis and Prolonged Turnover—in preliminary studies, the biochemical properties of PrP constructs lacking segments of different length in their N-terminal end were compared with those of wtPrP to exclude that conformational changes or overexpression might influence the outcome of the subsequent analysis. All of the mutants analyzed were properly glycosylated and did not aggregate. Therefore, significant alterations in their conformations were not likely. Our following analysis of internalization kinetics revealed delayed endocytosis for all PrP deletion constructs compared with

![Fig. 6. Kinetics of PrPΔ(68–91) and PrPΔ(23–51) transport through the secretory pathway monitored by pulse-chase experiments.](image-url)
Western blot analysis with antibody 3F4, used for PNGase F digestion. The resulting fractions were examined by analyzed for solubility and PK resistance. One aliquot of the lysate was lysates of N2a cells expressing the chimeric constructs Xen(23–69) were analyzed for solubility and PK resistance. One aliquot of the lysate was used for PNGase F digestion. The resulting fractions were examined by Western blot analysis with antibody 3F4.

![Figure 7](image)

**Fig. 7. Internalization and transport to the cell surface of Xen(23–69).** A, schematic representation of the chimeric construct Xen(23–69). For construction of Xen(23–69), residues 23–69 of the Xenopus PrP (vertical bars) were introduced into the murine PrPΔ(23–90), after residue 22. Black and open bars represent the signal peptide and GPI anchor, respectively; vertical bars are residues 23–69 of Xenopus PrP. B, biochemical characterization of Xen(23–69). Postnuclear lysates of N2a cells expressing the chimeric constructs Xen(23–69) were analyzed for solubility and PK resistance. One aliquot of the lysate was used for PNGase F digestion. The resulting fractions were examined by Western blot analysis with antibody 3F4. Lanes 1 and 2, detergent-soluble pellet (P) or supernatant (S), respectively, upon ultracentrifugation; lane 3, fraction treated with 20 μg/ml PK; lane 4, aliquot digested with PNGase F. C, upper panel, transiently transfected N2a cells expressing chimeric PrP were incubated with 0, 45, or 60 min at 37 °C after surface biotinylation on ice for 15 min. Cells were lysed immediately (−) or subjected to trypsin digestion (+) for 10 min on ice before lysis and were then precipitated with antibody 3F4. Samples were subjected to SDS-PAGE, and signals were detected by streptavidin. Molecular mass markers are shown in kDa on the left. Lower panel, mean values from two independent experiments represent the amount of internalized protein expressed as a percentage of total labeled protein without trypsin digestion (at the same chase time) and plotted as a function of different time points. D, upper panel, Xen(23–69) was transiently transfected into N2a cells, metabolically labeled with [35S]Met/Cys for 5 min on ice, and then chased in 35S-free culture medium for 0, 45, 60, and 75 min, respectively. One plate was harvested immediately after the pulse or treated with trypsin and then lysed. All other plates were subjected to digestion with trypsin before lysis. Proteins were immunoprecipitated with antibody 3F4, deglycosylated with PNGase F, and subjected to SDS-PAGE. The blot shows PrP signals before (−) and after (+) treatment with trypsin. Molecular mass markers are shown in kDa on the left. Lower panel, PhosphorImager evaluation of autoradiograms representing the amounts of protein detected after digestion with trypsin. Each point represents a mean value of two independent experiments. Quantities are calculated as a percentage of protein precipitated immediately after the pulse without trypsin treatment.

![Figure 8](image)

**Fig. 8. Endo-H digestion of wtPrP* and N-terminal deletion constructs.** Confluent N2a cells transfected with wtPrP*, PrPΔ(23–90), or PrPΔ(48–93) were metabolically labeled with [35S]Met/Cys for 5 min on ice and then either harvested immediately or incubated in culture medium without [35S]S at 37 °C for different chase periods before lysis. Proteins were then immunoprecipitated with antibody 3F4 and treated with (+) or without (−) Endo-H overnight at 37 °C before analyzing by SDS-PAGE. Bars on the right indicate deglycosylated PrP-specific bands. Molecular mass markers are shown in kDa on the left.

wtPrP* with a degree of impairment dependent on the length of the deletion. The effect of the N-terminal truncation on the endocytosis of PrP was particularly evident when we analyzed the internalization of PrPΔ(23–90) and PrPΔ(48–93). Our biotinylation studies support other work on endocytosis of chicken PrP* (24), although the murine PrP seems to have slower internalization kinetics. These differences are probably because of relatively poor identity between mammalian and avian PrP (~30%) and to the longer N terminus of chicken PrP, which contains eight hexapeptide repeats (25). The endocytosis of murine PrP has also been analyzed in other studies, and the binding of copper to the histidine residues within the octapeptide repeats of PrP* has been seen to affect the cell surface localization of the protein by stimulating endocytosis (11, 26, 27). Our studies show that the octapeptide repeats are not the only region in PrP* with an internalization-promoting function because we could detect considerable differences between the internalization kinetics of PrPΔ(23–90) and PrPΔ(48–93). This hypothesis was confirmed when we examined PrPΔ(23–51) because this construct also showed impairment in its endocytosis, although not as strong as PrPΔ(23–90). Because the PrP constructs analyzed were expressed on the cell surface and their biochemical behavior did not significantly differ from that of the wild type protein, we conclude that the effects described here on the trafficking of PrP mutants are not a consequence of folding abnormalities. An endoplasmic reticulum-based cellular quality control resulting in degradation by proteasomes in the cytosol as described for mutated secretory proteins does not seem to be the case (28). Interestingly, in our biotinylation assays, the glycosylated forms of wtPrP* and of the deletion constructs were particularly evident, compared with the unglycosylated one. This might be because the relative amount of glycosylation seems to be dependent on the cellular localization of PrP*. The labeled proteins localized at the plasma membrane could therefore represent a subpopulation with specific glycan ratios compared with the total cellular PrP (29). Endocytosis of most transmembrane proteins is mediated by direct recognition of tyrosine-based signal motifs in their cytoplasmic domain by specific sorters proteins. Different mechanisms are responsible for internalization of GPI-anchored proteins such as PrP because they are devoid of such domains. Lipid rafts where PrP* is localized on the cell surface appear to form a marginal boundary of semioriented lipids flanking the glycerolipid domain in which transmembrane proteins are embedded (30). Numerous transmembrane receptors have been shown to bind to GPI-anchored proteins and to translate between different

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plasma membrane domains (31, 32). Clustering of PrPc in an intermediate semiordered lipid domain could promote binding to transmembrane proteins leading to endocytic trafficking. Our results support a similar model in which the N-terminal half of PrPc binds, as a single motif or as part of a larger epitope, with other elements of the protein, to the extracellular part of a transmembrane receptor containing internalization motifs. Impairment in endocytosis seen with N-terminal deletion constructs could therefore be explained by reduced affinity of these PrPs for a putative transmembrane receptor caused by deletions within the binding epitope.

The extremely prolonged presence of PrP molecules with deletions in their N-terminal part on the outer leaflet of the plasma membrane led to the assumption that this phenomenon might influence the turnover of these proteins. Our results revealed a half-life of −2.6 h for wtPrPc, confirming published data on PrPc degradation in primary cell cultures derived from lymphoid and nervous tissues (33) and neuroblastoma cells (34). On the other hand, the half-life monitored for N-terminal deletion mutants was almost twice as long as that of wtPrPc. To our knowledge, these findings are new because we show that inefficient internalization of PrPc correlates with a significantly prolonged turnover. Studies done with PrP constructs encoding pathogenic mutations showed that these mutations can affect some biochemical properties and intracellular trafficking of PrPc (35), but prolonged half-life was not reported.

The N Terminus of PrPc and the Secretory Pathway—Given the importance of the N-terminal part of PrPc for internalization and rate of degradation, we next focused on the transport of PrPc along the secretory pathway. Because the targeting of proteins to the cell surface requires specific sorting determinants similar to those involved in endocytosis, we addressed whether the same PrP segment, having a significant role in endocytosis, could also display a targeting function in the transport to the cell surface. Our assays revealed a delay in the transport of the PrP mutants to the cell surface, and even relative small truncations within the N-terminal segment could impair trafficking through the secretory pathway. The N-terminal deletions affected PrP trafficking with different impacts, depending on the pathway analyzed: the strongest impairment was monitored in internalization, whereas the secretory pathway was less compromised. These findings argue for the specificity of our results.

In a first attempt to characterize this impairment in the secretory pathway in more detail we subjected immunoprecipitated proteins to an Endo-H digestion. This analysis revealed that complete resistance was achieved for all constructs after a pulse. Endo-H resistance implies that glycoproteins have left the secretory pathway, therefore representing a basic sorting station for transport of proteins to the cell surface (36). Here, sphingolipid- and cholesterol-rich rafts in the luminal leaflet of the Golgi membrane act as microdomains for inclusion of proteins destined for the plasma membrane (37). For proteins lacking cytoplasmic domains, sorting to specific cellular destination is not based exclusively on protein sequence: N- and O-glycosylations (38), lipid attachment (e.g., palmitoylation) (39), and the GPI anchor (40, 41) can act as sorting determinants for recruitment into vesicles targeted to the cell surface (42). Of note, the N-terminal segment of the phospholipid-binding protein annexin XIIIb was reported to contain a plasma membrane localization determinant (43). Despite the cell surface localization of the deletion mutants used in our assay, the impaired trafficking described indicates reduced capacity of the cell to deal with these proteins properly. The question therefore arises of whether prolonged permanence of these mutated PrPs on the cell surface and in the endocytic pathway has an impact on certain PrPc biogenesis scenarios. Indeed, studies done with transgenic mice expressing PrP lacking residues 32–93 showed that these were still susceptible to scrapie infection but showed altered pathology and longer incubation time. Prion titers and protease-resistant PrP were about 30-fold lower than in wild type mice, with no histopathology typical for scrapie (44). These results are in line with in vitro cell-free conversion studies performed with hamster PrPc, where a truncated form of PrPc lacking amino acids 32–94 influenced the quantity and the conformation of generated PrPSc (45). In light of the results reported here, these findings could also be explained by altered intracellular trafficking of the mutated PrPc because of the alterations in the N-terminal part. Clinical studies have shown that two to nine octapeptides in addition to the normal five segregate with familial forms of Creutzfeldt-Jakob disease (46, 47) and nontransmissible prion disease in transgenic mice (48). Studies addressing the effect of these mutations on subcellular trafficking are in progress. In general, the question remains as to whether alterations in the trafficking of PrP and of other neurodegenerative proteins is important in the pathogenesis of sporadic forms of neurodegenerative disorders.

Targeting Function of PrP N Terminus Is Conserved in Evolution—The involvement of the N terminus of murine PrP in targeting and internalization led us to the question whether the corresponding sequence in the PrPc of more remote species might exert the same functions. The recent analysis of X. laevis PrP (49) allowed the construction of a chimeric Xenopus/mouse prion protein and characterization of its trafficking. Analysis of the biochemical properties of this construct did not reveal any abnormalities compared with wtPrPc. Surprisingly, addition of the Xenopus N-terminal stretch to the truncated mouse PrPc almost restored the wild type trafficking phenotype, its internalization and secretory kinetics strongly differing from those of PrPc (32–90) as well as of PrPc (48–93). Xenopus PrPc is significantly shorter than the mammalian PrPc, mainly because of the complete lack of copper-binding repeat elements and only shows about 28% identity to murine PrPc. This segment was nevertheless able to direct subcellular trafficking properly when fused to PrP of another species. These data argue for a conserved, intrinsic sorting function of this segment.

Taken together, our results confirm the role of the N-terminal part of PrPc in mediation of endocytosis. Moreover, they extend its importance, as we show that this conserved segment exerts a more general function as a targeting signal through the secretory pathway, therefore representing a basic sorting motif for intracellular trafficking. Additionally, our finding that the trafficking of the chimeric Xenopus/mouse protein was not altered despite the absence of octapeptides argues in favor of a model in which copper binding and subcellular trafficking represent separate aspects in the life-cycle of PrP.

Acknowledgments—We are grateful to Ulrich Koszinowski for continuous support and to Christian Spielhaupter and Jenna Lemerise for editorial supervision.

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