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Prion protein-related proteins from zebrafish are complex glycosylated and contain a glycosylphosphatidylinositol anchor ☆

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Abstract

A hallmark of prion diseases in mammals is a conformational transition of the cellular prion protein (PrP^C) into a pathogenic isoform termed PrP^{Sc}. PrP^C is highly conserved in mammals, moreover, genes of PrP-related proteins have been recently identified in fish. While there is only little sequence homology to mammalian PrP, PrP-related fish proteins were predicted to be modified with N-linked glycans and a C-terminal glycosylphosphatidylinositol (GPI) anchor. We biochemically characterized two PrP-related proteins from zebrafish in cultured cells and show that both zePrP1 and zeSho2 are imported into the endoplasmic reticulum and are post-translationally modified with complex glycans and a C-terminal GPI anchor.

Keywords: Prion; Scrapie; GPI anchor; Zebrafish; Sho

Prion diseases are a group of transmissible neurodegenerative disorders including Creutzfeldt–Jakob disease (CJD) and Gerstmann–Sträussler–Scheinker syndrome (GSS) in humans, scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in deer. A key event in the pathogenesis is a conformational transition of the cellular prion protein (PrP^C) into a misfolded and partially proteinase K-resistant conformation designated PrP^{Sc} (reviewed in [1–4]).

The biogenesis of mammalian PrP^C is characterized by extensive post-translational modifications (reviewed in [5]). After import into the endoplasmic reticulum (ER), PrP is modified with two N-linked glycans [6], a disulfide bridge, and a GPI anchor at serine 230 [7]. Finally, the core glycans are converted into complex structures and PrP^C is targeted to the outer leaflet of the plasma membrane.

PrP^C is highly conserved among mammals and is also present in chicken and turtle. Only recently, PrP-related proteins have been identified in fish [8–10]. While there is little sequence homology between mammalian PrP and fish PrP-related proteins, in general, it appears that characteristic features of mammalian PrP^C, like repeats in the N-terminal domain, an internal hydrophobic domain, and a disulfide bridge in the C-terminal domain, are conserved. In addition, it was predicted that fish PrP-related proteins contain signal sequences for targeting to the ER and for GPI anchor attachment and that they harbor consensus sites for N-linked glycosylation. Experimental evidence for post-translational modifications and cellular targeting, however, are missing so far.

Interestingly, a PrP-related protein named Shadoo (Sho) is highly conserved between fish and mammals, and is characterized by an internal hydrophobic domain (HD)

☆ *Abbreviations:* ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; PrP, prion protein.

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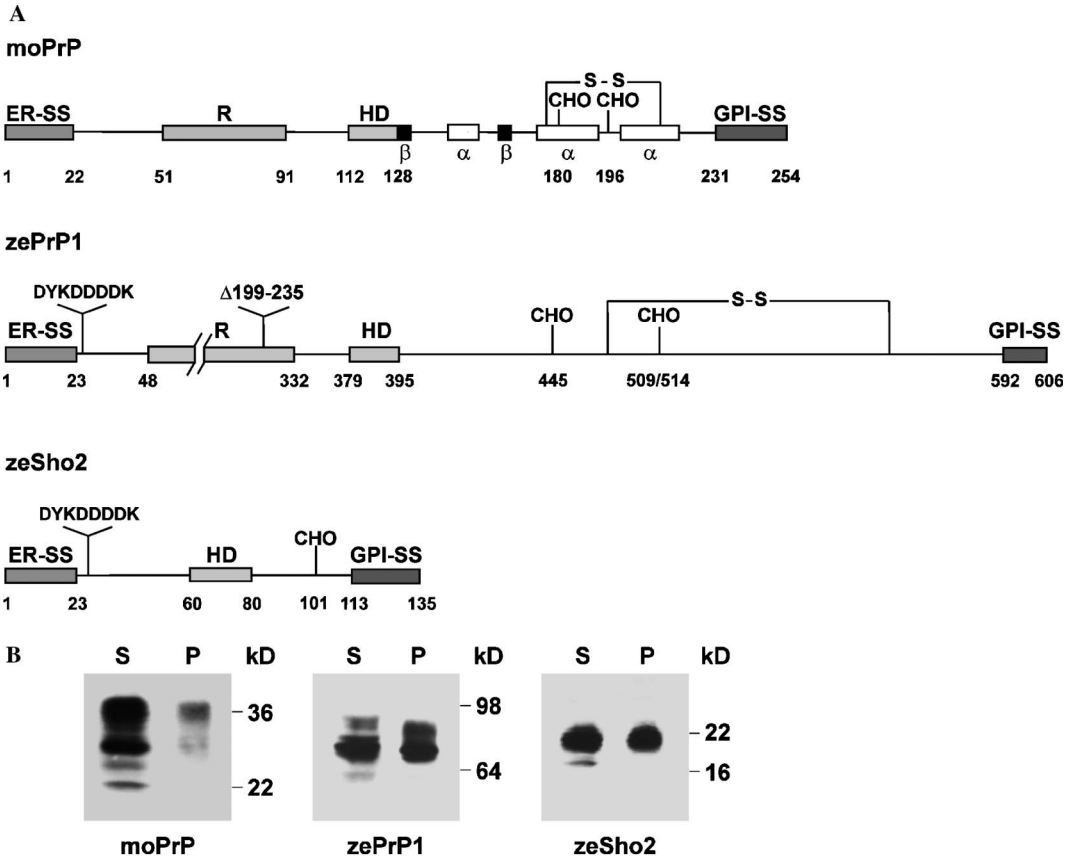

Fig. 1. Expression of PrP-related proteins from zebrafish in mouse neuroblastoma cells. (A) Schematic representation of mouse PrP^C (moPrP), zebrafish PrP 1 (zePrP1), and zebrafish Shadoo 2 (zeSho2). ER-SS, ER signal sequence; R, repeat; HD, hydrophobic domain; α, helical regions; β, β strands; CHO, putative N-linked glycan attachment sites; S–S, disulfide bond; GPI-SS, GPI signal sequence; DYKDDDDK, FLAG tag; Δ199–235, deletion of amino acids 199–235 in zePrP1. (B) N2a cells were transiently transfected with moPrP, zePrP1, and zeSho2. After cell lysis and centrifugation, PrP present in the detergent-soluble (S) and -insoluble (P) fractions was detected by Western blotting using the monoclonal antibody 3F4 (moPrP) or anti-FLAG mAb (zePrP1, zeSho2), respectively.

homologous to that of mammalian PrP^C (Fig. 1A). Moreover, it also was predicted that Sho is modified with N-linked glycans and a C-terminal GPI-anchor [11]. To study the biogenesis, maturation, and trafficking of fish PrP-related proteins, we cloned zePrP1 and zeSho2 into a mammalian expression vector for the expression in neuronal cells. We show that both proteins contain a functional ER signal sequence, are modified with complex glycans and a GPI anchor, and are targeted to the outer leaflet of the plasma membrane.

Materials and methods

Generation of DNA constructs. GenBank Accession Nos.: mouse PrP (M18070), zePrP1 (AJ850286), and zeSho2 (AJ583089). The generation of the mouse PrP^C (moPrP) and moPrP-CD4 constructs with a 3F4-epitope was described earlier [12]. For molecular cloning of zePrP1 and zeSho2 cDNAs, total RNA was extracted from zebrafish brains using the Trizol reagent (Gibco BRL). Total RNA (2 μg) was reverse transcribed with the First strand cDNA synthesis kit (Amersham Biosciences). zePrP1 and zeSho2 encoding cDNAs were subsequently amplified by standard polymerase chain reactions.

Antibodies and reagents. The following antibodies were used: mouse mAb 3F4 [13], anti-FLAG mAb (Sigma), rabbit polyclonal anti-parkin antiserum [14], HRP-conjugated anti-mouse and anti-rabbit IgG antibody (Amersham), and Cy3-conjugated anti-mouse and anti-rabbit IgG antibody (Dianova).

Cell culture, transfections, and N-linked glycosylation analysis. Mouse N2a cells (ATCC No. Ccl 131) were cultivated as described [15]. Cells were transfected by a liposome-mediated method using Lipofectamine Plus Reagent according to the manufacturer's instructions (Invitrogen). To analyze N-linked glycosylation, transfected cells were cultivated either with tunicamycin (0.5 μg/ml in DMSO, Sigma) or with swainsonine (10 μg/ml in PBS, Calbiochem) for 16 h at 37 °C prior to Western blot analysis. For endoglycosidase H (endoH) digestion, protein lysates were adjusted to 0.5% SDS, boiled for 10 min, and then treated with endoH (New England Biolabs) for 1 h at 37 °C, as specified by the manufacturer.

Trypsin and phospholipase C treatment. For trypsin treatment, intact cells were rinsed twice with ice-cold PBS and then incubated with trypsin (0.25%, w/v) for 5 min at 4 °C. The digestion was terminated by the addition of soy bean trypsin inhibitor (Invitrogen). Cells were collected by a brief centrifugation, washed with trypsin inhibitor, and analyzed by the detergent solubility assay. For phospholipase C treatment, cells were washed twice with ice-cold PBS. Phosphatidylinositol-specific phospholipase C (PIPLC; Sigma) in PBS (1 U/ml) was added to the cells for 3 h at 4 °C. Medium was collected, cells were washed twice with PBS and lysed in cold detergent buffer. Cellular PrP was analyzed by the detergent solubility assay. Proteins present in the cell culture medium were precipitated by trichloroacetic acid, boiled in Laemmli sample buffer, and analyzed by Western blotting.

Detergent solubility assay and Western blot analysis. As described earlier [16], cells were rinsed twice with ice-cold PBS, scraped off the plate,

pelleted by centrifugation, and lysed in cold detergent buffer (0.5% Triton X-100, 0.5% sodium deoxycholate in PBS). The lysate was centrifuged at 15,000*g* for 20 min at 4 °C and proteins present in the detergent-soluble and -insoluble fractions were examined by immunoblotting as described [15].

Indirect immunofluorescence experiments. Cells were grown on glass cover slips, rinsed twice with PBS, and then fixed with 3% paraformaldehyde for 10 min at room temperature. If indicated, cells were permeabilized with 0.1% Triton X-100 for 10 min. The fixed cells were incubated with the primary antibody for 45 min at 37 °C in PBS containing 1% BSA. After extensive washing with cold PBS, the secondary Cy3-conjugated antibody was added for 30 min at 37 °C. The washed coverslips were mounted onto glass slides and examined by phase contrast and fluorescence microscopy using a Zeiss Axiovert 200 M microscope.

Results

Cloning and expression of zePrP1 and zeSho2 in mammalian cells

Based on the published sequences, (GenBank Accession Nos.: AJ850286, AJ583089) primers were designed, zePrP1 and zeSho2 were cloned from a zebrafish cDNA library into a pcDNA3 vector. To allow immunodetection, a FLAG epitope (DYKDDDDK) was inserted downstream of the ER signal sequence after aa 24 for zePrP1 and after aa 26 for zeSho2 (Fig. 1A). Sequencing analysis revealed that zePrP1 has an internal in-frame deletion (amino acids 199–235) indicating the existence of splice variants, which were similarly described for Fugu rubripes PrP [9]. For the initial characterization, mouse neuroblastoma (N2a) cells were transiently transfected, lysed in detergent buffer (0.5% Triton X-100, 0.5% sodium deoxycholate in PBS), and fractionated by centrifugation. Proteins present in the detergent-soluble (S) and -insoluble (P) fractions were analyzed by Western blotting using a monoclonal anti-FLAG antibody. As a control for this and all subsequent experiments, transfected cells expressing mouse PrP (moPrP) containing a 3F4 epitope [17] were analyzed in parallel. This initial analysis revealed that both zebrafish proteins can be efficiently expressed in mouse cells; in comparison to moPrP, a larger fraction of zePrP1 and zeSho2 is present in the detergent-insoluble fraction (Fig. 1B).

ZePrP1 and zeSho2 are imported into the ER and are modified with N-linked complex glycans

A characteristic feature of PrP^C are the extensive co- and post-translational modifications and the cellular

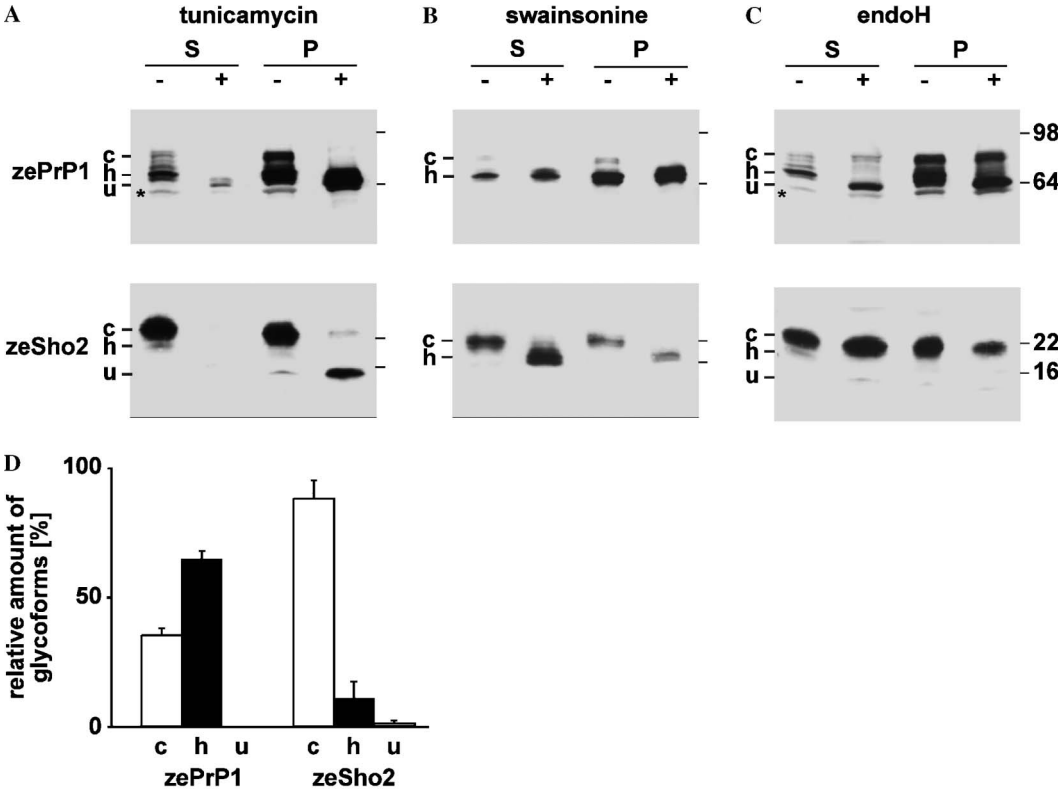

Fig. 2. PrP-related proteins from zebrafish are complex glycosylated. (A,B) Transiently transfected N2a cells were cultivated for 16 h at 37 °C in the presence of (A) tunicamycin (0.5 μg/ml) to inhibit core glycosylation or (B) swainsonine (10 μg/ml) to inhibit α-mannosidases or mock-treated. Proteins in the detergent-soluble (S) or -insoluble (P) fraction were analyzed by Western blotting using the anti-FLAG antibody. (C) To detect N-linked high mannose glycans, cell lysates prepared from transiently transfected cells were either incubated with or without endoglycosidase H (endoH ±) prior to the Western blot analysis. Different glycoforms are indicated on the left side of the panels: c, N-linked glycans with complex structure; h, high mannose glycans; and u, unglycosylated protein. The asterisk indicates a non-specific band present in the zePrP1 panels. (D) Quantitative analysis of the experiments shown under A, B, and C. The relative amounts of the different glycoforms present in the detergent-soluble (S) and -insoluble (P) fractions of untreated cells were calculated from three independent experiments.

trafficking to the outer leaflet of the plasma membrane (reviewed. in [5]). In this context, it is important to note that both zebrafish proteins were cloned with their authentic N-terminus, predicted to contain a signal sequence for ER import. We first addressed the question whether the zebrafish PrP-related proteins are modified by N-linked glycosylation which would indicate that they are imported into the ER. Each aspargine residue present in the tripetide motif N-X-T/S (where X is any amino acid except P) can theoretically be modified with core glycans. Based on the amino acid sequence, three putative glycosylation sites are present in zePrP1 (Asn 445, 509, and 514), and one in zeSho2 (Asn 101). A fourth putative site for N-linked glycosylation at Asn 367 was predicted for zePrP1 previously [10], however, this asparagine cannot serve as an acceptor site because the tripeptide motif contains a proline. To inhibit the addition of core glycans, in general, transiently transfected cells were cultivated in the presence of tunicamycin. The increase in the electrophoretic mobility of zePrP1 and zeSho2 after

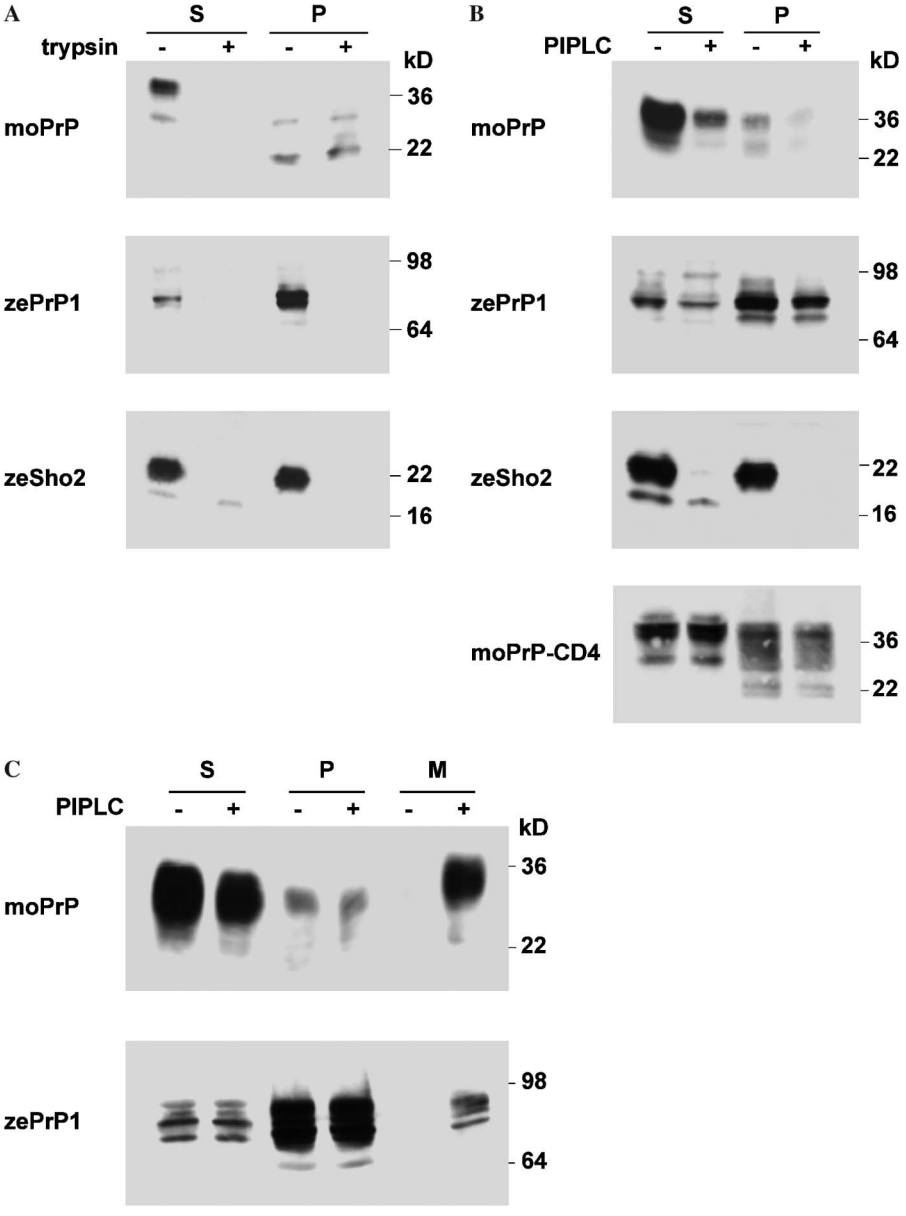

Fig. 3. ZePrP1 and zeSho2 are GPI-anchored and present on the outer leaflet of the cell surface. N2a cells were transiently transfected with the indicated constructs. In moPrP-CD4, the authentic GPI anchor is replaced by the heterologous transmembrane domain of the CD4 protein. (A) To remove cell surface proteins, in general, cells were incubated with trypsin (trypsin +) for 5 min at 4 °C. After cell lysis and centrifugation, residual PrP present in the detergent-soluble (S) or -insoluble (P) fraction was detected by immunoblotting. As a control, mock-treated cells (trypsin −) were analyzed in parallel. (B,C) Transfected cells were cultivated in the presence or absence of phosphatidylinositol-specific phospholipase C (PIPLC ±) for 2 h at 4 °C to release specifically GPI-anchored proteins from the cell surface. PrP present in the detergent-soluble (S) and -insoluble (P) fraction, as well as in the cell culture medium (M) was detected by Western blot analysis.

tunicamycin treatment indicated that both proteins had been N-glycosylated (Fig. 2A). To specifically investigate formation of complex glycans, cells were exposed to swainsonine, which blocks processing of high mannose to complex type oligosaccharides. In the presence of swainsonine, a high mannose glycoform appeared in cells expressing zeSho2 with a molecular weight between the mature form and the unglycosylated form generated in the presence of tunicamycin. Inhibition of complex glycosylation interfered with the formation of the upper band of zePrP1 and increased the amount of the lower band, indicating that the faint upper band represents complex glycosylated zePrP1 (Fig. 2B). To enzymatically remove N-linked glycans, protein extracts were incubated with endoglycosidase H (endoH), which is active only on high-mannose and hybrid structures. While the majority of zeSho2 was not affected by endoH digestion, indicating complex glycosylation, the major fraction of zePrP1 showed an increased electrophoretic mobility after endoH treatment, indicating modification by high mannose glycans (Fig. 2C).

In summary, this analysis revealed that both PrP-related proteins can be modified with glycans. While zeSho2 is almost exclusively complex glycosylated, two major glycoforms are present after the expression of zePrP1, representing high mannose and complex glycoforms.

ZePrP1 and zeSho2 contain a GPI anchor and are targeted to the outer leaflet of the plasma membrane

Shortly after mammalian PrP^C is fully translocated into the ER lumen, a GPI anchor is attached to serine 230 [7], which targets PrP to the plasma membrane. To investigate a possible cell surface localization of the two zebrafish PrP-related proteins, live cells were incubated with trypsin to remove all proteins present at the cell surface. After inactivation of trypsin by an inhibitor, cells were lysed and analyzed by Western blotting. As expected, mature complex glycosylated moPrP was no longer detectable in extracts prepared from trypsin-treated cells. Notably, both zePrP1 and zeSho2 were also degraded by extracellular trypsin treatment, indicating cell surface localization (Fig. 3A). To specifically analyze attachment to the plasma membrane via a GPI anchor, live cells were incubated with phosphatidylinositol-specific phospholipase C (PIPLC), which liberates GPI-anchored proteins. As a negative control, we included moPrP-CD4 in our analysis, a PrP mutant in which

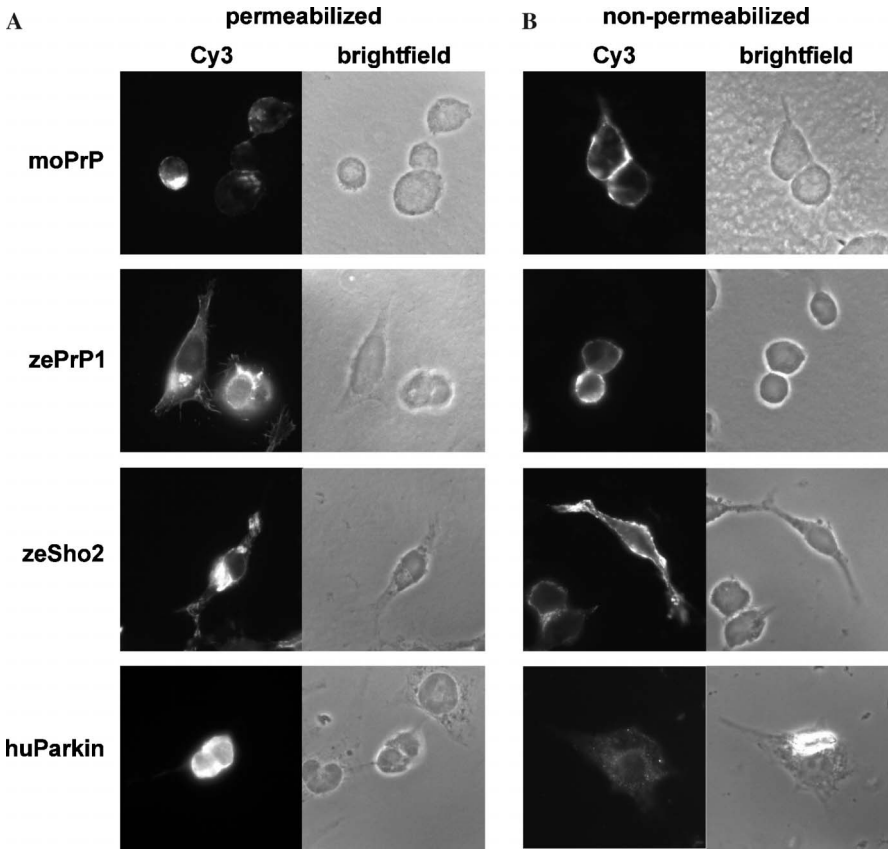

Fig. 4. Cellular localization of zePrP1 and zeSho2 in neuroblastoma cells. N2a cells growing on glass coverslips were transiently transfected with moPrP, zePrP1, zeSho2 or human parkin (cytosolic protein, control). Localization of the proteins was visualized by indirect immunofluorescence of permeabilized or non-permeabilized cells. For the detection of moPrP the mAb 3F4, for the fish PrPs the anti-FLAG mAb and for parkin a polyclonal anti-parkin antiserum was used.

the GPI anchor signal sequence was replaced by a heterologous transmembrane domain [12]. ZeSho2 was nearly quantitatively removed from the plasma membrane by PIPLC, similarly to moPrP, while zePrP1 seemed to be almost resistant to PIPLC treatment (Fig. 3B). To investigate this phenomenon in more detail, we analyzed the cell culture medium after PIPLC treatment for the presence of zePrP1. MoPrP could be detected in the supernatant medium of PIPLC-treated cells, and also a small fraction of zePrP1 could be liberated from the plasma by PIPLC, indicating that zePrP1 does contain a GPI anchor (Fig. 3C). The observed partial resistance of zePrP1 to PIPLC digestion could be due to sterical problems of the enzyme to access the GPI anchor. Notably, the size of zePrP1 is significantly larger than those of zeSho2 and moPrP.

Finally, we analyzed transiently transfected cells by indirect immunofluorescence. To distinguish between intracellular and plasma membrane localization, we analyzed non-permeabilized cells and cells permeabilized after fixation as well. The positive staining of non-permeabilized cells revealed that both zebrafish proteins were localized at the cell surface, similarly to moPrP (Fig. 4, non-permeabilized).

Discussion

Taken together, our study provides for the first time experimental evidence that the two PrP-related proteins zePrP1 and zeSho2 are (i) imported into the ER, (ii) are modified with complex glycans (Fig. 2), and (iii) are GPI anchored cell surface proteins (Figs. 3 and 4). Hence, essential maturation steps of fish prion proteins occur similarly to mammalian PrP^C, and notably of its paralogue doppel as well [18]. In addition to amino acid sequence and repeat motif similarities between fish and tetrapod PrPs the previously predicted conservation of important structural features is therefore substantiated by the data presented here. However, the open question remains whether mammalian PrP^C and fish PrP-related proteins have indeed similar functions or activities in their respective hosts. Our eukaryotic expression system for fish PrP-related proteins may facilitate a comparison of functional aspects to tetrapod PrPs.

The outstanding pathophysiological feature of mammalian PrP is the formation of misfolded, self-replicating, and possibly neurotoxic conformers. Fish species farmed for human consumption have been, and in all likelihood still are, exposed to prions of mammalian origin by feeding contaminated meat and bone meal. Based on sequence homologies alone it appears unlikely that fish could contract prion diseases. However, tissue distribution of fish PrP expression is clearly reminiscent of expression patterns seen for mammalian PrPs and, as shown in the present study, structural features are also functionally conserved. Availability of authentically post-translationally modified fish PrPs could aid in future investigations concerning the possible conversion of fish PrPs into an infectious isoform.

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