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Establishment of a Madin–Darby bovine kidney cell line expressing anchorless bovine prion protein

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ABSTRACT. Enzyme-linked immunosorbent assay (ELISA) performed using extensively purified bacterially expressed bovine prion protein (PrP) shows decreased cross-reactivity. We generated a transduced Madin–Darby bovine kidney (MDBK) cell line continuously expressing glycosylphosphatidylinositol (GPI)-anchorless bovine PrP (designated as MDBK ΔGPI protein) by using a lentiviral expression system. The present study also described the method for purifying bovine PrP through sequential culturing without the need for complex purification protocol. Our results showed that the purified bovine PrP could be used as an immunogen for developing anti-PrP monoclonal antibodies. Together, our results suggest that the new GPI-anchorless bovine PrP and its purification method can be used for performing basic studies for employing a cell-based approach.

KEY WORDS: bovine, GPI anchorless, lentivirus, PrP

Abbreviations: ELISA, Enzyme-linked immunosorbent assay; PrP, prion protein; MDBK, Madin-Darby bovine kidney; GPI, glycosylphosphatidylinositol; TSEs, Transmissible spongiform encephalopathies; PrP^{Sc}, disease-specific prion protein; PrP^{C}, normal cellular PrP; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WB, western blotting; mAb, monoclonal antibody; IFA, indirect fluorescence assay; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PNGase F, peptide-*N*-glycosidase F, HRP, horseradish peroxidase, PVDF, polyvinylidene fluoride.

Transmissible spongiform encephalopathies (TSEs), including scrapie in sheep and goats, bovine spongiform encephalopathy in cattle, chronic wasting disease in deer and elk, and Creutzfeldt–Jakob disease in humans, are fatal neurodegenerative diseases. TSEs are caused by disease-specific prion proteins (PrP^{Sc}) that are generated from host-encoded normal cellular PrP (PrP^{C}) through a conformational change [11, 16]. PrP^{C} is a glycoprotein that anchors to the cell membrane through a glycosylphosphatidylinositol (GPI) moiety [2, 11]. Thus far, recombinant PrPs purified from *Escherichia coli* have been used extensively in several studies [6, 12, 15, 19]. Previously, we generated an elk recombinant PrP [7] and bovine recombinant PrP by using a similar strategy. Briefly, the PrP gene was amplified from bovine genomic DNA by performing PCR with gene-specific primers (M-BoPrP-F: 5′-AAGATCTAAGAAGCGACCAAAACCTGGAG-3′ and GSx-BoPrP-R: 5′-GCTCGAGTCATGCCCCTCGTTGGTAATAAGCC TGGG-3′) and was cloned into pRSETB expression vector (Invitrogen, Carlsbad, CA, U.S.A.). Recombinant bovine PrP (designated as bovine-recPrP; amino acids 25–241; GenBank accession no. AJ298878) was eluted, and the eluted fractions were monitored by performing sodium dodedyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; left panel) and western blotting (WB) analysis (Fig. 1A). bovine-recPrP was used as an antigen for performing ELISA and showed low immunological activity against monoclonal antibody (mAb) 6H4 (Prionics, Zurich, Switzerland) (Fig. 1B), which was in contrast to that expected. This result indicated the need for a new expression system to produce bovine PrP with high yield and easy purification. Therefore, we established a Madin–Darby bovine kidney (MDBK) cell line by using a recombinant lentiviral expression system, as described previously [8, 17]. The recombinant lentiviral expression system was developed by cloning a gene fragment encoding a truncated soluble form of bovine PrP (amino acids 1–241) with a native signal peptide but lacking the GPI anchor into PLEX MCS vector (Open Biosystems, Huntsville, AL, U.S.A.). MDBK cells were transduced with the recombinant lentiviral expression system that continuously expressed GPI-anchorless bovine PrP^{C} and were selected using puromycin (2.5 μg/m*l*). PrP expression was confirmed by performing an indirect fluorescence assay (IFA) [8]. We observed that that GPI-anchorless bovine PrP (designated as MDBK ΔGPI protein) was expressed in puromycin-resistant transduced MDBK cells compared with that in control MDBK cells. Results

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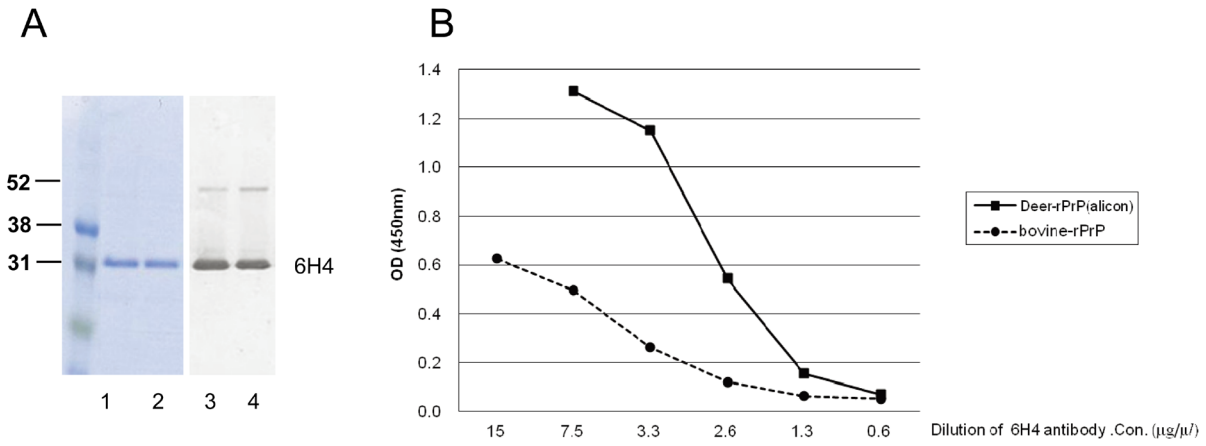

Fig. 1. Generation of recombinant bovine PrP (bovine-recPrP). (A) Purification of bovine-recPrP from *E. coli* Rosetta2 (DE3) pLysS cells. SDS-PAGE was stained with SimplyBlue SafeStain (lanes 1 and 2), and resolved proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. Results of WB analysis showing purified bovine-recPrP (lanes 3 and 4). (B) Antigen reactivity of the purified bovine-recPrP was detected by mAb 6H4.

of IFA showed that the MDBK ΔGPI protein was localized around the nucleus, particularly in the perinuclear endoplasmic reticulum, in transduced MDBK cells cultured in a medium containing Triton X-100 (data not shown). These results were similar to those of previous studies [1, 5, 13]. Cell viability was determined by performing an MTS-based assay by using CellTiter 96® Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, U.S.A.), according to the manufacturer's instructions. Cells were plated in 96-well plates at a density of 1×10^4 cells/well in triplicate. Control MDBK cells and MDBK ΔGPI-expressing transduced MDBK cells did not show any morphological differences. Moreover, results of the cell viability assay did not show significant differences (data not shown) in the viability of MDBK cells and MDBK ΔGPI protein-expressing transduced MDBK cells. Next, we described a method for purifying bovine PrP by optimizing culture conditions by using a serum-free medium and without the need for complex purification protocol. Briefly, MDBK control cells and MDBK ΔGPI protein-expressing cells were cultured in T-75 flasks containing high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/m*l*), streptomycin (100 μg/m*l*), non-essential amino acids, and fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 incubator. On the first day, MDBK ΔGPI protein-expressing cells showing 50% growth were cultured in high-glucose DMEM supplemented with 1% FBS. On the next day when the cells showed 60–70% growth, the culture medium was replaced with a fresh culture medium containing 0.5% FBS. On the last day when the cells showed 80% growth, the culture medium was replaced with an FBS-free medium. Thus, 5×10^6 cells/m*l* seeded in a medium supplemented with 1% FBS produced 8×10^6 cells/m*l* with sustained high viability (>80%) in the serum-free medium after a 3-day culture. The cells were counted every 24 hr and lysed in a buffer containing 150 mM NaCl, 1% Zwittergent 3–14, 50 mM Tris-HCl (pH 7.5), and 2 mM EDTA. The MDBK ΔGPI protein was characterized by performing WB analysis with a polyclonal S1 antibody, which was obtained by immunizing rabbits with a synthetic bovine PrP106–122 peptide (CTHGQWNKPSKPKTNMK). WB analysis detected the MDBK ΔGPI protein in cell lysates and cell culture medium, indicating that the recombinant lentiviral expression system-transduced MDBK cells secreted the MDBK ΔGPI protein into the culture medium (Fig. 2A, left panel). Molecular weight of the MDBK ΔGPI protein present in cell lysates was 28–37 kDa and that of the MDBK ΔGPI protein present in the FBS-free culture medium was approximately 30 kDa (Fig. 2A, right panel). The bovine PrP was secreted in the culture medium (>2 μg/20 m*l* medium) and purified using Amicon® Ultra-15 Centrifugal Filter 50K Device. Moreover, analysis of the secreted bovine PrP by using NanoDrop 2000 spectrophotometer (Thermo Scientific, Middlesex, MA, U.S.A.) indicated its high purity. Next, the MDBK ΔGPI protein was digested with peptide-*N*-glycosidase F (PNGase F), with normal bovine brain homogenates as control. For PNGase F treatment, the samples were resuspended, boiled for 10 min at 100°C, and incubated with PNGase F for 1 hr at 37°C. PNGase F-induced digestion of the MDBK ΔGPI protein suppressed its N-glycosylation and produced a single band corresponding to the non-glycosylated form of bovine PrP, as that present in the normal bovine brain homogenates (Fig. 2B). ELISA, which was performed as described previously [7], detected the MDBK ΔGPI protein by using PrP-specific mAbs 6H4 and N18-5 (Fig. 2C). ELISA was also performed using three different mAbs, namely, 6H4 (156–164; Prionics), N18-5 (152–155), and 8E63 (152–168). Bovine PrP epitopes, 156–164, 152–155, positively reacted with antisera raised against anchorless bovine PrP on ELISA, compared with sera from elk PrP epitope, 152–168. For immunizing PrP gene-knockout mice, 200 μg MDBK ΔGPI protein was emulsified in complete Freund's adjuvant (Sigma, St. Louis, MO, U.S.A.) at a ratio of 1:1. The mice were subcutaneously injected with the indicated protein preparation three times consecutively at 0, 2, and 4 weeks. Serum samples of the mice were obtained from the heart and were stored at −70°C. To evaluate the development of an immune response, ELISA wells were coated with a bovine recombinant PrP (0.2 μg/well; Prionics) and were incubated with the antisera obtained from immunized mice (gradient dilutions, 1:100, 1:200, 1:500 and 1:1,000) for 1 hr. Antibody reactivity was determined using horseradish peroxidase (HRP)-conjugated goat anti-mouse

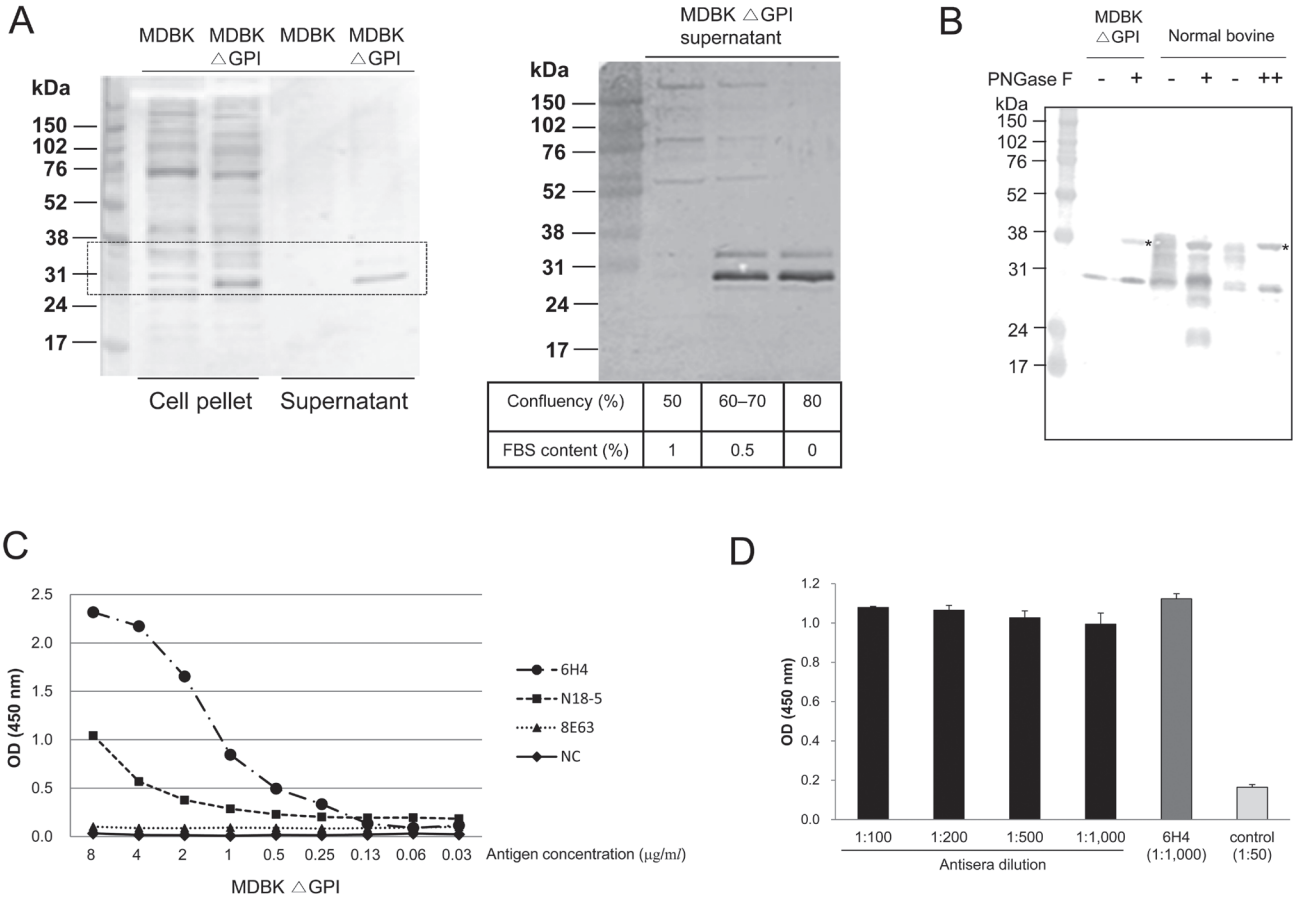

Fig. 2. Characterization of the purified MDBK ΔGPI protein. (A) WB analysis of the MDBK ΔGPI protein purified from transduced MDBK cells. The MDBK ΔGPI protein was immunoblotted using polyclonal S1 antibody (APQA, the Republic of Korea). (B) Glycosylation of the MDBK ΔGPI protein. Cell culture supernatant (medium fraction) and bovine brain homogenates were incubated with or without PNGase F for 1 hr. The asterisk indicates the molecular weight of PNGase F. +=1 μl PNGase F (500 NEB units), ++=2 μl PNGase F (500 NEB units). (C) Antibody reactivity of the MDBK ΔGPI protein was determined using mAbs 6H4 and N18-5 that recognized only bovine PrP and could not be determined using mAb 8E63 that showed specificity for elk PrP. (D) Reactivity of the antisera obtained from immunized mice against the purified MDBK ΔGPI protein was analyzed by performing ELISA to recognize the MDBK ΔGPI protein as an immunogenic bovine protein. Positive control well, anti-PrP 6H4 antibody; negative control well, PrP k/o mouse serum.

IgG secondary antibody and its substrate ABTS and is expressed as OD values in triplicate. Prion gene-knockout mice were immunized with the recombinant MDBK ΔGPI protein and the strength of the immune response was examined by performing ELISA (Fig. 2D). Thus, we first verified whether FBS present in cell culture medium interfered with the purification of the recombinant GPI-anchorless PrP and described a method for purifying GPI-anchorless PrP by optimizing culture conditions without the need for a complicated purification protocol. We detected GPI-anchorless PrP in recombinant lentiviral vector-transduced MDBK cells. Results of biochemical assays showed that the GPI-anchorless PrP was not present at the cell membrane but was present around the nucleus of transduced cells. WB analysis detected the GPI-anchorless PrP in both cell lysates and cell culture medium. The molecular weight of the glycosylated GPI-anchorless PrP in the cell lysates and culture medium was approximately 30 kDa. Furthermore, the GPI-anchorless PrP was more efficiently secreted into the culture medium (Fig. 2A) and produced its characteristic non-glycosylated form, as that present in control MDBK cells, after PNGase F treatment. Second, we used the lentiviral expression system for establishing recombinant bovine PrP-expressing MDBK cells because this system is highly efficient for transfecting exogenous genes, as described previously [8, 17], and used these cells for efficiently producing the recombinant GPI-anchorless PrP. Thus, we produced a soluble bovine PrP by establishing a recombinant lentiviral expression system. The obtained GPI-anchorless bovine PrP can be used for performing structural, functional, and biochemical analyses of normal PrPs.

Several studies suggest that GPI-anchorless PrP has a long biological half-life and indicate that GPI-anchorless PrP produced in the transgenic mouse brain and in cell culture medium is present in an unglycosylated form [1, 3, 10, 14]. An *in vitro* study showed that an artificially produced PrP mutant lacking the GPI anchor at its C-terminal can adopt a PrP^{Sc} conformation [9, 18]. However, Fisher rat thyroid cells contain a highly glycosylated GPI-anchorless PrP mutant that is tethered to the cell membrane and that does

not assume a transmembrane topology [1]. Moreover, another study showed that the GPI anchor and not glycosylation are essential for PrP secretion [13]. In contrast, prion-infected mice expressing GPI-anchorless PrP developed a new type of prion disease that did not result in typical sponge-like brain damage [4]. Thus, the convenient expression system developed in the present study can be used to investigate the effect for GPI-anchorless PrP on mechanisms underlying the normal functions and accumulation of PrP^{Sc}. ELISA by using mAbs 6H4 and N18-5 detected the recombinant GPI-anchorless bovine MDBK ΔGPI protein. Interestingly, the mAb N18-5 produced in our laboratory recognized only bovine PrP. Moreover, the MDBK ΔGPI protein produced in the present study did not react with mAb 8E63 that showed reactivity against Elk PrP. The immunogenicity of the MDBK ΔGPI protein was determined by performing ELISA, as described above, by using different dilutions (1:100, 1:200, 1:500 and 1:1,000) of the antisera obtained from MDBK ΔGPI-immunized mice and 1:2,000 dilution of HRP-conjugated goat anti-mouse IgG secondary antibody. Together, these results suggest that purified bovine PrP maintains its antigenicity and immunogenicity and can be used for producing glycosylated bovine PrP-specific mAbs. In conclusion, our results showed that GPI-anchorless PrP expressed in MDBK cells was secreted in its soluble form into the culture medium. The GPI-anchorless PrP isolated from the FBS-free culture medium was also present in its soluble form. However, further studies should be performed to determine the association between the expression level of GPI-anchorless bovine PrP and formation of PrP^{Sc}.

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