

Binding of Recombinant but Not Endogenous Prion Protein to DNA Causes DNA Internalization and Expression in Mammalian Cells*

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Recombinant prion protein, rPrP, binds DNA. Both the KKRPK motif and the octapeptide repeat region of rPrP are essential for maximal binding. rPrP with pathogenic insertional mutations binds more DNA than wild-type rPrP. DNA promotes the aggregation of rPrP and protects its N terminus from proteinase K digestion. When rPrP is mixed with an expression plasmid and Ca^{2+} , the rPrP-DNA complex is taken up by mammalian cells leading to gene expression. In the presence of Ca^{2+} , rPrP by itself is also taken up by cells in a temperature- and pinocytosis-dependent manner. Cells do not take up rPrP^{ΔKKRPK}, which lacks the KKRPK motif. Thus, rPrP is the carrier for DNA and the KKRPK motif is essential for its uptake. When mixed with DNA, a pentapeptide KKRPK, but not KKKKK, is sufficient for DNA internalization and expression. In contrast, whereas the normal cellular prion protein, PrP^C, on the cell surface can also internalize DNA, the imported DNA is not expressed. These findings may have relevance to the normal functions of PrP^C and the pathogenic mechanisms of human prion disease.

Human prion diseases constitute a group of fatal neurodegenerative diseases (1, 2). The majority of human prion diseases are sporadic, in which the pathogenic mechanisms are not known. Human prion diseases, such as Kuru and iatrogenic and variant Creutzfeldt-Jacob disease, are contracted by an infectious mechanism. On the other hand, inherited human prion disease, which accounts for about 10% of human prion disease, is caused by mutation of the germline prion gene, *PRNP*.

More than 30 different pathogenic mutations in the human *PRNP* gene have been identified (3). These mutations are either insertional or point mutations. Insertion mutation occurs solely in the octapeptide repeat region; wild-type human (PrP^C)² has five octapeptide repeats. Point mutations occur

along the entire PrP^C molecule. It is thought that the mutant prion protein, PrP^M, is inherently unstable, leading to self-association to produce an oligomeric structure (4, 5). This structure acts as a “seed” recruiting additional PrP^M, eventually leading to the formation of PrP^{Sc}. Accumulated evidence suggests that the conversion process may require the participation of other macromolecules, such as glycosaminoglycans (6–8), nucleic acids (9, 10), lipids (11, 12), cellular proteins, such as chaperone proteins (13, 14), or divalent cations (15, 16). The mechanism by which a PrP^M causes neuropathology remains unclear. PrP^M may cause disease because of a gain of toxic function, loss of normal function, or both.

Bacteria-produced recombinant prion proteins, rPrPs, have been used extensively as model systems to study the differences between wild-type rPrP and rPrP^M (17–19). Biophysical studies suggest that thermoinstability is not the major contributing factor in the conversion process (20, 21). Recently, we reported that rPrP^Ms with pathogenic mutations have a more exposed N terminus and bind more glycosaminoglycan (GAG) (22, 23). Binding of GAG also promotes the aggregation of rPrP^M (24). The dominant GAG binding site in rPrP is located at the N terminus, the first five amino acids, KKRPK (25). This motif also functions as a nuclear localization signal (26). rPrPs also bind DNA and RNA (27–33). However, the motif on PrP that is involved in binding nucleic acids remains unclear. Some studies suggest that the N terminus is essential (29, 34), whereas others suggest that the C terminus is also important for binding (28, 35, 36).

In this article, we describe our most recent findings showing that both the KKRPK motif and octapeptide repeat region of the rPrP are essential for the binding of rPrP to DNA. Furthermore, in comparison to wild-type rPrP, rPrP^Ms with pathogenic insertional mutations bind more DNA. DNA promotes the aggregation of rPrP and renders rPrP partially proteinase K resistant. Binding of rPrP to DNA promotes the uptake of the rPrP-DNA complexes by mammalian cells, resulting in gene expression. On the other hand, cell surface PrP^C also internalizes DNA but the imported DNA is not expressed. The significance of these findings with respect to the normal functions of PrP^C and the pathogenesis of prion diseases is discussed.

rPrP with 10 octapeptide repeats; rPrP^{ΔKKRPK}, rPrP lacking the KKRPK motif; rPrP^{ΔOR}, rPrP lacking the octapeptide repeats; rPrP^{Δ23–90}, rPrP lacking residues 23 to 90; rPrP^{23–145}, rPrP with residues 23 to 145; Alexa-rPrP, Alexa Fluor 546-labeled rPrP; PBS, phosphate-buffered saline; GAG, glycosaminoglycans; mAb, monoclonal antibody; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; GPI, glycosylphosphatidylinositol; PK, proteinase K.

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² The abbreviations used are: PrP^C, cellular form of native normal prion protein; PrP^{Sc}, the infectious and pathogenic scrapie prion; PrP^M, prion protein with pathogenic mutations; rPrP, bacterial produced, recombinant mature full-length wild type PrP; rPrP^{8OR}, rPrP with 8 octapeptide repeats; rPrP^{10OR},

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies—The generation and characterization of anti-PrP^C mAbs 8B4, 7A12, and 8H4 have been described in detail (37).

Cell Culture—Chinese hamster ovary cells (CHO) were maintained in α -minimal essential medium (Invitrogen) and human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen). Each medium was supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μ g/ml penicillin, and 100 μ g/ml streptomycin. Primary cultures of normal human astrocytes were obtained from Cambrex (Cambrex BioScience, Walkersville, MD) and cultured in Astrocyte Growth Medium, AGMTM, obtained from the same vendor. Cells were cultured at 37 °C in a humidified atmosphere of air and 5% CO₂.

Plasmid Constructions and Proteins Preparation—The expression vectors for human rPrP corresponding to the mature full-length fragment (23–231) pET-rPrP, pET-rPrP ^{Δ 23–90}, pET-rPrP ^{Δ KKRPK}, pET-rPrP ^{Δ OR}, pET-rPrP^{8OR}, and pET-rPrP^{10OR} have been described previously (22, 23). All sequences were verified using an Applied Biosystems 373A Automated Sequencer. The generation, purification, and characterization of the recombinant proteins has been described in detail (22, 38). Protein concentration was determined with a Bio-Rad Protein Assay kit.

Binding of rPrPs to DNA—To detect rPrP-DNA binding, a synthetic single-stranded oligonucleotide was used: 5'-GTAA-CCGAAATCGGTTGA-3' (18-nucleotide long DNA). DNA (0.5 μ M) was coated onto 96-well ELISA plates at 4 °C overnight, and blocked with 3% bovine serum albumin in PBS at room temperature for 3 h. Appropriate dilutions of rPrP or rPrP^Ms were added to individual wells in triplicates and incubated for 2 h. After three washes with PBS-T (PBS, pH 7.4, containing 0.05% Tween 20), bound rPrP was detected with anti-PrP mAb 8H4 or SAF32 (39). Plates were washed three times with PBS-T, and horseradish peroxidase-conjugated streptavidin at 1:10,000 was added for 1 h. ELISA plates were washed three times with PBS-T before adding the substrate *p*-nitrophenyl phosphate at 0.5 mg/ml. ELISA plates were read at 405 nm on a Beckman Coulter AD340 micro-ELISA plate reader. In some experiments, double-stranded plasmid pEYFP-GPI was used for detection of DNA-rPrP binding. To study the effects of DNA size on rPrP-DNA interaction, two other synthetic single-stranded oligonucleotides were also used in some experiments: 5'-ACCGGAATTCCTAGTCACTGCCGAA-TGTATGATG-3' (35-nucleotide long DNA) and 5'-TGTC-GAC-3' (7-nucleotide long DNA).

Turbidity Measurement—Aggregation reactions were performed as previously described (24). The assays were performed at room temperature in a volume of 100 μ l in 96-well plates. Wild type rPrP or rPrP^M (5 μ M) were suspended in 100 μ l of PBS, pH 7.4. DNA at the indicated size was added into the reactions at a final concentration of 2.5 μ M. After adding DNA, turbidities were monitored as quickly as possible (during 15 s) by reading the absorbance at 405 nm in a Beckman Coulter AD340 micro-ELISA plate reader, using a kinetic photometric model (interval time 30 s, 30 cycles with 1-s shaking before

every cycle). To study the blocking effects of 7-nucleotide long DNA in the aggregation experiments, 5 μ M wild-type rPrP was preincubated with 2.5 μ M 7-nucleotide long DNA for 30 min at room temperature, then 35-nucleotide long DNA was added to start the aggregation reaction.

Gel Shift Assay—In the agarose gel shift assay, 0.2 μ g of plasmid pcDNA3 (5.4 kb) was incubated with 1, 2.5, and 12.5 μ g of wild-type rPrP or rPrP ^{Δ KKRPK} in a 10- μ l total volume of PBS for 1.5 h at room temperature, respectively. After incubation, each individual sample was loaded on a 1% agarose gel, and electrophoresed at 100 V. Gels were stained with ethidium bromide after electrophoresis to visualize DNA bands.

Radioactivity Counting of rPrP/DNA Aggregates—Wild type rPrP, rPrP ^{Δ KKRPK}, rPrP^{8OR}, or rPrP^{10OR} (10 μ M of each) were incubated with ³²P-labeled 18-nucleotide long DNA (5 μ M) in 100 μ l of PBS for 30 min at room temperature, then centrifuged at 13,000 \times g for 10 min. After extensive washing by PBS, the pellets were dissolved in 100 μ l of 1.0 M NaOH, 1% Triton X-100, and transferred to vials for scintillation counting in a PerkinElmer 1450 LCS counter. Bovine serum albumin was mixed with labeled DNA, processed similarly, and used as a negative control.

PK Digestion of rPrP-DNA Complexes—Wild type rPrP or rPrP^{10OR} (1 μ M of each) were incubated in 18-nucleotide long DNA (5 μ M) in a 20- μ l total volume of PBS for 30 min at 37 °C, then proteinase K was added at 0, 5, 10, and 20 μ g/ml and further incubated for 30 min at 37 °C. rPrPs without DNA were used as a control. The digested mixtures were then separated by SDS-PAGE (12% Tris glycine, Bio-Rad), electrotransferred onto nitrocellulose membranes, and immunoblotted with different anti-PrP mAbs. A horseradish peroxidase-conjugated goat anti-mouse IgG Fc region-specific antibody was used as the secondary antibody. rPrP detection was performed using the chemiluminescence blotting system (Roche).

Cell Transfection and Assay—Cell transfection experiments were performed as described previously (40) with modification. Cells were seeded in 24-well tissue culture plates at a density of 1×10^4 cells per well and grown overnight at 37 °C in a CO₂ incubator. The growth medium was removed and replaced with 0.5 ml/well of fresh growth medium for 2 h before the addition of rPrP-DNA complexes. Plasmid pEYFP-GPI (1 μ g) in 100 μ l of PBS was mixed with different amount of rPrPs or KKRPK peptide and 0.5 μ l of 0.5 M CaCl₂ at room temperature for 1 h. After addition of 500 μ l of growth medium containing 10% fetal bovine serum and 6 μ l of 0.5 M CaCl₂, the transfection mixture was used to replace the culture medium of the confluent cells. The mixture was removed after 4 h of incubation and replaced with 0.5 ml of fresh medium. After 24 h, the cells were transferred to 6-well plates and cultured in the presence of 10 μ g/ml blasticidin. The antibiotic resistant cell colonies were fixed with 100% methanol and stained with 4% trypan blue and counted under a microscope. Normal human primary astrocytes were grown on 4-well chamber slides and transfected by the rPrP-DNA complexes as described above. Cells were observed in a fluorescence microscopy at 18 h after transfection. For flow cytometry analysis, 1×10^6 cells in 6-well tissue culture plates were transfected using the optimized rPrP concentration (5 μ g/ml) and pEYFP-GPI (4 μ g/well). After 48 h, cells were ana-

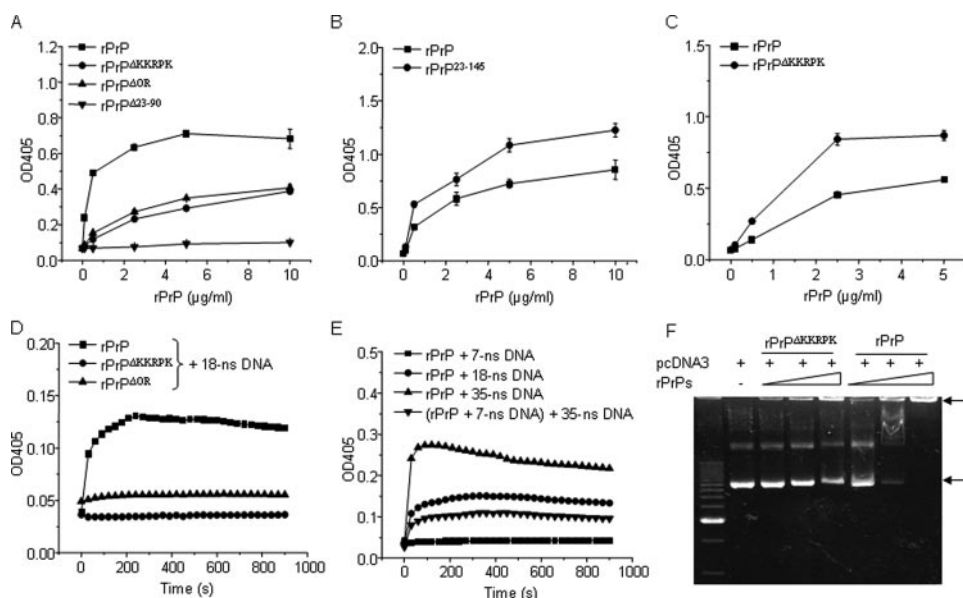


FIGURE 1. The N-terminal domain of rPrP is essential for binding DNA. A, ELISA plates were coated with 0.5 μ M 18-nucleotide long DNA. Various concentrations of wild-type rPrP, rPrP Δ KKRPK, rPrP Δ OR, and rPrP Δ 23–90 were added to the respective wells. After washing, bound rPrPs were detected using mAb 8H4 followed by horseradish peroxidase-conjugated goat anti-mouse IgG-Fc specific antiserum. The data presented are the mean \pm S.E. of triplicate wells. B, the experiment was carried out as described in A, but mAb SAF32 was used to detect bound wild-type rPrP and rPrP Δ 23–145. C, the experiment was carried out as described in A, except that double-stranded plasmid pEYFP-GPI (2.5 nM) was coated onto the ELISA plates. D, wild-type rPrP, rPrP Δ KKRPK, or rPrP Δ OR (5 μ M of each) were incubated with 2.5 μ M 18-nucleotide long (18-ns) DNA, respectively. The kinetics of aggregation was monitored by measurement at OD405. E, wild-type rPrP (5 μ M) was incubated with 2.5 μ M DNA of the indicated size. Turbidities were monitored by absorbance at 405 nm. The data presented are the mean \pm S.E. The aggregation of rPrP induced by DNA is DNA size-dependent. Although 7-nucleotide long (7-ns) DNA did not cause aggregation of rPrP, it could block the larger DNA-induced aggregation. F, DNA binding was analyzed by agarose gel electrophoresis of plasmid pcDNA3 in the absence of rPrPs, or after incubation with increased amounts of wild-type rPrP or rPrP Δ KKRPK. The positions of supercoiled DNA and rPrPs-DNA complexes are indicated by arrows. All experiments were performed at least three times with comparable results.

lyzed in a FACSscanTM (BD Bioscience). CHO cells do not express endogenous PrP^C; a stable CHO cell line (CHO-PrP^C) was established as described (41). To detect cell surface PrP^C by fluorescence-activated cell sorter, CHO-PrP^C was incubated with mAb 8H4 (1 μ g/ml) in ice-cold PBS. The cells were washed and further incubated with a fluorescein isothiocyanate-labeled goat anti-mouse IgG antibody (1:1000).

Confocal Fluorescence Microscopy—rPrP was labeled with Alexa Fluor 546 using an Alexa Fluor 546 labeling kit (Invitrogen). Plasmid pEYFP-GPI was labeled with a Label IT Tracker Cy3 labeling kit (Mirus, Madison, WI). Cells were seeded on chamber slides and allowed to grow overnight. For rPrPs internalization assay, Alexa Fluor 546-labeled wild-type rPrP (Alexa-rPrP) or Alexa-rPrP Δ KKRPK (5 μ g/ml of each) was mixed 0.5 μ l of 0.5 M CaCl₂ in 100 μ l of PBS at room temperature for 1 h. After addition of 500 μ l of growth medium containing 10% fetal bovine serum and 6 μ l of 0.5 M CaCl₂, the mixture was used to replace the culture medium of confluent cells in chamber wells and incubated for 1 h at 37 °C. For testing the temperature effects on uptake of rPrPs, some of the incubation was also performed at 4 °C for 1 h. In some experiments, cells were treated with the macropinocytosis inhibitor amiloride (2.5 mM) for 30 min prior to incubation and during uptake experiments.

For peptide-mediated DNA internalization assay, Cy3-labeled pEYFP-GPI plasmid (0.5 μ g/ml) was mixed with KKRPK or KKKKK peptide (33 μ g/ml of each) and 0.5 μ l of 0.5 M CaCl₂

in 100 μ l of PBS at room temperature for 1 h. After addition of 500 μ l of growth medium containing 10% fetal bovine serum and 6 μ l of 0.5 M CaCl₂, the mixture was used to replace the culture medium of confluent cells in chamber wells and incubated for 1 h at 37 °C.

For the cell surface PrP^C-mediated DNA internalization assay, Cy3-labeled pEYFP-GPI plasmid (0.5 μ g/ml) was directly added into 500 μ l of Opti-MEM (Invitrogen) and incubated with CHO or CHO-PrP^C cells for 1 h at 37 °C. All cells were rinsed with PBS six times, fixed with 4% (w/v) paraformaldehyde, and incubated with the fluorescent dye 4',6-diamidino-2-phenylindole (1:1000) to stain the nucleus. Cells were washed extensively and mounted in Fluoromount-G (Southern Biotech) and analyzed on a LSM 510 META confocal microscopy.

RESULTS

Binding of rPrPs to DNA—We quantified the binding of wild-type rPrP and three truncated rPrPs to single strand DNA by ELISA. rPrP binds DNA in a protein concentration-dependent manner (Fig. 1A). Compared with wild-type rPrP, rPrP Δ KKRPK, which lacks the KKRPK motif and rPrP Δ OR, which lacks the octapeptide repeats have only half of the DNA binding activity. On the other hand, rPrP lacking the entire N terminus, rPrP Δ 23–90, is unable to bind DNA. Conversely, an rPrP Δ 23–145, which lacks the C-globular domain, binds even higher levels of DNA than wild-type rPrP (Fig. 1B). Binding to double strand DNA revealed similar results (Fig. 1C). Thus, both the KKRPK motif and the octapeptide repeat region are required for maximum binding of DNA. The smallest DNA, which rPrP binds is 7-nucleotide long (not shown).

Aggregation of PrP^C is an important step in the PrP^C to PrP^{Sc} conversion process (1, 42, 43). DNA promotes the aggregation of wild-type rPrP but not rPrP Δ KKRPK or rPrP Δ OR (Fig. 1D), and the degree of aggregation is proportional to the length of the DNA (Fig. 1E). Although rPrP binds 7-nucleotide long DNA in ELISA, this DNA does not cause rPrP aggregation. However, DNA with 7 nucleotides did block the aggregation of rPrP induced by larger DNA, albeit only by ~50% (Fig. 1E). We speculate that simply binding to DNA is insufficient to cause rPrP to aggregate; aggregation of rPrP requires longer DNA, which can simultaneously bind multiple rPrP molecules. Binding of rPrP to DNA is further confirmed by gel shift assay (Fig. 1F). In the presence of rPrP the migration of the DNA into the gel is retarded, proportional to the amount of rPrP added (see

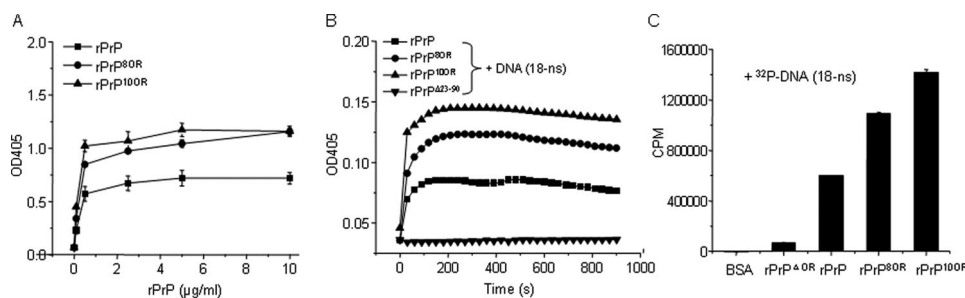


FIGURE 2. rPrP^Ms with pathogenic insertional mutations bind more DNA. A, ELISA plates were pre-coated with 0.5 μM 18-nucleotide long DNA. Various concentrations of wild-type rPrP, rPrP^{80R}, or rPrP^{100R} were added to the respective wells. After washing, bound rPrPs were detected using mAb 8H4 followed by horseradish peroxidase-conjugated goat anti-mouse IgG-Fc specific antiserum. The data presented are the mean ± S.E. of the triplicate wells. B, wild-type rPrP, rPrP^{80R}, rPrP^{100R}, and rPrP^{Δ23-90} (5 μM each) were incubated with 2.5 μM 18-nucleotide long (18-ns) DNA. Turbidities were monitored by the absorbance at 405 nm. C, wild-type rPrP, rPrP^{ΔOR}, rPrP^{80R}, or rPrP^{100R} (10 μM of each) were incubated with ³²P-labeled 18-nucleotide long DNA (5 μM). After incubation, the aggregates were pelleted by centrifugation. The levels of radioactivity in the pellets were counted. The data are the mean ± S.E. of the triplicate tubes.

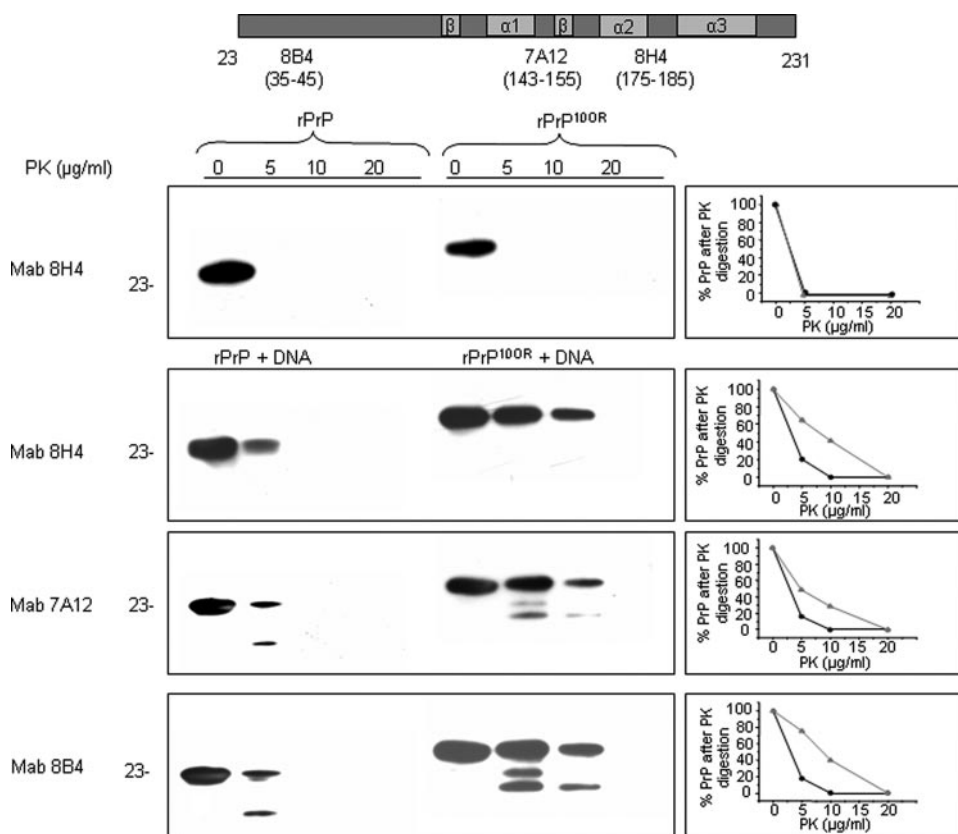


FIGURE 3. The rPrP-DNA complex is more resistant to PK digestion and binding of DNA protects the N terminus of rPrP from PK digestion. Wild-type rPrP or rPrP^{100R} (1 μM of each) was incubated with 18-nucleotide long DNA (5 μM) for 30 min at 37 °C; then PK was added at 0, 5, 10, and 20 μg/ml and further incubated at 37 °C for 30 min. rPrPs without DNA were used as controls. The digested mixtures were separated by SDS-PAGE and immunoblotted with mAb 8H4, 8B4, or 7A12. The remaining percents of wild-type rPrP (●) or rPrP^{100R} (▲) after PK digestion were estimated by densitometry measurement of the individual bands in the right panel. The epitopes recognized by mAbs 8B4, 7A12, and 8H4 are shown in the top panel.

arrows). The retardation is greatly reduced if the DNA is incubated with rPrP^{ΔKKRPK} or rPrP^{ΔOR} (not shown).

rPrP^Ms with Pathogenic Insertional Mutations Bind More DNA—rPrP^M with pathogenic mutations binds more GAG compared with wild-type rPrP because of a more exposed N terminus (22, 23). Hence, we next investigated whether rPrP^M also binds more DNA. We found that two insertional mutants, rPrP^{80R} and rPrP^{100R} (Fig. 2A), bind more DNA than wild-type

rPrP. In the presence of an 18-nucleotide long DNA, rPrPs with insertional mutations also aggregate more rapidly and form more aggregate than wild-type rPrP (Fig. 2B).

The presence of both rPrP and DNA in the aggregate was further demonstrated by radioactive labeling experiments. DNA (18-nucleotide long) was labeled with ³²P, mixed with rPrP, and the aggregates were pelleted by centrifugation. The levels of radioactivity in the pellets and in the supernatants were then determined (Fig. 2C). We found that the rPrP^{100R}/DNA pellet has the highest ³²P activity followed by rPrP^{80R}/DNA and then wild-type rPrP/DNA. The rPrP^{ΔKKRPK}/DNA pellet has the lowest activity and the bovine serum albumin/DNA pellet only has background activity. Accordingly, the levels of radioactivity in the supernatant fractions were reversed: the supernatant from the bovine serum albumin/DNA sample has the highest activity, whereas the supernatant from the rPrP^{100R}/DNA has the lowest (not shown).

Binding of DNA Protects the N Terminus of rPrP from Proteinase K (PK) Digestion—Resistance to PK digestion is a hallmark of infectious PrP^{Sc} (44). We next investigated whether rPrP-DNA complexes are PK resistant. rPrP alone or rPrP-DNA complexes were treated with different concentrations of PK. After digestion, samples were immunoblotted with anti-PrP mAb 8H4. Both wild-type rPrP and rPrP^{100R} immunoreactivities were eliminated after treatment with 5 μg/ml PK (Fig. 3). When bound to DNA, rPrP acquired some degree of PK resistance. After PK digestion (5 μg/ml), mAb 8H4 reacts with a single rPrP species of about 23 kDa, which is similar to untreated full-length rPrP; ~20% of the total rPrP is PK resistant as estimated by densitometry. In contrast, the rPrP^{100R}-DNA complex is more PK resistant. After treatment with 10 μg/ml PK, more than 40% of the total rPrP^{100R} is still detectable. Again, the PK-digested samples have one full-length rPrP^{100R} species. At 20 μg/ml PK all rPrP^{100R} immunoreactivity is eliminated. These results suggest that the rPrP-DNA complexes may be heterogeneous; some of the rPrP may be relatively more PK

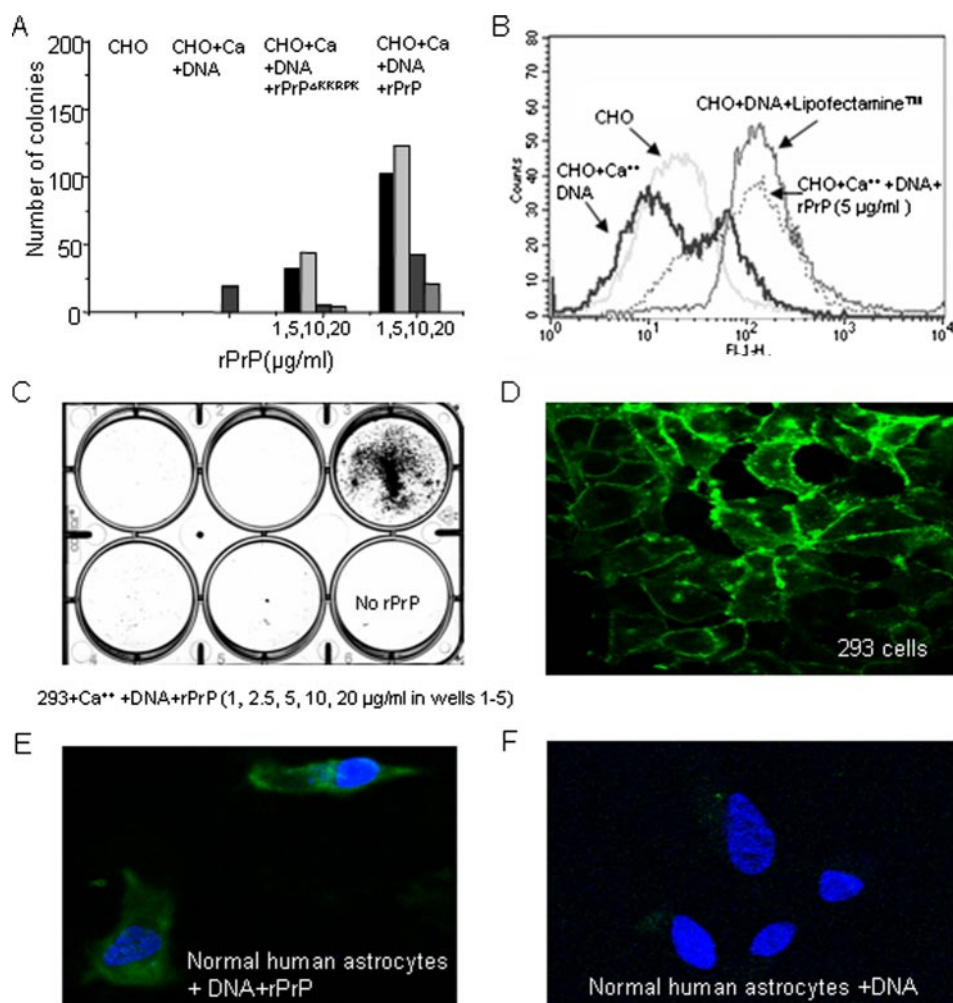


FIGURE 4. rPrP mediates gene delivery and expression in mammalian cells. *A*, CHO cells were transfected by incubating the indicated amounts of wild-type rPrP or rPrP^{ΔKKRPK} with plasmid pEYFP-GPI and CaCl₂ and further cultured in the presence of blasticidin. The surviving colonies were fixed and stained with trypan blue and counted with a microscope. *B*, CHO cells were transiently transfected as described and the expression of the pEYFP-GPI was analyzed by flow cytometry. *C*, 293 cells were transfected with the rPrP-DNA complexes. After selection with blasticidin, cellular colonies were fixed and stained with trypan blue. *D*, confocal microscopic image of 293 cells transfected with the rPrP-DNA complexes. *E*, normal human primary astrocytes were grown on 4-well chamber slides and transfected with the rPrP-DNA complexes. Cells were observed on a fluorescence microscope 24 h after transfection. *F*, non-transfected control human primary astrocytes. Cell nuclei were stained by 4',6-diamidino-2-phenylindole (blue). In our experiments, the yellow fluorescent protein appears green due to the filter used.

resistant. This interpretation is consistent with our finding that after PK digestion some full-length rPrP is still detectable. Alternatively, because the epitope of mAb 8H4 is at the C-terminal globular domain between residues 175 and 185, PK digestion might have eliminated this region of the rPrP, rendering the protein non-reactive to mAb 8H4. To test this possibility, we used additional anti-PrP mAbs, which react with epitopes either at the central region, such as mAb 7A12 (residues 143 to 155), or the N terminus, such as mAb 8B4 (residues 35 to 45), to investigate whether there are C-terminal truncated rPrP species in the PK-digested samples. In the rPrP/DNA sample, after treatment with PK, mAb 7A12 reacted with two different rPrP species: one is full-length, 23-kDa rPrP; the other is an 18-kDa species. Because this smaller species is not detected with mAb 8H4, it is likely to be a C-terminal-truncated rPrP species. Results from the rPrP^{100R}-DNA complex revealed that after digestion with 5 µg/ml PK, in addition to the full-length

rPrP^{100R} species, there are two additional smaller species, an intermediate one at about 20 kDa and a smaller one at 18 kDa. After treatment with 10 µg/ml PK, only the full-length and the 18-kDa rPrP^{100R} species were detected. All rPrP^{100R} immunoreactivity disappeared after digestion with 20 µg/ml PK. Results using mAb 8B4 were similar to those using mAb 7A12. Collectively, these results suggest that PK digestion starts from the C terminus resulting in the elimination of the mAb 8H4 reactive epitope and then proceeds to the N terminus. The N terminus of rPrP is more resistant to PK because it is protected by the bound DNA.

Internalization of rPrP/DNA Leading to Gene Expression—Because of its ability to bind and aggregate with DNA, we hypothesized that rPrP may function as a carrier for DNA in gene delivery. To test this hypothesis, we first mixed different amounts of wild-type rPrP or rPrP^{ΔKKRPK} with a plasmid, which encodes for an enhanced yellow fluorescent protein fused with the N-terminal signal sequence and C-terminal GPI anchor sequence from PrP, designated pEYFP-GPI, and a blasticidin selectable marker (45). The rPrP-DNA complexes were then added to CHO cells *in vitro* in the presence of CaCl₂ (40). Cells were then incubated for 4 h, rinsed gently, and then re-incubated with fresh medium with blasticidin.

After selection, the number of colonies in each well were counted. Cells incubated with DNA, rPrP, and CaCl₂ have the most colonies (Fig. 4A). Cells cultured with DNA, rPrP^{ΔKKRPK}, and CaCl₂ have fewer colonies, and cells cultured with DNA and CaCl₂ but without rPrP have even fewer colonies. The optimal concentration for rPrP is 5 µg/ml. The efficiency is reduced with either higher or lower rPrP concentrations. Mutant rPrP^{80R}, which binds more DNA, is also more efficient than wild-type rPrP in mediating gene delivery and expression (not shown).

We next compared the efficiency of wild-type rPrP-mediated gene delivery with commercial LipofectamineTM. CHO cells were transiently transfected with the pEYFP-GPI plasmid under the optimized conditions by either rPrP or Lipofectamine. After transfection, cells were allowed to rest for 24 h and the expression of EYFP-GPI on the cell surface was then evaluated by flow cytometry. As expected, high levels of fluorescent signal were uniformly detected on the surface of cells

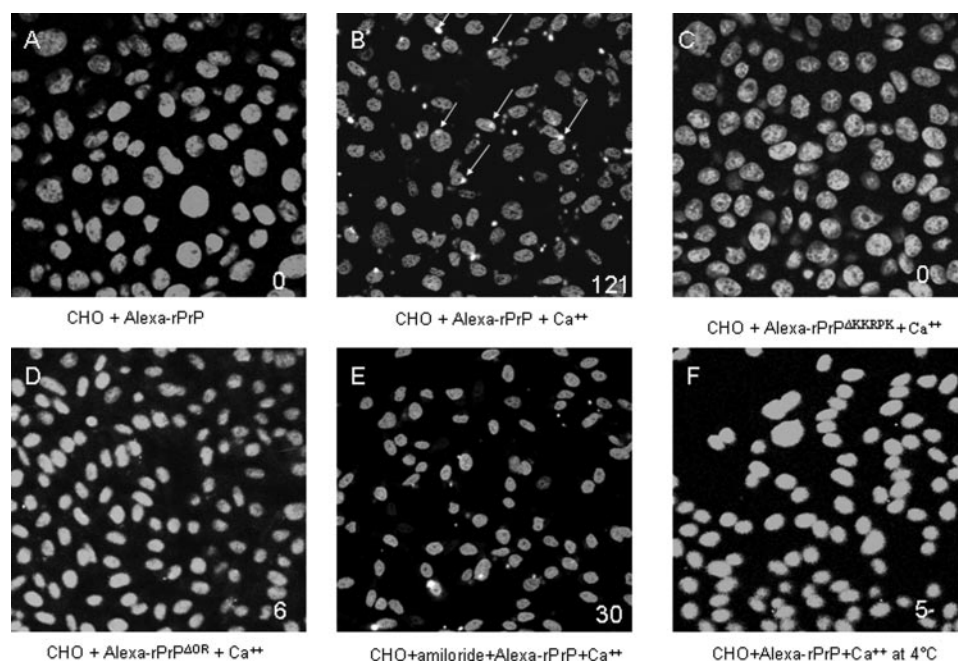


FIGURE 5. Uptake of wild-type rPrP into mammalian cells is calcium-dependent and requires the KKRPPK motif. Confocal microscopic images of CHO cells incubated with 5 $\mu\text{g}/\text{ml}$ of different Alexa-rPrPs. *A*, CHO cells incubated Alexa-rPrP. *B*, CHO cells incubated Alexa-rPrP in the presence of CaCl_2 . *C*, CHO cells incubated with Alexa-rPrP $^{\Delta\text{KKRPPK}}$ and CaCl_2 . *D*, CHO cells incubated with Alexa-rPrP $^{\Delta\text{OR}}$ and CaCl_2 . *E*, CHO cells pretreated with amiloride and then incubated with Alexa-rPrP and CaCl_2 . *F*, CHO cells incubated with Alexa-rPrP and CaCl_2 at 4 °C. Cell nuclei were stained by 4',6-diamidino-2-phenylindole (appear as white). The locations of some of the fluorescent-labeled rPrP are indicated by arrows.

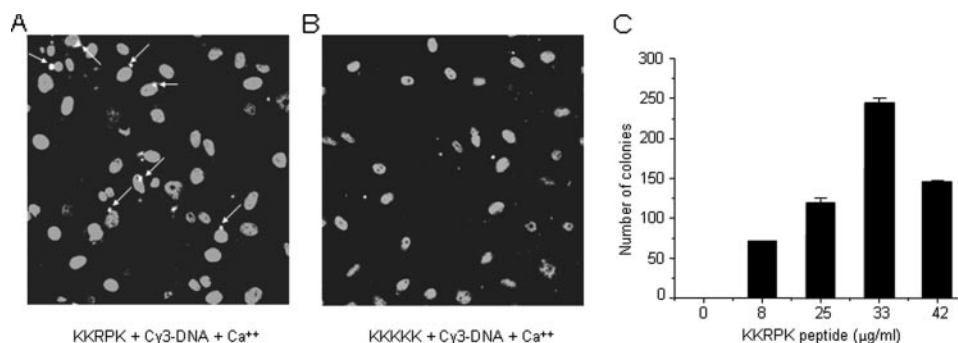


FIGURE 6. The KKRPPK peptide mediates gene delivery and expression in mammalian cells. Confocal microscopic images of CHO cells incubated with 33 $\mu\text{g}/\text{ml}$ KKRPPK peptide (*A*) or KKKKK peptide (*B*) in the presence of Cy3-labeled pEYFP-GPI (0.5 $\mu\text{g}/\text{ml}$) and CaCl_2 . The locations of some of the Cy3-labeled DNA are indicated by arrows. *C*, CHO cells were transfected by incubating the indicated amounts of KKRPPK peptide with plasmid pEYFP-GPI and CaCl_2 and further cultured in the presence of blasticidin. The surviving colonies were stained with trypan blue and counted.

transfected with Lipofectamine (Fig. 4*B*). Significant levels of fluorescent signals were also detected on the surface of a subpopulation of cells transfected with rPrP, CaCl_2 , and plasmid, but there are also cells, which have lower fluorescent signals. Therefore, it appears that cells are more uniformly transfected with Lipofectamine than rPrP in this transient transfection protocol. Much less signal is detected in cells incubated with plasmid and CaCl_2 without rPrP. Furthermore, the usage of rPrP as a gene carrier is applicable to another human tumor cell line, 293 cells (Fig. 4, *C* and *D*), and primary cultures of normal human astrocytes (Fig. 4, *E* and *F*). Collectively, these results provide unequivocal evidence that rPrP-DNA complexes are effectively taken up by mammalian cells resulting in gene expression in more than one cell type.

rPrP Can Enter Cells in the Presence of Ca^{2+} without DNA, and Uptake of rPrP Requires the KKRPPK Motif—To determine whether wild-type rPrP is the carrier of DNA, we investigated whether rPrP by itself enters cells without DNA. CHO cells were incubated with Alexa-rPrP, Alexa-rPrP $^{\Delta\text{KKRPPK}}$, or Alexa-rPrP $^{\Delta\text{OR}}$, either with or without Ca^{2+} . Without Ca^{2+} , rPrP is not detected in CHO cells (Fig. 5*A*). In the presence of Ca^{2+} , most CHO cells take up rPrP (Fig. 5*B*). Many of the internalized rPrP are present near the nucleus (Fig. 5*B*, see arrows). On the other hand, no signal is detected in cells incubated with Alexa-rPrP $^{\Delta\text{KKRPPK}}$ under similar conditions (Fig. 5*C*). Cells incubated with Alexa-rPrP $^{\Delta\text{OR}}$ have much less signal compared with cells incubated with rPrP, and the sizes of the fluorescent particles also appear to be smaller than the wild-type rPrP (Fig. 5*D*).

Next, we investigated whether uptake of rPrP is mediated by pinocytosis and is temperature dependent. In the presence of amiloride (2.5 mM), an inhibitor of macropinocytosis, the uptake of rPrP is reduced by >50%, as revealed by counting the total number of cells and the cells with fluorescent signals (Fig. 5*E*). rPrP uptake is also reduced by >80% when the cells are incubated at 4 °C (Fig. 5*F*).

Subsequently, we found that cells take up rPrP as quickly as 10 min, but not 5 min after incubation (not shown). Based on these observations, we suggest that rPrP is the carrier for DNA and the presence of

the KKRPPK motif is essential for the uptake of rPrP via a macropinocytosis- and temperature-dependent mechanism. Furthermore, because CHO cells do not express normal cellular prion, PrP^C, this process is cell surface PrP^C independent.

A Pentapeptide, KKRPPK, Is Sufficient for Gene Delivery—Because KKRPPK is one of the major DNA binding motifs, we investigated whether a pentapeptide, KKRPPK, is sufficient to facilitate uptake of DNA. Another pentapeptide, KKKKK, was used as a control. CHO cells were incubated with a mixture of Cy3-labeled pEYFP-GPI, the pentapeptide, and CaCl_2 . After 1 h, cells were processed and viewed under confocal microscopy. It is obvious that many cells incubated with DNA and KKRPPK have labeled DNA; most of them are in the perinuclear region (Fig. 6*A*). By contrast, cells incubated with DNA and

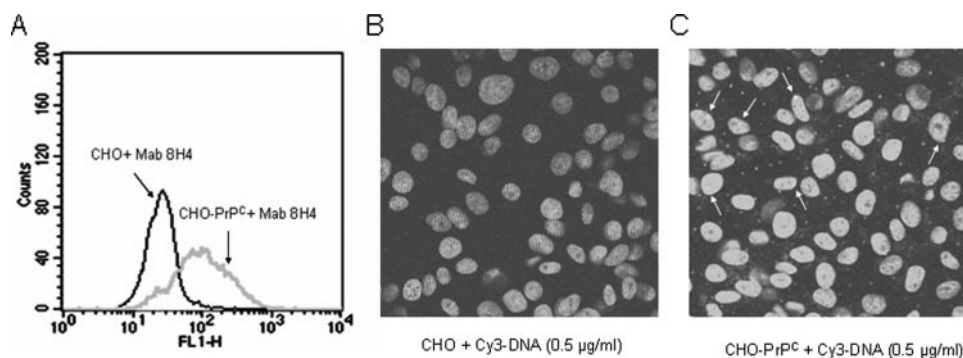


FIGURE 7. **PrP^C mediates exogenous DNA internalization.** A, flow cytometry analysis of cell surface PrP^C expression in wild-type CHO cells and CHO-PrP^C cells. Confocal microscopic images of CHO cells (B) or CHO-PrP^C cells (C) incubated with Cy3-labeled pEYFP-GPI plasmid (0.5 µg/ml). Cell nuclei were stained by 4',6-diamidino-2-phenylindole (appear as white). The locations of some of the fluorescent-labeled plasmid are indicated by arrows.

KKKKK have less Cy3-labeled DNA and most are either outside the cell or in the cytoplasm (Fig. 6B). We next repeated the same experiment for cell transfection. After drug selection, the number of colonies were counted. KKRPK mediates gene transfer and expression in a peptide concentration-dependent manner (Fig. 6C). No viable colonies were detected with cells incubated with KKKKK.

Cell Surface PrP^C Can Internalize Exogenous DNA, but the Internalized DNA Is Not Expressed—We next investigated whether cell surface PrP^C can serve as a carrier for exogenous DNA. CHO cells do not express endogenous PrP^C, but when transfected with a human *Prnp* expression plasmid they do express high levels of cell surface human PrP^C (Fig. 7A). Parental CHO cells or CHO-PrP^C cells were incubated with different amounts of Cy3-labeled pEYFP-GPI plasmid DNA. One h later, cells were processed and analyzed by confocal microscopy. No signal was detected in CHO cells cultured with DNA (Fig. 7B). On the other hand, significant levels of labeled DNA were detected in CHO-PrP^C cells cultured under identical conditions (Fig. 7C). These results suggest that cell surface PrP^C can also bind and internalize DNA. However, we were unable to detect the expression of the internalized plasmid either by flow cytometry or after drug selection (0 to 3 colonies in three experiments). Therefore, whereas cell surface PrP^C can bind and internalize exogenous DNA, the internalized DNA is either not expressed or expressed at very low level.

DISCUSSION

Both the KKRPK motif and the octapeptide repeat region at the N terminus of rPrP are essential for maximal DNA binding (Fig. 1A). Furthermore, rPrP^{23–145}, which lacks most of the C-globular domain, binds even more DNA than wild-type full-length rPrP (Fig. 1B). This increase is likely due to the charge difference between wild-type rPrP and rPrP^{23–145}. Charge property of rPrP is important in DNA binding. Wild-type rPrP has a theoretical pI of 9.39; rPrP^{23–145} has a theoretical pI of 10.84. Although our results suggest that the N terminus of PrP is involved in binding nucleic acids, other laboratories have reported that the C-terminal globular domain of PrP also binds nucleic acids (28, 35, 36). The reasons for these divergent results are not clear. In addition to experimental approaches,

there are other differences between these studies, such as the concentrations of PrP; the nature of the nucleic acids (single strand *versus* double strand; DNA *versus* RNA); and the length of the nucleic acids as well as binding conditions.

rPrP binds single strand and double strand DNA equally well (Fig. 1C). Although rPrP binds DNA as small as 7 nucleotides long, this DNA does not cause rPrP aggregation. Aggregation is detected with 18-nucleotide long DNA (Fig. 1D). We speculate that DNA with 7 nucleotides can only accommodate one or two rPrP molecules, but

aggregation of rPrP requires binding of multiple rPrP molecules to DNA. rPrP^Ms with pathogenic insertional mutations also bind more DNA (Fig. 2). rPrP acquires some degree of PK resistance after binding DNA (Fig. 3). Because a full-length rPrP species is always detected after PK digestion, therefore some rPrP-DNA complexes may be more PK resistant than others. Alternatively, because each DNA can bind multiple rPrP molecules, some bound rPrP molecules may be more PK resistant. Others have reported that under certain conditions, the PrP N terminus can become PK resistant (46).

Whether binding of DNA has any role in prion disease is not clear. *In vivo* derived PrP^{Sc} does contain DNA (47), but the PrP^{Sc} is much more PK resistant, and it is the N terminus of the PrP^{Sc} that is sensitive to PK digestion (44). Accumulated evidence suggests that cellular factors, such as GAG or nucleic acid may facilitate the conversion of PrP^C to PrP^{Sc} (32). In the *in vitro* protein misfolding cyclic amplification process, addition of nucleic acid enhances the amplification efficiency (48). However, these macromolecules can also interfere with the conversion processes. Treatment of animals with GAG (49) or CpG (50) prolongs the incubation period in PrP^{Sc}-infected animals. In an *in vitro* cell model, treating cells with phosphorothioate DNA inhibits PrP^{Sc} replication (51). The minimum length of DNA required is 18 nucleotides long (51), which is similar to the length of DNA required for rPrP aggregation (Fig. 1C).

We found that in the presence of CaCl₂, the rPrP-DNA complex is taken up by CHO cells, 293 cells, and normal human astrocytes, resulting in gene expression (Fig. 4). Under similar conditions, rPrP without DNA is also taken up (Fig. 5). CaCl₂ was included in our initial protocol based on an earlier report using a bacterial protein as a gene delivery vehicle (40). Although calcium phosphate-based mammalian cell gene delivery has been widely used for gene transfection (52), CaCl₂ is less frequently used. Because CaCl₂ also facilitates the internalization of rPrP without DNA, it is unlikely that CaCl₂ is mediating its effect by simply co-precipitating with the DNA. On the other hand, CaCl₂ may be required for the binding of rPrP to cell surface proteoglycan, which is essential for internalization. Alternatively, CaCl₂ can also act on the cell membrane, for example, by destabilizing the membrane. However, this effect is not nonspecific because rPrP^{ΔKKRPK} is not taken up

under similar conditions (Fig. 5C). A much more detailed study will be needed to clarify the roles of CaCl_2 in this process.

The composition of the rPrP-DNA complex is a determining factor in the efficiency of gene delivery. Too little or too much rPrP reduces transfection efficiency. The amounts of rPrP bound to DNA may influence the size, charge, and stability of the aggregate, which then influence the uptake, translocation, and unloading of the DNA for transcription. Similar results have also been reported for two other proteins that function as vehicles for gene delivery, namely histone (53) and HMGB2 (54).

In general, uptake of macromolecules is by either receptor-mediated endocytosis or nonspecific phagocytosis (55, 56). CHO cells will take up exogenous DNA only if the DNA is first incubated with CHO cell-conditioned medium (57). It was thought that the DNA has to be bound by proteins in the conditioned medium prior to being transported. This process is proteoglycan-dependent, and mediated by macropinocytosis (57). The pathway is PrP^C independent because CHO cells do not express PrP^C.

An N-terminal peptide, which contains the leading signal peptide sequence of bovine rPrP as well as the first five amino acids KKRPK, has cell penetrating capacity (58). This peptide can transport DNA into cells by macropinocytosis resulting in gene expression (58). It was concluded that the leading peptide sequence of PrP is critical for this process. However, whether the KKRPK motif is important was not evaluated. On the other hand, we found that a pentapeptide KKRPK without the leading peptide sequence is sufficient for DNA uptake and gene expression. In contrast, a pentapeptide KKKKK is unable to do so under identical conditions (Fig. 6). Because the KKRPK motif is important in DNA binding as well as internalization of rPrP, one would expect that binding to DNA could have prevented DNA internalization. It is possible that in the rPrP-DNA complexes some of the KKRPK motifs may still be available for subsequent binding and internalization.

Because the uptake of rPrP is inhibited by amiloride and low temperature (Fig. 5), it is likely that the uptake of the rPrP-DNA complex is also mediated by macropinocytosis. We speculate that KKRPK or rPrP is able to "coat" the plasmid, enable the plasmid to be taken up, protect it from degradation, transport the cargo into the nucleus and release the DNA resulting in gene expression. KKRPK or rPrP accomplishes this task by having a DNA binding motif, a GAG/proteoglycan binding motif, as well as a nuclear localization signal.

When DNA is internalized by cell surface PrP^C, the internalized DNA is not expressed (Fig. 7). It is possible that the complexes are funneled into different cellular compartments. In the case of rPrP/DNA, they are being taken up by macropinocytosis, as has been suggested earlier (57, 58) and reported here. On the other hand, the cell surface PrP^C-DNA complexes are being internalized by receptor-mediated endocytosis, which may cause the DNA to be nicked or degraded, rendering it unsuitable for transcription.

The protein translocation domain of HIV-1, Tat, also functions as a carrier for DNA internalization in a caveolae-dependent manner (59). PrP^C has been reported to reside in caveolae in

some cell types (60, 61). However, whether internalization of DNA by cell surface PrP^C is caveolae-dependent is not known.

The mammalian cell membrane imposes a strong barrier to the uptake of exogenous DNA (62). However, horizontal transfer of DNA between mammalian cells does occur *in vivo* (63). *In vivo* injection of DNA has been used to deliver antigen for immunization (64). Phagocytic cells take up DNA from apoptotic cells (65). It has been suggested that lateral transfer of DNA encoding oncogenes between eukaryotic cells may cause aneuploidy and accumulation of genetic changes that are necessary for tumor formation (65). Soluble PrP^C and PrP^C fragments are present in body fluids, such as blood and urine. Binding of soluble PrP^C to DNA may facilitate the lateral transfer of DNA *in vivo*. On the other hand, the ability of PrP^C to enter cells may also facilitate PrP^C to PrP^{Sc} conversion and spreading of PrP^{Sc} from cell to cell in prion diseases.

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Binding of Recombinant but Not Endogenous Prion Protein to DNA Causes DNA Internalization and Expression in Mammalian Cells

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