

Prion Infection of Muscle Cells In Vitro[∇]

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The prion agent has been detected in skeletal muscle of humans and animals with prion diseases. Here we report scrapie infection of murine C2C12 myoblasts and myotubes in vitro following coculture with a scrapie-infected murine neuroblastoma (N2A) cell line but not following incubation with a scrapie-infected nonneuronal cell line or a scrapie brain homogenate. Terminal differentiation of scrapie-infected C2C12 myoblasts into myotubes resulted in an increase in the expression of the disease-specific prion protein, PrP^{Sc}. The amount of scrapie infectivity or PrP^{Sc} in C2C12 myotubes was comparable to the levels found in scrapie-infected N2A cells, indicating that a high level of infection was established in muscle cells. Subclones of scrapie-infected C2C12 cells produced high levels of PrP^{Sc} in myotubes, and the C-terminal C2 polypeptide fragment of PrP^{Sc} was found based on deglycosylation and PrP^{Sc}-specific immunoprecipitation of cell lysates. This is the first report of a stable prion infection in muscle cells in vitro and of a long-term prion infection in a nondividing, differentiated peripheral cell type in culture. These in vitro studies also suggest that in vivo prion infection of skeletal muscle requires contact with prion-infected neurons or, possibly, nerve terminals.

Prion infection of neuronally derived cell lines has provided insightful information on the cell biology of the disease-specific isoform of the prion protein, PrP^{Sc}, in vitro. The standard method to establish prion infection involves incubating a culture of dividing cells with a crude brain homogenate from a prion-infected host or with preparations enriched for PrP^{Sc} (7, 12, 45). After several serial passages in vitro, in which the prion agent in the inoculum is removed by dilution, PrP^{Sc} is stably produced in these cell lines and prion infectivity can be detected by bioassay in mice (12, 45). Many important contributions toward understanding the biology of prion diseases have been made by studying prion-infected cell lines, including the cellular trafficking of PrP^{Sc} (10, 14, 50), mechanism of PrP^{Sc} formation and inhibition (14, 44), identification of compounds with antiprion activity (13), and pathways of prion-induced alterations in cellular function or cell death (28, 42, 51).

The majority of cell lines susceptible to prion infection are neuronal in origin or are derived from a prion-infected brain. These include a murine neuroblastoma (N2A) cell line (12, 45), a murine hypothalamic (GT1) cell line (49), a murine cholinergic septal neuronal (SN56) cell line (37), rat PC12 cells (48), and other, less well-defined cell lines isolated from a scrapie-infected mouse brain (26). More complex manipulations have also led to prion infection of cell lines derived from murine Schwann cells (3), rabbit epithelial cells (54), and deer brain cells (46) as well as primary neuronal and astrocyte cultures (21). One feature of prion infection in these cell lines is that infection is stably maintained in dividing cells. However, these cultures have not proven effective for investigating the role of prion infection in differentiated neurons, which is the main target cell in the nervous system. The murine cell line SN56 can be readily infected with the scrapie agent in vitro and

induced to differentiate into mature neurons, but one disadvantage is that there is a reduction in PrP^{Sc} levels upon induction of cellular maturation (7). The other major target for prion infection in mammalian hosts is the follicular dendritic cell (32, 38), but these long-lived cells have proven difficult to isolate and culture in vitro.

Recently, prion infection in skeletal muscle has been described for a number of natural and experimental prion diseases of animals and humans. PrP^{Sc} or prion infectivity is found in skeletal muscle homogenates from rodents experimentally infected with the transmissible mink encephalopathy (TME) or scrapie agent (8, 11, 39, 52), sheep with scrapie or bovine spongiform encephalopathy infections (1, 36), mule deer with chronic wasting disease (2, 29), and humans and macaques with Creutzfeldt-Jakob disease (27, 43). Detection of PrP^{Sc} by immunohistochemistry reveals localization to nerve fibers that transverse muscle or, in a few cases, deposition in skeletal muscle cells. In one study, PrP^{Sc} was localized to nerve fibers, muscle cells, and the neuromuscular junction in hamsters infected with the HY strain of the TME agent (39). It was postulated that the HY TME agent could spread from the motor nerve endings into the muscle cell via the synapse at the neuromuscular junction. Ultrastructural localization of the cellular isoform of the prion protein, PrP^C, determined that it is present on both the presynaptic nerve terminal and postsynaptic muscle cell membrane (25), suggesting that both locations are potential sites of PrP^{Sc} formation. Since the prion agent can undergo transsynaptic spread between neurons, the distribution of the prion protein in muscle is consistent with the neuromuscular junction acting as a peripheral synapse for cell-to-cell spread of the prion agent.

There is clinical and experimental evidence that PrP^C plays a role in muscle physiology and disease. In several human neuromuscular diseases it has been found that there is an upregulation and/or redistribution of PrP^C in skeletal muscle cells. These changes include increased PrP^C expression in muscle of individuals with inclusion body myositis and neurogenic muscle atrophy (5, 60) as well as increased PrP^C immunostain-

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ing in regenerating and atrophic muscle fibers in chronic neurogenic lesions, regenerating muscle fibers in muscular dystrophy, the rim of vacuoles in inclusion body myositis, and focal PrP^C deposits in myofibrillar myopathy inclusions (34). Furthermore, in transgenic mice, overexpression of wild-type PrP^C, or a mutant PrP^C transgene that is linked to human prion disease, can induce a necrotizing myopathy (18, 57). It is noteworthy that in sheep scrapie and chronic wasting disease of cervids there is a myopathy and loss of body mass, but the roles of PrP^C and PrP^{Sc} in these pathologies are unclear. Presently, there is no *in vitro* model to investigate the effect(s) of prion infection on skeletal muscle cells.

In this study, we report establishment of scrapie infection in a murine myoblast cell line that can undergo differentiation into nondividing, elongated myotubes, which can remain viable for several weeks *in vitro*. To establish scrapie infection in C2C12 myoblasts, coculture with scrapie-infected N2A cells was necessary, followed by growth in media selective for C2C12 cell survival. Subclones of scrapie-infected C2C12 myoblasts that express high levels of PrP^{Sc} upon differentiation into myotubes were identified, and they rapidly produce disease in a mouse scrapie bioassay. The scrapie-infected myotubes produced a C-terminal PrP^{Sc} polypeptide fragment characteristic of calpain-dependent cleavage. These are the first studies to demonstrate prion infection of a muscle cell line and long-term maintenance of prion infection *in vitro* in a terminally differentiated peripheral cell type that also is a target for prion infection *in vivo*.

MATERIALS AND METHODS

Cell culture. Murine C2C12 skeletal myoblast cells (American Type Culture Collection, Manassas, VA) were cultured in high-glucose Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 2 mM glutamine (Invitrogen, Carlsbad, CA), 100 IU/ml of penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (CellGro Mediatech, Inc., Herndon, VA). Differentiation to myotubes was achieved by allowing myoblast cultures to grow until confluent, which resulted in cell fusion to produce multinucleated, elongated myotubes. Murine neuroblastoma cells (N2A) cured of scrapie infection were a generous gift from Suzette Priola (Rocky Mountain Laboratories, Hamilton, MT) and were maintained in OptiMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml of penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. An antibiotic-resistant line of C2C12 (C2C12/Zeo^r) was generated by transfection of muscle cells with the pcDNA3.1/Zeo plasmid (Invitrogen, Carlsbad, CA) and selection in DMEM supplemented with 200 µg/ml Zeocin. Scrapie murine brain (SMB) cells (TSE Resource Center, Institute of Animal Health, Edinburgh, Scotland) were cultured in high-glucose DMEM supplemented with 10% newborn calf serum (Invitrogen, Carlsbad, CA), 5% FBS, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. All cell lines were grown at 37°C with 5% CO₂.

Scrapie infection of cultured cells. N2A and C2C12 cells were grown in culture medium supplemented with a 0.1% or 1% (wt/vol) brain homogenate from a scrapie strain 22L-infected mouse for 48 h. When cells reached 90% confluence, they were reseeded at a 1:8 dilution, and after the fourth serial passage they were screened for PrP^{Sc} by Western blotting. 22L scrapie-infected N2A cells and C2C12 myoblasts were cocultured by plating one in the bottom of a six-well plate while the other cell type was grown on PET membranes mounted above the well bottom (Thin Certs, Bellco Biotechnology, Vineland, NJ). The range in pore size of the PET membrane was 1 to 8 µm. Cells were allowed to grow for 1 week, after which they were detached by trypsinization from either the membrane or plate bottom and were screened for PrP^{Sc} by Western blotting at every other passage. To establish scrapie infection by direct coculture, 22L scrapie-infected N2A cells (280,000 cells) were plated with C2C12/Zeo^r cells (140,000 cells) in 100-mm dishes. Cocultures were grown in C2C12 complete medium that was changed every 2 to 3 days for 8 days, at which time myotubes were visibly present. The cocultures were then trypsinized and reseeded at a 1:2 dilution with C2C12

complete medium containing 200 µg/ml Zeocin. 22L scrapie-infected N2A cells did not survive under these conditions, and neuronal cells were not observed after two additional serial passages. The Zeocin-resistant C2C12 cells were screened for PrP^{Sc}, and the purity of the muscle cells was determined with antibodies specific for neuronal and muscle cells by Western blotting. Mock-infected C2C12 cells were established by coculturing C2C12 myoblasts with scrapie-cured N2A cells instead of 22L scrapie-infected N2A cells as described above. 22L scrapie-infected SMB cell and C2C12/Zeo^r cell cocultures were also performed as described above for 22L N2A and C2C12/Zeo^r cell cocultures.

Immunofluorescence staining for desmin. C2C12 myoblasts and myotubes were grown on Nunc Lab-Tek II chamber slides (Fisher Scientific, Hampton, NJ), fixed with cold ethanol, permeabilized with 0.1% Triton X-100, and incubated with rabbit antidesmin polyclonal antibody at a 1:20 dilution (DAKO Corp., Carpinteria, CA) followed by anti-rabbit Alexa Fluor 568 at a 1:200 dilution (Molecular Probes, Eugene, OR). Slides were mounted with ProLong Gold antifade reagent (Molecular Probes, Eugene, OR) and imaged with a Nikon E600 microscope with epi-illumination.

Preparation of lysates for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Cell culture lysates were collected in cell lysis buffer containing 1 mM Tris-HCl (pH 7.4), 140 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, and 0.5% Triton X-100 at 4°C (56). Lysates were subject to centrifugation at 11,000 × *g* for 10 min at 4°C and the supernatant retained for Western blot analysis. The protein concentration in cell lysates was determined using the Pierce Micro BCA assay kit (Pierce, Rockford, IL). For PrP^{Sc} analysis, proteinase K (PK) (Sigma Aldrich, St. Louis, MO) was added to cell lysates to a final concentration of 0.6 units/ml. Samples were incubated at 37°C for 1 h, and phenylmethylsulfonyl fluoride (Sigma Aldrich, St. Louis, MO) was added to a final concentration of 3 mM in order to inhibit PK activity. Following ultracentrifugation at 55,000 rpm for 2 h at 10°C in a TLA-55 rotor (Beckman Inc., Palo Alto, CA), pellets were resuspended in NuPAGE sample buffer and boiled for 10 min prior to polyacrylamide gel electrophoresis.

For removal of carbohydrate residues, cell lysates were precipitated in methanol and the pellet dissolved in 30 µl *N*-endoglycosidase F (PNGase F) denaturation buffer (New England Biolabs, Beverly, MA.). Samples were boiled for 10 min and subjected to centrifugation in a microcentrifuge for 10 min. To one-third of the supernatant, 75 units of PNGase F was added, and the digestion was performed at 37°C for 16 h. NuPAGE sample buffer was added to samples and analyzed by electrophoresis and Western blotting.

Samples were analyzed on a 12% WOST (morpholinepropanesulfonic acid) NuPAGE gel (Invitrogen, Carlsbad, CA), and proteins were transferred to a polyvinylidene difluoride membrane and incubated with one of the following antibodies: mouse anti-PrP monoclonal 6H4 antibody (Prionics, Zurich, Switzerland) at a dilution of 1:10,000, mouse anti-PrP monoclonal SAF-32 antibody (Cayman Chemical Company, Ann Arbor, MI) at 1 µg/ml, rabbit anti-desmin polyclonal antibody (DAKO Corp., Carpinteria, CA) at a 1:2,000 dilution, or mouse anti-tubulin III monoclonal antibody (TU-20; Abcam Inc., Cambridge, MA) at a 1:1,000 dilution. The detection system included incubation with either an anti-mouse immunoglobulin (IgG) alkaline phosphatase conjugate (Promega, Madison, WI) at a dilution of 1:20,000 (for PrP antibodies) or 1:5,000 (for TU-20 antibody) or an anti-rabbit IgG alkaline phosphatase conjugate (Cell Signaling, Danvers MA) at a 1:2,000 dilution. Blots were developed using CDP-Star substrate (Applied Biosystems, Foster City, CA) and imaged with a Kodak Image Station 2000MM (Eastman Kodak Company, Rochester, NY).

Subcloning of scrapie strain 22L-infected C2C12 cells. For subcloning of C2C12 muscle cells, the plating density of C2C12 myoblasts was determined at limiting dilution using standard cell counting and plating techniques. 22L scrapie-infected C2C12 myoblasts at passage 12 (the number of serial passages in Zeocin selection medium following establishment of a coculture of the 22L scrapie-infected N2A cells and uninfected C2C12 cells) were plated at a density of 18 cells per well in a 48-well plate. After cells reached confluence, they were trypsinized and transferred into one well of a six-well plate (a 1:10 split). Upon reaching confluence, the cells were passaged into three 60-mm culture dishes (a 1:6 split). Two of the culture dishes were screened for PK-resistant PrP^{Sc} by Western blotting, one as a C2C12 myoblast culture and the other after differentiation into day 5 myotubes. A third culture was passed into a 100-mm dish in order to maintain cultures until the initial screening for scrapie infection was complete.

Immunoprecipitation of PrP^{Sc}. Monoclonal anti-PrP 15B3 IgM antibody was a gift from Alex Rüber (Prionics, Zurich, Switzerland) and was used to selectively immunoprecipitate PrP^{Sc} as previously described (33, 41). Dynabeads M-450 (rat anti-mouse IgM) (Invitrogen, Carlsbad, CA) were coated with either anti-PrP 15B3 IgM antibody or buffer alone, and standard immunoprecipitation procedures were used as described by the manufacturer (Prionics Inc., Zurich, Swit-

erland). Briefly, 10 μ l of 15B3 IgM-coated Dynabeads was used to immunoprecipitate PrP^{Sc} from cell lysates (2.0-cm² tissue culture equivalents) from N2A cells or C2C12 myotubes. Immunoprecipitates of PrP^{Sc} were separated from cell lysates using a Dynal MPC-S magnetic particle concentrator (Invitrogen, Carlsbad, CA) and eluted from the Dynabeads by boiling in NuPAGE sample buffer (Invitrogen, Carlsbad, CA). NuPAGE gels and Western blots were performed with anti-PrP IgG SAF-32 or 6H4 monoclonal antibodies and visualized by CDP-Star chemiluminescence on a Kodak Image Station 2000MM as described above.

Preparation of C2C12 myoblasts and myotubes for scrapie infectivity assay. Cells from mock- and 22L scrapie-infected N2A cells, C2C12 myoblasts, and C2C12 myotubes were prepared for mouse bioassay. Cells for scrapie infectivity assay were serially passaged greater than 13 times following the scrapie infection step as described above. Based on dilution analysis, the scrapie infectivity from the original scrapie inoculum would have been removed by this passage number. Duplicate 100-mm dishes of each cell line were trypsinized, subject to centrifugation at 200 \times g for 5 min, and washed two times in cold phosphate-buffered saline, and each duplicate cell suspension was combined together in 400 μ l phosphate-buffered saline. Two hundred microliters was set aside for bioassay, and the remaining cells were screened for PK-resistant PrP^{Sc} by Western blotting as described above. Prior to inoculation, samples were submitted to three cycles of sonication at 4°C for 1 minute and several rounds of freezing and thawing in order to lyse the cells.

Animal inoculations and scrapie infectivity bioassay. Weanling C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were intracerebrally inoculated with 30 μ l of a cell lysate (56-cm² culture plate equivalents per three to six mice) from mock- or scrapie-infected N2A cells, C2C12 myoblasts, or C2C12 myotubes. Following inoculation, mice were observed three times per week for the onset of clinical symptoms of scrapie. The time to the onset of scrapie was reported, and the animals were euthanized during the early stages of clinical disease. All procedures involving animals were approved by the Montana State University Animal Care and Use Committee and were in compliance with the *Guide for the Care and Use of Laboratory Animals* (40).

RESULTS

Endogenous prion protein (PrP^C) expression in C2C12 myotubes and myoblasts. Murine C2C12 muscle cells were previously derived from dystrophic mouse hind limb muscle (59), and the morphologies of the myoblast and myotube cells following immunofluorescent staining for desmin are illustrated in Fig. 1. The C2C12 myoblasts are mononucleated cells with a morphology that is similar to that of fibroblasts, and, under subconfluent conditions, they divide every 14 to 16 h (Fig. 1A). Differentiation to myotubes was achieved by allowing the myoblasts to grow to confluence, at which time cell fusion resulted in multinucleated and elongated muscle cells with a morphology similar to that of myofibrils in vivo (Fig. 1B). Once they undergo differentiation, the C2C12 myotubes do not continue to divide, but they remain viable for approximately 21 days in vitro, after which time spontaneous contraction can occur, causing detachment from the culture surface (6, 20, 35).

In order to determine the relative abundance of the cellular prion protein (PrP^C) in the C2C12 cells, Western blotting was performed on cell lysates from myoblasts and myotubes at days 3, 7, 10, and 14 in vitro (Fig. 1C). Four major immunoreactive polypeptides that have molecular masses of 19, 23 to 25, 29, and 34 kDa were found. This PrP^C profile was distinct from that found in N2A cells, but the majority of the polypeptides immunoreactive with anti-PrP 6H4 antibody were between 18 and 35 kDa for both cell types. The PrP^C expression level in C2C12 muscle cells exhibited an increase upon differentiation of myoblasts into myotubes. Typically there was a two- to fourfold increase in PrP^C levels in D7 myotubes, but after this

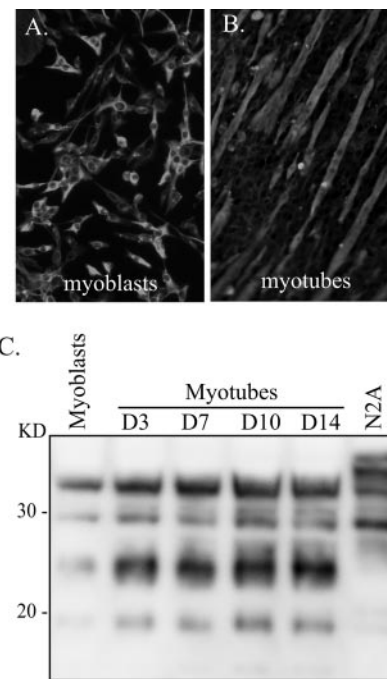


FIG. 1. Morphology and prion protein expression in C2C12 myoblasts and myotubes. (A and B) The morphologies of C2C12 myoblasts (A) and D5 myotubes (B) are illustrated by immunofluorescence staining for the cytoskeletal protein desmin. (C) Lysates from N2A cells (63 μ g protein), C2C12 myoblasts (126 μ g protein), and C2C12 myotubes (126 μ g protein) at days 3, 7, 10, and 14 in vitro were analyzed for total PrP in the absence of PK digestion by Western blotting using anti-PrP 6H4 monoclonal antibody as described in Materials and Methods. Molecular masses are indicated to the left.

time there was little additional increase of PrP^C in myotubes between days 7 and 21 in vitro (Fig. 1C and data not shown).

Scrapie infection of C2C12 myoblasts and myotubes. Three approaches were used to establish scrapie infection in C2C12 cells (Table 1). For the first method, murine C2C12 and N2A cells were cultured with brain homogenates (0.1% or 1% [wt/vol] in culture medium) from mice infected with the 22L strain of scrapie, as previously described for establishment of scrapie infection in vitro (12, 45). Despite four attempts, PrP^{Sc} was not detected by Western blotting in C2C12 myoblasts and myotubes following 8 to 10 serial passages after the initial plating with infectious scrapie tissue, but PrP^{Sc} was consistently found following infection of N2A cells (Table 1 and data not shown). In the second method, 22L scrapie-infected N2A cells were cocultured with C2C12 myoblasts, but the cell types were separated by a membrane with pores that ranged in diameter from 1 to 8 μ m. This attempt to enable the 22L scrapie agent to pass through the membrane and infect the C2C12 cells did not result in PrP^{Sc} production in the C2C12 myoblasts even though PrP^{Sc} was maintained in the 22L scrapie-infected N2A cells (Table 1 and data not shown). In the third method, C2C12 myoblasts were stably transfected with a plasmid expressing Zeocin resistance (C2C12/Zeo^r) prior to coculture with 22L scrapie-infected N2A cells at a ratio of 1:2 (Table 1). The cocultures were grown for 1 week in medium without Zeocin, after which the cocultures were switched to medium containing 200 μ g/ml Zeocin in order to eliminate the antibiotic-suscep-

TABLE 1. Approaches to establish 22L scrapie infection in C2C12 myoblasts and myotubes

Method to establish infection in C2C12 cells	Outcome in:	
	N2A cells	C2C12 cells
Add 22L scrapie brain homogenate ^a	Scrapie infection established	Scrapie infection not established
Coculture with 22L scrapie-infected N2A cells separated by a barrier ^b	Scrapie infection maintained	Scrapie infection not established
Coculture with 22L scrapie-infected N2A cells and Zeocin-transfected C2C12 cells	Death due to Zeocin sensitivity	Scrapie infection established in Zeocin-resistant myoblasts and myotubes

^a Homogenates at 0.1% to 1% (wt/vol).

^b One- or 8- μ m-pore-diameter membranes.

tible 22L scrapie-infected N2A cells. The C2C12/*Zeo*^r myoblasts and myotubes were serially passaged and screened at every other passage for PrP^{Sc} by Western blotting. Figure 2A illustrates the presence of PK-resistant PrP^{Sc} in C2C12/*Zeo*^r day 7 myotubes at passage 10 after addition of Zeocin selection medium. There was no difference in the total PrP polypeptide pattern (i.e., in the absence of experimental PK digestion) from cell lysates of C2C12/*Zeo*^r day 7 myotubes cocultured with either mock-infected N2A cells or 22L scrapie-infected N2A cells (Fig. 2A, lanes 1 to 3). However, following PK digestion of the cell lysates, the myotubes cocultured with mock-infected N2A cells showed no PrP^{Sc} signal, while the myotubes cocultured with 22L scrapie-infected N2A cells had PK-resistant PrP^{Sc} polypeptides of between 19 and 30 kDa (Fig. 2A, lanes 4 to 6). The first appearance of PK-resistant PrP^{Sc} polypeptides was observed in cell lysates of both myoblasts and myotubes after four serial passages in Zeocin selection medium (data not shown), indicating that PrP^{Sc} production was due to de novo synthesis and not to contamination with the scrapie inoculum. These findings were confirmed in duplicate coculture experiments. The outcome of C2C12 cell infection with either 22L scrapie brain homogenates or 22L scrapie-infected N2A coculture was not due to exposure of C2C12 cells to different doses of scrapie infectivity, since both scrapie sources induced relatively short incubation periods following intracerebral inoculation of mice (Table 2).

To investigate whether transfer of scrapie infection to C2C12 cells from other scrapie-infected cell lines could be achieved, cocultures between C2C12/*Zeo*^r cells and a 22L SMB cell line was performed as described above. The SMB cell line is a nonneuronal cell line, and following coculture and growth in Zeocin selection medium, C2C12/*Zeo*^r myoblasts and myotubes did not show evidence of PrP^{Sc} production by the eighth serial passage (Fig. 2B, lanes 1 and 2). In the control cocultures using 22L N2A cells, PrP^{Sc} was detected in C2C12/*Zeo*^r cells by the sixth serial passage (Fig. 2B, lanes 3 and 4). These findings indicate that a scrapie-infected neuronal cell line was able to transfer infection to C2C12 cells, while a scrapie-infected nonneuronal cell line did not establish infection in C2C12 cells upon coculture.

To determine whether cocultures of 22L scrapie-infected N2A cells and C2C12/*Zeo*^r cells had any remaining N2A cells after the fourth serial passage in Zeocin-containing growth medium, we tested for the presence of a muscle marker (desmin) and neuronal marker (tubulin III) in cell lysates by Western blotting. The Zeocin-resistant 22L scrapie-infected day 5 myotubes were immunopositive for desmin at 54 kDa

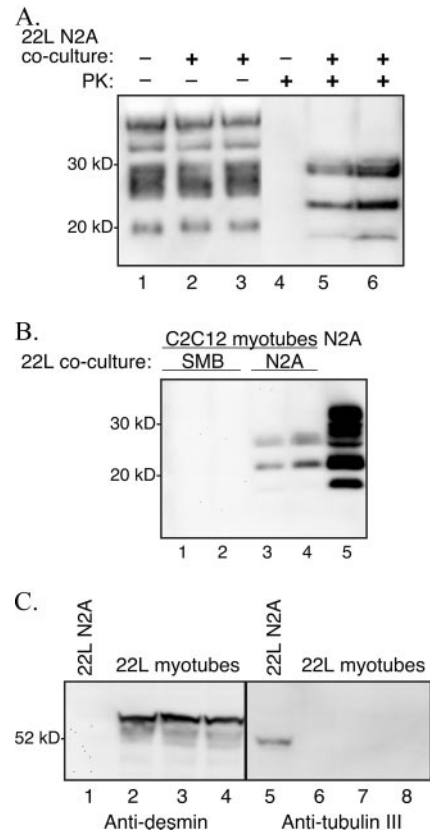


FIG. 2. Coculture of C2C12 myoblasts and 22L scrapie-infected N2A cells. C2C12 myoblasts containing a plasmid expressing Zeocin resistance and 22L scrapie-infected N2A cells were cocultured at a ratio of 1:2 for 1 week prior to selection in Zeocin-containing culture medium. The surviving C2C12/*Zeo*^r myotubes were assayed for PrP^{Sc} by Western blotting after serial passage in vitro. (A) Cell lysates from C2C12 D5 myotubes at passage 10 in Zeocin-containing selection medium following coculture with either uninfected N2A cells (lanes 1 and 4) or 22L scrapie-infected N2A cells (lanes 2, 3, 5, and 6). The cellular lysates were either undigested (-) or digested (+) with 0.6 U/ml of PK prior to analysis. (B) Western blot of PK-digested lysates from C2C12 D5 myotubes at passage 6 in Zeocin-containing selection medium following coculture with either 22L scrapie-infected SMB cells (lanes 1 and 2) or 22L scrapie-infected N2A cells (lanes 3 and 4). Control 22L N2A cells (lane 5) were in the absence of C2C12 cell coculture and Zeocin selection. (C) Western blotting for desmin and tubulin III was performed on cell lysates from C2C12 D6 myotubes following coculture of 22L scrapie-infected N2A cells and C2C12 myoblasts at passage 4 in Zeocin selection medium (lanes 2 to 4 and 6 to 8). Eighty-six micrograms of 22L scrapie-infected N2A cells (lanes 1 and 5) and 22L scrapie-infected C2C12 D6 myotubes was analyzed using anti-PrP 6H4 monoclonal antibody. Molecular mass markers are shown on the left.

TABLE 2. Scrapie incubation period in murine neuroblastoma (N2A) and C2C12 myoblast and myotube cell lysates

Sample ^a	Trial 1 ^b			Trial 2		
	Incubation period, days (mean \pm SD) ^c	No. affected/no. inoculated	No. of times cultures were passed after scrapie infection	Incubation period, days (mean \pm SD)	No. affected/no. inoculated	No. of times cultures were passed after scrapie infection
22L scrapie-infected brain	137 \pm 7 ^d	5/5	NA ^e	ND ^f	NA	NA
22L scrapie-infected N2A cells	125 \pm 7.6*	5/5	19	138 \pm 2.2	5/5	15
22L scrapie-infected C2C12 myoblasts	138 \pm 6.2*	6/6	17	145 \pm 4.3	6/6	14
22L scrapie-infected C2C12 myotubes	132 \pm 2.2	5/5	17	137 \pm 1.7	4/4	14
22L A3 scrapie-infected C2C12 myoblasts	ND	NA	NA	139 \pm 6.4	4/4	18
22L A3 scrapie-infected C2C12 myotubes	ND	NA	NA	133 \pm 0.6	3/3	18
C2C12 myoblasts	>250	0/5	17	>205	0/3	14
C2C12 myotubes	ND	NA	NA	>205	0/3	14

^a For the brain sample, a 1% (wt/vol) homogenate was used. For all other samples, a 56-cm² tissue culture at ~90% to 100% confluence was trypsinized and resuspended in phosphate-buffered saline, and the total cell lysate was inoculated into each group of mice.

^b Trials represent C2C12 muscle cells and N2A cells from independent scrapie infection experiments.

^c Brain homogenates or tissue lysates were intracerebrally inoculated into C57Bl/6 mice and the time to onset of clinical disease was measured. *, Tukey's Studentized range test, $P < 0.05$.

^d The presence of PrP^{Sc} in the brains of all clinically ill mice was confirmed by Western blotting.

^e NA, not applicable.

^f ND, not done.

(Fig. 2B, lanes 2 to 4) but not for tubulin III (Fig. 2B, lanes 6 to 8), indicating an absence of the neuronal marker in these PrP^{Sc}-positive cells. Control cultures of 22L scrapie-infected N2A cells that were not grown in Zeocin-containing medium did not immunoreact for desmin (Fig. 2B, lane 1) but did contain tubulin III at 52 kDa (Fig. 2B, lane 5). Similar results were obtained in a duplicate coculture experiment. No viable cells were found in control cultures of 22L scrapie-infected N2A cells after growth in culture medium containing Zeocin at 200 μ g/ml (data not shown).

Subcloning of 22L scrapie-infected C2C12 myoblasts. To identify a population of 22L scrapie-infected myoblasts that expressed high levels of PrP^{Sc}, 22L scrapie-infected myoblasts were plated at limiting dilution in a 48-well plate, and when ~70% confluent they were serially passaged three times into larger culture plates. Five PrP^{Sc}-expressing subclones were identified by screening of cell lysates from myoblasts and day 5 myotubes by Western blotting (Fig. 3A). While two prominent PrP^{Sc} polypeptides were found in subclones of 22L scrapie-infected myoblasts at 22 kDa and 26 to 28 kDa, in the myotubes a third, lower-molecular-mass PrP^{Sc} polypeptide at 18 kDa was also apparent (Fig. 3A, lanes 6 to 9). Subclone 22L A3 had the highest level of PK-resistant PrP^{Sc} polypeptides in day 5 myotubes (Fig. 3A, lane 6), even though 22L A3 scrapie-infected myoblasts had PrP^{Sc} levels comparable to those of the other myoblast subclones (Fig. 3A, lanes 1 to 4). Additional immunoreactive PrP^{Sc} polypeptides were found in the 22L A3 myotube subclone at higher molecular masses (Fig. 3A, lane 6), which are likely due to the larger quantity of PrP^{Sc} analyzed in this sample. A second round of subcloning starting with the 22L A3 myoblasts resulted in identification of seven additional PrP^{Sc}-positive clones out of 12 limiting-dilution groups. These subclones expressed comparable or lower levels of PrP^{Sc} than the original 22L A3 C2C12 subclone (data not shown).

The amount of PrP^{Sc} increases in scrapie-infected C2C12 cells upon differentiation of myoblasts into myotubes. Based

on the higher expression level of PrP^C in C2C12 myotubes versus myoblasts (Fig. 1C), the amount of PrP^{Sc} in 22L A3 scrapie-infected C2C12 cells was measured. Lysates of scrapie-infected 22L A3 myotubes at days 7, 14, and 18 in vitro were analyzed for PrP^{Sc} by Western blotting (Fig. 3B). Approximately 700 μ g of protein from each cell lysate was digested with 0.6 units/ml of PK for 1 hour at 37°C. There was a progressive increase in PrP^{Sc} levels of approximately two- to four-fold in myotubes between days 7 and 18 (Fig. 3B). Despite the increase in PrP^{Sc} accumulation in 22L A3 scrapie-infected C2C12 cells upon differentiation, no overt cytopathology was observed in these cultures (data not shown).

22L scrapie-infected C2C12 myotubes are highly resistant to PK digestion. To compare the relative resistance of PrP^{Sc} to PK degradation from the neuronal and muscle cell types, lysates (containing 580 μ g of protein) from 22L scrapie-infected N2A cells and 22L A3 scrapie-infected C2C12 day 5 myotubes were digested with either 0.6, 2, 4, or 8 units/ml of PK at 37°C for 1 hour and analyzed for PrP^{Sc} by Western blotting (Fig. 3C). For comparison, 300 μ g of protein from a 22L scrapie-infected mouse brain was also digested with PK under similar conditions (Fig. 3C, left panel). In the 22L scrapie-infected N2A cells and the 22L scrapie-infected A3 myotubes, the PrP^{Sc} signal did not diminish considerably as the concentration of PK was increased from 0.6 to 8 U/ml (Fig. 3C, middle and right panels), but there was a loss of PrP^{Sc} signal in the 22L scrapie-infected murine brain following digestion with 8 U/ml of PK. These findings indicate that PrP^{Sc} derived from these cell lines is highly resistant to PK digestion and suggests that PrP^{Sc} in 22L scrapie-infected myotubes and PrP^{Sc} in scrapie-infected N2A cells have similar biochemical properties.

22L scrapie-infected C2C12 myoblasts and myotubes are infectious in mouse bioassay. In order to measure the amount of scrapie infectivity in the 22L scrapie-infected C2C12 myoblasts and myotubes, cell lysates were intracerebrally inoculated into C57Bl/6 mice and the incubation period was deter-

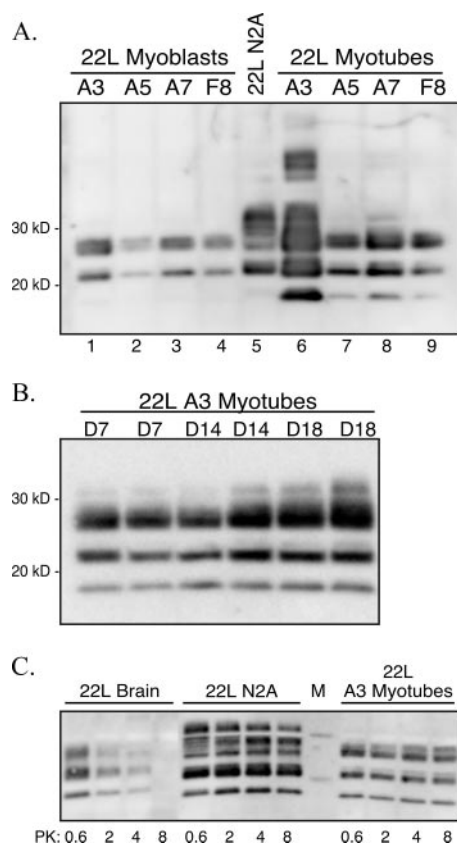


FIG. 3. Subcloning of 22L scrapie-infected C2C12 cells. (A) 22L scrapie-infected C2C12 myoblasts were subcloned by limiting dilution, and cell lysates ($\sim 48\text{-cm}^2$ tissue culture plate equivalents at $>90\%$ confluence) from myoblasts (lanes 1 to 4) and D5 myotubes (lanes 6 to 9) were digested with proteinase K prior to screening for PrP^{Sc} by Western blotting with monoclonal anti-PrP 6H4 antibody. PK-digested lysates from 22L scrapie-infected N2A cells are in lane 5 ($\sim 24\text{-cm}^2$ tissue culture plate equivalents at $>90\%$ confluence). (B) Western blot for PrP^{Sc} in PK-digested lysates ($\sim 7.5\text{-cm}^2$ tissue culture plate equivalents at $>90\%$ confluence) from 22L C2C12 myotubes at days 7, 14, and 18 in vitro. (C) PrP^{Sc} Western blot of 22L N2A cells ($\sim 12\text{-cm}^2$ tissue culture plate equivalents at $>90\%$ confluence), 22L C2C12 day 5 myotubes ($\sim 12\text{-cm}^2$ tissue culture plate equivalents at $>90\%$ confluence), and 22L scrapie-infected mouse brain (300 μg protein prior to PK digestion) following digestion with 0.6, 2, 4, or 8 U/ml of PK.

mined using a mouse bioassay. In trial 1, lysates from both uncloned 22L scrapie-infected C2C12 myoblasts and myotubes induced clinical disease in recipient mice between 130 and 140 days postinoculation (Table 2). This incubation period was similar to that caused by inoculation of 30 μl of a 1% (wt/vol) brain homogenate from a 22L scrapie-infected mouse at clinical disease. The 22L scrapie-infected N2A cell lysate produced a statistically different incubation period compared to the 22L scrapie-infected C2C12 myoblasts (125 ± 7.6 days versus 138 ± 6.2 days) but not compared to 22L scrapie-infected C2C12 myotubes (125 ± 7.6 days versus 132 ± 2.2 days) in the mouse bioassay (Table 2). By the 12th serial passage of the C2C12 myoblasts after establishment of cocultures, the original 22L scrapie-infected N2A cell inoculum was diluted by a factor of greater than 10^9 . Since C2C12 cell lysates from serial passage 14 or later were tested in the mouse bioassay, the ability of 22L

scrapie-infected C2C12 myoblast and myotube lysates to cause prion disease in mice was not due to residual scrapie inoculum (Table 2). C2C12 myoblasts that were not infected with the 22L scrapie agent did not cause disease in recipient mice after 250 days postinoculation. In trial 2, which represents a mouse bioassay using C2C12 cell lysates from an independent scrapie coculture experiment, there was a 1-week difference in incubation period between the uncloned 22L scrapie-infected C2C12 myoblasts and myotubes (Table 2). The 22L A3 C2C12 subclone derived from the uncloned 22L scrapie-infected myoblasts had a slightly shorter incubation period than the uncloned cell lysates; however, there was no statistical difference in incubation period between these two sources of inoculum or between the 22L A3 C2C12 myoblasts and myotubes. These findings indicate that 22L scrapie-infected C2C12 cells can maintain a high level of scrapie infection.

N-terminal truncation of PrP^C and PrP^{Sc} in C2C12 myotubes. An immunoblot for total PrP in mock-infected C2C12 myotubes, uncloned 22L scrapie-infected C2C12 myotubes, and the A3 subclone of the 22L scrapie-infected C2C12 myotubes revealed a distinct polypeptide pattern in the A3 subclone (Fig. 4A). The PrP^{Sc} polypeptide patterns of the mock-infected and uncloned 22L scrapie-infected myotubes were similar to that shown in Fig. 2A and were characterized by four major immunoreactive polypeptides at approximately 19, 23 to 25, 29, and 34 kDa (Fig. 2A and 4A). For the 22L A3 scrapie-infected C2C12 myotubes, the major PrP polypeptides were found at approximately 18, 22, and 26 to 28 kDa. Hence, there appeared to be truncation of the PrP polypeptides in the 22L A3 scrapie-infected myotubes compared to the mock and uncloned 22L scrapie-infected myotubes. The total PrP profile of the 22L scrapie-infected N2A cells also had three major polypeptides that were slightly larger than those in the 22L A3 scrapie-infected myotubes (Fig. 4A). Since it has previously been demonstrated that PrP^{Sc} can be truncated in lysosomes or by calpain-dependent endoproteolytic cleavage in scrapie-infected cell lines in vitro or in vivo (15, 16, 58) and since disintegrins can truncate PrP^C (19, 55), additional studies were designed to investigate cellular processing of PrP^C and PrP^{Sc} in the mock-infected and 22L A3 scrapie-infected C2C12 myotubes.

To further assess the molecular masses of PrP^C and PrP^{Sc} in the C2C12 myotubes, and specifically the cleavage into C1 or C2 polypeptides (16), the N-linked carbohydrates were removed following enzymatic digestion with PNGase F. In mock-infected C2C12 myotubes two PrP^C polypeptides were observed, at approximately 16 and 27 kDa, following PNGase F digestion and Western blotting with anti-PrP 6H4 monoclonal antibody (Fig. 4B, lane 3). While the larger PrP^C polypeptide is consistent with the full-length unglycosylated PrP^C molecule, the 16-kDa polypeptide is characteristic of the C1 PrP^C fragment (55). Following PK digestion, the PrP^C polypeptides were degraded and immunoreactivity to anti-PrP 6H4 antibody was lost (Fig. 4, lane 4). Analysis of the A3 subclone of the 22L scrapie-infected C2C12 myotubes revealed the 16- and 27-kDa polypeptides that were present in the mock-infected cells and an additional polypeptide band at 18 kDa (Fig. 4, lane 1). While the PrP^C polypeptides were no longer immunoreactive after PK digestion in the 22L A3 scrapie-infected myotubes, the 18-kDa polypeptide band was not further reduced in mo-

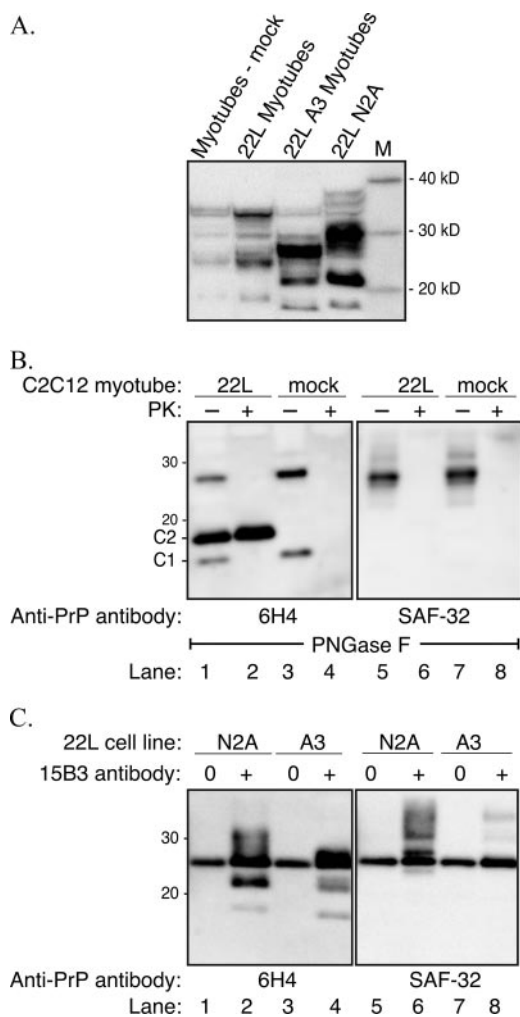


FIG. 4. Deglycosylation and immunoprecipitation of the prion protein reveal N-terminal truncation in C2C12 myotubes. (A) Total PrP polypeptides in lysates (200 μ g protein) of mock-infected myotubes, uncloned 22L scrapie-infected C2C12 myotubes, 22L A3 scrapie-infected C2C12 myotubes, and 22L scrapie-infected N2A cells were immunoblotted with anti-PrP 6H4 monoclonal antibody. (B) PNGase F deglycosylation of mock-infected myotubes (lanes 3, 4, 7 and 8) and 22L A3 scrapie-infected C2C12 myotubes (lanes 1, 2, 5 and 6) in the absence (-) or presence (+) of PK digestion. Cell lysates were immunoblotted with anti-PrP 6H4 (lanes 1 to 4) or anti-PrP SAF-32 (lanes 5 to 8) monoclonal antibodies. (C) 22L scrapie-infected cell lysates immunoprecipitated with anti-PrP 15B3 IgM monoclonal antibody (+) or uncoated Dynabeads (0) were immunoblotted with anti-PrP 6H4 (lanes 1 to 4) or anti-PrP SAF-32 (lanes 5 to 8) monoclonal antibodies. Immunoprecipitation of PrP^{Sc} was performed on 22L scrapie-infected N2A cells (lanes 1, 2, 5, and 6) and 22L A3 scrapie-infected C2C12 myotubes (lanes 3, 4, 7, and 8). A 25-kDa nonspecific polypeptide was immunoprecipitated by Dynabeads that were not coated with anti-PrP 15B3 IgM monoclonal antibody and from uninfected cell lysates (data not shown). Lane M, molecular mass markers.

lecular mass after PK digestion (Fig. 4, lane 2) indicating that it was truncated in the muscle cells. This 18-kDa PrP^{Sc} polypeptide is consistent with the previously described C2 PrP^{Sc} fragment, but this C-terminal polypeptide was reported to have a molecular mass of 21 kDa (16).

Western blotting of a set of deglycosylated cell lysates sim-

ilar to that illustrated in Fig. 4B, lanes 1 to 4, was performed using anti-PrP SAF-32 monoclonal antibody, which recognizes the octapeptide repeat region of murine prion protein located between amino acids 52 and 92 of the N-terminal region of the prion protein. This octapeptide region is partially degraded following limited PK digestion of PrP^{Sc} and results in a reduction or loss of SAF-32 antibody immunoreactivity. For both the PNGase F-treated mock-infected and 22L A3 scrapie-infected myotubes, only the 27-kDa PrP^C polypeptide was immunoreactive in the non-PK-digested samples, indicating that the 16-kDa C1 PrP^C polypeptide is truncated at the N-terminal end (Fig. 4B, lanes 5 and 7). Following PK digestion, the 27-kDa PrP^C polypeptide is degraded (Fig. 4B, lanes 6 and 8). Interestingly, the 18-kDa C2 PrP^{Sc} polypeptide in the 22L scrapie-infected myotubes also does not immunoreact with anti-PrP SAF-32 antibody (Fig. 4B, lanes 5 and 6), indicating that it too is truncated at its N terminus. Therefore, in C2C12 myotubes, in addition to full-length PrP^C, both PrP^C and PrP^{Sc} undergo N-terminal truncation.

A second approach was used to examine the molecular mass of PrP^{Sc} polypeptides in 22L A3 scrapie-infected C2C12 myotubes. PrP^{Sc} was selectively immunoprecipitated from 22L scrapie-infected myotubes and 22L scrapie-infected N2A cell lysates with anti-PrP 15B3 antibody, which does not recognize PrP^C (33, 41). These PrP^{Sc} polypeptides were further analyzed by immunoblotting with either anti-PrP 6H4 antibody (Fig. 4C, lanes 1 to 4) or anti-PrP SAF-32 antibody (Fig. 4C, lanes 5 and 8). In the anti-PrP 6H4 immunoblot, PrP^{Sc} polypeptides were found at approximately 18, 22, and 26 kDa (Fig. 4C, lane 4), excluding the nonspecific immunoprecipitation of a 25-kDa polypeptide band (Fig. 4C, lane 3). An immunoblot of the 15B3-immunoprecipitated polypeptides from 22L A3 scrapie-infected C2C12 myotubes with anti-PrP SAF-32 antibody revealed no immunoreactivity with these polypeptide bands and weak detection of two PrP^{Sc} polypeptides at 31 and 35 kDa (Fig. 4C, lane 8). This reduction in immunoreactivity with SAF-32 antibody indicates that the majority of PrP^{Sc} in the 22L scrapie-infected myotubes was truncated at the N-terminal end. A comparison with 22L scrapie-infected N2A cells reveals a similar pattern of immunoreactivity with anti-PrP 6H4 and SAF-32 monoclonal antibodies following immunoprecipitation with anti-PrP^{Sc} 15B3 monoclonal antibody (Fig. 4, lanes 2 and 6).

DISCUSSION

In an attempt to develop a cell culture model for scrapie infection in muscle, we investigated the murine C2C12 myoblast cell line, since it can undergo myogenic differentiation into nondividing myotubes, which are similar to myofibrils in vivo (59). In this study we demonstrate that scrapie infection and PrP^{Sc} production can be established in murine C2C12 myoblasts and myotubes in vitro. The properties of C2C12 cells infected with 22L scrapie were similar to those found in scrapie-infected N2A cells, including a high level of PrP^{Sc} resistance to PK degradation, the production of C1 and C2 PrP polypeptide fragments, and the ability to induce scrapie in mice upon intracerebral inoculation. Based on the similarities of scrapie infection in these cell lines, we propose that 22L scrapie-infected C2C12 myoblasts and myotubes will provide a

useful tissue culture model for studying prion infection of skeletal muscle *in vivo*. The recent report of prion infection in muscle of deer with chronic wasting disease indicates a food safety risk to humans who consume venison (2).

In order to establish scrapie infection in C2C12 cells, it was necessary to coculture myoblasts with 22L scrapie-infected N2A cells and then selectively grow the myoblasts in Zeocin-containing growth medium, which does not support N2A cell survival. Attempts to establish scrapie infection in C2C12 myoblasts and myotubes by direct incubation with a scrapie brain homogenate were unsuccessful in four independent experiments even though homogenates contained high scrapie titers that were able to establish scrapie infection of N2A cells. These findings suggested that the 22L scrapie agent, or induction of PrP^{Sc} formation, was directly transferred between the infected N2A cells and uninfected myoblasts or myotubes. It is likely that scrapie infection requires direct contact between these cell types, since separation of these cell types by a porous membrane did not result in scrapie infection of the C2C12 myoblasts. The type of cell that can transfer scrapie infection to C2C12 myoblasts and myotubes also appears to be an important factor, since coculture with 22L scrapie-infected SMB cells did not result in infection of C2C12/Zeo^r cells. However, previous coculture studies demonstrated that SMB cells infected with the 79A strain of the scrapie agent can transfer infection to uninfected SMB cells (31); cell-to-cell contact is not needed to infect SMB cells, since infection can be established using the standard method of incubating the dividing cells with a scrapie brain homogenate (31). These findings suggest that the ability of a scrapie-infected neuronal cell line, but not a nonneuronal cell line, to infect C2C12 myoblasts or myotubes may be due to the formation of a neuromuscular junction *in vitro*. Both N2a cells and C2C12 myotubes have been demonstrated to form neuromuscular junctions in nerve-muscle cell line cocultures (17, 61). Transynaptic spread of the prion agent has been demonstrated along motor pathways (9). Localization of PrP^{Sc} to the neuromuscular junction in prion-infected muscle cells *in vivo* (39) and of PrP^C to both the presynaptic and postsynaptic membranes in the neuromuscular junction provides support for a role of the neuromuscular junction in prion agent spread. The transfer of scrapie infection via exosomes (47) and release of scrapie infectivity into the supernatant (7, 49) represents another possible mechanism for prion infection of C2C12 cells, but this is less likely to have been the case in the current study since we observed an absence of measurable PrP^{Sc} in C2C12 cells when they were cocultured with 22L scrapie-infected N2A cells that were separated by a porous membrane. However, we cannot exclude the possibility that these mechanisms of cell-to-cell spread are involved in scrapie infection of C2C12 muscle cells.

In C2C12 muscle cells there was evidence of N-terminal truncation of the full-length prion protein into either a 16-kDa PrP^C polypeptide in both mock- and 22L scrapie-infected C2C12 cells or an 18-kDa PrP^{Sc} polypeptide in 22L scrapie-infected C2C12 myotubes. The size and cleavage properties of these truncated PrP polypeptides are consistent, but not precisely matched, with the 17-kDa C1 and 21-kDa C2 PrP polypeptide fragments, respectively, that have been described in mock- and prion-infected brain and cell lines (16, 30, 55, 58). The apparent discrepancy in the size of the C2 PrP^{Sc} polypep-

tide between 22L C2C12 myotubes and other scrapie-infected cells or brain tissue could be due to the use of different polyacrylamide gel separation systems or, perhaps, to differences in cleavage specificity among tissue types. Prior studies demonstrated that N-terminal truncation of PrP^{Sc} into the C2 polypeptide is mediated by calpain-dependent endoproteolytic cleavage to generate the C2 PrP^{Sc} polypeptide (58). Calpains play an essential role in muscle cell differentiation and, in myoblasts, they have been associated with cell adhesion and spreading (23). In C2C12 cells, inhibition of calpains prevented myocyte migration and fusion into multinucleated myotubes (22). High calpain activity during myogenic differentiation is consistent with cleavage into the C2 PrP^{Sc} polypeptide in 22L scrapie-infected C2C12 myotubes.

Our findings that scrapie infection can be established in both dividing myoblasts and nondividing myotubes *in vitro* directly illustrates that the scrapie agent can replicate in muscle cells. This study supports previous reports of direct detection of PrP^{Sc} in myocytes of sheep with scrapie, in skeletal muscle cells of the tongue in rodents experimentally infected with the prion agent (24, 39, 53), and in cardiomyocyte fibers of humans with Creutzfeldt-Jakob disease (4) or deer with chronic wasting disease (29). The C2C12 cell model for scrapie infection could be used to investigate prion infection of muscle, including the mechanism of scrapie infection in muscle cells, the pathway of PrP^{Sc} formation and trafficking in a terminally differentiated cell type, and the relationship of prion infection in muscle cells to prion-induced myopathy that is found in scrapie of sheep and chronic wasting disease of deer and elk.

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