R E P O R T S

The cellular prion protein PrPc is a glycosylphosphatidylinositol-anchored cell-surface protein whose biological function is unclear. We used the murine 1C11 neuronal differentiation model to search for PrPc-dependent signal transduction pathways mediated by antibody-mediated cross-linking. A caveolin-1-dependent coupling of PrPc to the tyrosine kinase Fyn was observed. Clathrin might also contribute to this coupling. The ability of the 1C11 cell line to trigger PrPc-dependent Fyn activation was restricted to its fully differentiated serotonergic or noradrenergic progeny. Moreover, the signaling activity of PrPc occurred mainly at neurites. Thus, PrPc may be a signal transduction protein.

Although much progress has been made over the past few years regarding the involvement of the scrapie prion protein (PrPSc) in transmissible spongiform encephalopathies (TSEs) (1), the biological function of the cellular, nonpathogenic isoform of PrP, PrPc, still remains enigmatic. PrPc is an ubiquitous glycoprotein expressed strongly in neurons (2). PrPc-deficient mice are viable and develop normally, but they display minor defects that differ according to the null strain (3). In contrast, mice expressing an NH2-terminally truncated version of PrPc in a null background show neuronal degeneration soon after birth, suggesting that PrPc may play an important role in the maintenance and/or regulation of neuronal functions (4). Recent data have focused on the copper-binding ability of PrPc (5), and an involvement of PrPc in the regulation of the presynaptic copper concentration and of synaptic transmission has been proposed (6). The attachment of PrPc to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor may also be consistent with a role in cell-surface signaling or cell adhesion. Indeed, the 37-kD laminin receptor binds PrPc (7).

Because PrPc may act as a cell-surface receptor, we investigated whether signal transduction pathways coupled to PrPc after antibody-mediated cross-linking (8). Our experimental strategy relies on the neuronal differentiation model 1C11 (9). The 1C11 clone is a committed neuroectodermal progenitor with an epithelial morphology that lacks neuron-associated functions (9). Upon induction, 1C11 cells develop a neural-like morphology, acquire neuronal markers, and convert into either 1C11*5-HT (serotonergic) cells or 1C11**NE (noradrenergic) cells. The choice between the two differentiation pathways depends on the set of inducers used (9). Within 4 days, 1C11*/ 5-HT cells implement a complete serotonergic phenotype, including 5-HT synthesis, storage, transport, catabolism, as well as three functional serotonergic receptors of the 5-HT1B/D, 5-HT2B, and 5-HT2A subtypes (9). The noradrenergic phenotype of 1C11**NE cells is complete within 12 days, with a progressive onset of catecholamine synthesis, storage, transport, catabolism, and an a1D-adrenergic receptor (9). The differentiation events involve almost 100% of the cells and follow a well-characterized time course.

PrPc is constitutively expressed in the 1C11 progenitor and throughout differentiation (10). Thus, the 1C11 cell line offers the opportunity to study PrPc in relation to the onset of neuronal functions and within an integrated neuronal context. The effects of antibody-mediated ligation of PrPc at the cell surface of the 1C11 progenitor or its fully differentiated 1C11*5-HT and 1C11**NE progeny were followed. PrPc cross-linking did not induce any specific phosphoinositide (PI) hydrolysis or nitric oxide production, nor did it promote the activation of p21ras or phospholipase A2 in the 1C11 cell line within 30 min of cross-linking (11). In contrast, ligation of PrPc with specific antibodies induced a marked decrease in the phosphorylation level of the tyrosine kinase Fyn in

Michigan Agricultural Experiment Station. We thank S. J. Halstead, K. D. Baergen, A. T. Corbin, C. M. Easley, and G. R. Ponciroli for technical assistance in the field and laboratory, M. A. Halvorson for agronomic management, and C. P. McSwiney for fuel use calculations. We also thank A. R. Mosier, K. A. Smith, and P. M. Vitousek for many insightful comments on an earlier draft.

2 May 2000; accepted 28 July 2000
both 1C11*/5-HT and 1C11**/NE cells (Fig. 1A). The effect became measurable 10 min after antibody-mediated ligation. Similar decreases were obtained with two distinct antibodies to PrP (1A8 and SAF 61), but not with irrelevant antibodies directed against the membrane serotonin transporter. Fyn immunoprecipitates were subjected to immunocomplex kinase assay. As expected (2), the dephosphorylation of Fyn monitored in 1C11*/5-HT and 1C11**/NE cells after PrP<sup>+</sup> cross-linking increased kinase activity (Fig. 1B). The activation of the Fyn kinase was abolished in the presence of competing Fab fragments of SAF61 antibodies (Fig. 1B).

In 1C11 precursor cells, the phosphorylation level of the tyrosine kinase Fyn was not sensitive to PrP<sup>+</sup> cross-linking (Fig. 1A). However, 1C11 cells contain similar amounts of PrP<sup>+</sup> as found in 1C11**/NE cells (10). Also, PrP<sup>+</sup> is equally well released by PI-specific phospholipase C (PI-PLC) from undifferentiated and differentiated cells (11). Moreover, the levels of Fyn expression, as assessed through immunoprecipitation, were roughly the same in the 1C11 clone and its differentiated progenies (Fig. 1B). Therefore, the signaling competence of the 1C11 cell line toward PrP<sup>+</sup> activation appeared to depend on the conversion of 1C11 cells into either 1C11*/5-HT, or 1C11**/NE cells.

Because Fyn is an intracellular protein but PrP<sup>+</sup> is anchored to the outer membrane, the PrP<sup>+</sup>-dependent signaling mechanism causing Fyn activation is likely to involve intermediate factor(s). To identify candidate proteins, we performed coimmunoprecipitation experiments with antibodies to PrP using metabolically labeled lysates of 1C11*/5-HT or 1C11**/NE cells. In addition to heterogeneously glycosylated PrP<sup>+</sup> molecules, two bands of 21 and 22 kD, respectively, were revealed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the precipitates (Fig. 2A). When the samples receive a pre-treatment of enzymatic deglycosylation with N-glycosydase F (PNGase), the monoglycosylated PrP<sup>+</sup> molecules (migrating to 28 to 30 kD) and the highly glycosylated isoforms (migrating as a band spanning 33 to 40 kD) all resolve into a single species of 25 kD. In contrast, p21 and p22 do not shift in molecular weight after PNGase treatment. Microsequence analysis (Fig. 2B) allowed us to unambiguously identify p21 and p22 as caveolin-1<sub>B</sub> (cav-1<sub>B</sub>) and caveolin-1<sub>A</sub> (cav-1<sub>A</sub>), respectively. These two isoforms of caveolin-1 differ in their respective initiation sites only (13).

In the gel analysis of an immunoprecipitate from 1C11 undifferentiated cells, the bands corresponding to p21 and p22 were barely detectable (Fig. 2A). This observation could not be explained by a defect in caveolin-1 expression in 1C11 precursor cells because all cell types studied here express caveolin. The level of caveolin expression, as measured by Western blot, increased only faintly in differentiated cells compared with 1C11 cells (Fig. 2C). The capacity of PrP<sup>+</sup> to interact with caveolin-1 therefore appeared to depend on the differentiation of 1C11 cells toward either a serotonergic or a noradrenergic program.

To assess whether caveolin-1 takes part in the PrP<sup>+</sup>-mediated Fyn activation, we introduced antibodies to caveolin-1 to 1C11*/5-HT or 1C11**/NE cells before PrP<sup>+</sup> cross-linking with the use of the cell bombardment technique (14). Immunosequestation of caveolin-1 in live cells blocked the PrP<sup>+</sup>-dependent activation of Fyn (Fig. 2D). Thus, caveolin-1 appears to be one of the protagonists involved in PrP<sup>+</sup> coupling to the tyrosine kinase Fyn. Antibodies to clathrin, which were used as a control, were unable to cancel the Fyn response (Fig. 2D), although the amplitude of Fyn activation was somewhat reduced under these conditions. Such a partial reduction
upon clathrin immunosequestration may reflect indirect interference with the caveolin-dependent signaling pathway through changes in membrane properties. However, it may also indicate a direct involvement of clathrin in the PrP\textsuperscript{c}-mediated signal.

The difference in signaling competence of 1C11 versus 1C11\textsuperscript{***}/HT and 1C11\textsuperscript{**}/NE cells prompted us to examine whether the response to PrP\textsuperscript{c} cross-linking was related to the sequential acquisition of neurotransmitter-associated functions. PrP\textsuperscript{c} cross-linking was applied at specific time points during each differentiation program. Antibody-mediated ligation of PrP\textsuperscript{c} failed to induce any activation of the tyrosine kinase Fyn by day 2 of either program (Fig. 3A). Because neurite outgrowth and the onset of neuronal markers are seen as early as 1 day after either induction, simple engagement of 1C11 cells in a neuronal-like program was not sufficient to confer responsiveness to PrP\textsuperscript{c} stimulation. Instead, not until day 4 of the serotonergic and day 12 of the noradrenergic differentiation programs were the signaling cascades triggered by PrP\textsuperscript{c} cross-linking (Fig. 3A). Thus, full differentiation of 1C11 cells and the induction of a functional bioaminergic uptake are a prerequisite for responsiveness to PrP\textsuperscript{c} activation.

Because the two identified partners of the cascade (Fyn and caveolin) are available in the cells from the beginning of differentiation, several hypotheses can be made. First, PrP\textsuperscript{c} signaling may involve other yet-to-be-identified cellular partner(s) whose expression is strictly related to the differentiation stage of the cells. Second, the interaction of all cellular partners within a signaling complex may depend on the overall acquisition of neuronal and neurotransmitter-associated functions, specific to the ultimate stage of differentiation. Because caveolin-1 appears to interact with PrP\textsuperscript{c} from day 2 of either the serotonergic or the noradrenergic program (Fig. 2A), it may be that the recruitment of Fyn to the PrP\textsuperscript{c}–caveolin-1 complex occurs only at terminal stages of differentiation. Third, the onset of the overall functions of bioaminergic neurons may be a prerequisite to the proper structural organization of the signaling partners within subcellular compartments or microdomains.

Because of the neuronal polarity of 1C11\textsuperscript{***}/HT and 1C11\textsuperscript{**}/NE cells, we performed cell fractionation to separate neurite extensions from cell bodies (Fig. 3B) and we evaluated the relative contributions of either compartment to PrP\textsuperscript{c} signaling. In 1C11\textsuperscript{***}/HT and 1C11\textsuperscript{**}/NE cells, PrP\textsuperscript{c} was abundant at the surface of cell bodies. Prion proteins were also clustered along the neurites (Fig. 3C). The fraction of PrP\textsuperscript{c} located at the cell body only weakly contributed to the activation of Fyn upon antibody-mediated ligation (Fig. 3D). In contrast, cross-linking of neuritic PrP\textsuperscript{c} induced a marked increase in Fyn kinase activity. The amplitude of the response was similar to that observed with total cell lysates (Fig. 3D). In 1C11\textsuperscript{***}/HT serotonergic and 1C11\textsuperscript{**}/NE noradrenergic cells, the signaling activity of PrP\textsuperscript{c} may essentially be attributable to those PrP\textsuperscript{c} molecules located on neurites.

Thus, in the differentiating 1C11 cell system, coupling of PrP\textsuperscript{c} to the tyrosine kinase Fyn is closely related to the maturation of the cells. Neurotransmitter-associated functions, as well as the structural morphology of the cells, appear to be involved. It is likely that the sequential onset of functional bioaminergic receptors and transporters is accompanied by a spatial organization of membrane components within specialized domains, possibly including cell-surface receptors, membrane adaptors, and signaling molecules. How could this PrP\textsuperscript{c}-dependent signal functionally interact with other transduction pathways or contribute to cell homeostasis? Brief exposure of fully differentiated 1C11\textsuperscript{***}/HT or 1C11\textsuperscript{**}/NE cells to antibodies to PrP does not induce any noticeable morphological change. PrP\textsuperscript{c} is not required for the expression of a critical cell function (3). Instead, PrP\textsuperscript{c} may be involved in the modulation of neuronal functions at the cellular level.

The identification of PrP\textsuperscript{c} as a signaling molecule opens new directions for unraveling PrP\textsuperscript{c} function. It also implies the existence of extracellular signal(s) capable of triggering the activation of this protein and provides a foundation for uncovering such signal(s). In the context of prion infection, an important question to resolve is how PrP\textsuperscript{c} accumulation may interfere with the signaling activity of PrP\textsuperscript{c}. The 1C11 cell system, which supports prion replication in vitro (15), may help to illuminate this issue.

References and Notes
A Link Between RNA Interference and Nonsense-Mediated Decay in Caenorhabditis elegans

Mary Ellen Domeier,1 Daniel P. Morse,2 Scott W. Knight,2 Michael Portereiko,1 Brenda L. Bass,2 Susan E. Mango1*

Double-stranded RNA (dsRNA) inhibits expression of homologous genes by a process involving messenger RNA degradation. To gain insight into the mechanism of degradation, we examined how RNA interference is affected by mutations in the smg genes, which are required for nonsense-mediated decay. For three of six smg genes tested, mutations resulted in animals that were initially silenced by dsRNA but then recovered; wild-type animals remained silenced. The levels of target messenger RNAs were restored during recovery, and RNA editing and degradation of the dsRNA were identical to those of the wild type. We suggest that persistence of RNA interference relies on a subset of smg genes.

Epigenetic silencing by dsRNA is a widespread phenomenon for regulating gene expression (1, 2). This process, termed RNA interference, or RNAi, is thought to involve targeted degradation of homologous mRNAs (3–7). In C. elegans, seven genes have been shown to be important for RNAi: the RNA-directed RNA polymerase homolog ego-1 (8), mut-7 (9, 10), rde-2, rde-3, rde-4, mut-2, and rde-1, which encodes a member of the elf2c/zwille family (10). At present, it is unclear how the products of these genes function in RNAi, why some of the genes are required for silencing in the germ line but not the soma, or what roles the genes play in other processes such as transposition (8–11).

Based on the observation that both RNAi and nonsense-mediated decay involve RNA degradation, we examined whether proteins required for nonsense-mediated decay also functioned during RNAi. Seven smg genes have been identified, each of which is involved in nonsense-mediated decay (12, 13). Mutations in five of these genes produce identical phenotypes, emphasizing that the SMG proteins act in a common pathway [smg-1 through smg-5 (12, 14)].

To compare the effects of RNAi in wild-type (WT) and smg animals, we injected dsRNA corresponding to the unc-54 gene, which encodes myosin heavy chain B and is expressed in body wall muscles (15). We chose unc-54 because it generates a robust RNAi phenotype in which animals are paralyzed (16) and also because the severity of paralysis correlates with mRNA levels (14, 17).

We observed that mutant smg-2 animals recovered rapidly from unc-54 dsRNA-induced paralysis, whereas WT worms did not (Fig. 1A) (18). Progeny of injected mothers were examined daily for 4 days after injection. On days 1 and 2, both WT and smg-2 larvae were severely paralyzed. However, smg-2 mutants showed increased motility as they aged and moved almost as well as uninjected controls by day 4. We also observed recovery from RNAi in smg-2 mutants carrying a sur-5:GFP transgene (Fig. 1B) (18). Thus, recovery was not specific to unc-54 RNA or to body wall muscles, but occurred in many cell types and for at least two transcripts. GFP expression also rebounded in the neurons of WT worms, indicating that neurons have an intrinsic recovery mechanism that is independent of the smg genes.

To rule out the possibility that smg-2 mutants recovered from RNAi for nonspecific reasons, such as being poor injection hosts, we injected smg-2(+/−) heterozygous mothers with unc-54 dsRNA, scored recovery of individual offspring on day 3, and then determined the genotype of each scored animal. We found that progeny that failed to recover were rarely smg-2 homozygotes (12% of paralyzed animals, n = 158). Conversely, siblings that recovered from RNAi were often smg-2 homozygotes (55% of moving animals, n = 131). If segregation were random, 25% of paralyzed or moving animals would be expected to be smg-2 homozygotes. These experiments demonstrate that recovery from RNAi depends on smg-2 activity in the zygote and therefore does not reflect the inability of smg-2 mutants to function as good injection hosts.

To examine whether smg-2 mutations affected RNAi-mediated mRNA degradation, we measured endogenous RNA levels using a real-time semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) assay (19, 20). Controls with WT and mutant unc-54 animals demonstrated that our assay accurately reflected transcript levels (21). Furthermore, smg-2 mutations altered unc-54 RNA levels in ways that paralleled the phenotypic recovery. In WT larvae, unc-54 RNA levels were reduced about 10-fold compared with uninjected controls and remained low throughout the time course. In smg-2 animals, unc-54 levels were reduced 10-fold on day 1, but rebounded rapidly, eventually reaching levels close to those of uninjected controls (Fig. 2). The reduction seen on day 1 was comparable to that seen in WT worms, indicating that the initial response was robust. These data demonstrate that smg-2 mutants attenuate RNAi-mediated mRNA degradation.

Our data predict that for the effects of the smg genes on RNAi to be observed, the targeted mRNA must be transcribed continuously and RNAi must not induce lethality, or the animals will not be able to recover. These requirements explain why recovery in smg mutants was not observed previously (7). Earlier studies targeted mex-3, which is maternally transcribed and essential for embryogenesis (22). In addition, these studies assayed smg-3 mutants, which fail to recover well from RNAi (see below).

1Huntsman Cancer Institute Center for Children and Department of Oncological Sciences, University of Utah, Salt Lake City, UT 84112, USA. 2Department of Biochemistry and Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT 84132, USA.

*To whom correspondence should be addressed. E-mail: susan.mango@hci.utah.edu