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Identifying critical sites of PrP^{c}-PrP^{Sc} interaction in prion-infected cells by dominant-negative inhibition

Yuzuru Taguchi[1] and Hermann M Schätzl[1,2,*]

[1]Department of Comparative Biology & Experimental Medicine; Faculty of Veterinary Medicine; University of Calgary; Calgary, AB Canada;

[2]Departments of Molecular Biology and of Veterinary Sciences; University of Wyoming; Laramie, WY USA

A direct physical interaction of the prion protein isoforms is a key element in prion conversion. Which sites interact first and which parts of PrP^{c} are converted subsequently is presently not known in detail. We hypothesized that structural changes induced by PrP^{Sc} interaction occur in more than one interface and subsequently propagate within the PrP^{C} substrate, like epicenters of structural changes. To identify potential interfaces we created a series of systematically-designed mutant PrPs and tested them in prion-infected cells for dominant-negative inhibition (DNI) effects. This showed that mutant PrPs with deletions in the region between first and second α-helix are involved in PrP-PrP interaction and conversion of PrP^{C} into PrP^{Sc}. Although some PrPs did not reach the plasma membrane, they had access to the locales of prion conversion and PrP^{Sc} recycling using autophagy pathways. Using other series of mutant PrPs we already have identified additional sites which constitute potential interaction interfaces. Our approach has the potential to characterize PrP-PrP interaction sites in the context of prion-infected cells. Besides providing further insights into the molecular mechanisms of prion conversion, this data may help to further elucidate how prion strain diversity is maintained.

Keywords: PrP^{C}-PrP^{Sc} interaction, prion conversion, dominant-negative inhibition, interaction interface, epicenter of structural changes

Abbreviations: PrP, prion protein; PrP^{C}, normal isoform of PrP; PrP^{Sc}, abnormal isoform of PrP; H1, first α-helix of PrP; H2, second α-helix of PrP; H1~H2, region between H1 and H2; DNI, dominant-negative inhibition; GPI, glycosylphosphatidylinositol; PK, proteinase K; ER, endoplasmic reticulum; N2a, Neuro2a mouse neuroblastoma cells; 22L-ScN2a, N2a cells persistently infected with 22L prions

*Correspondence to: Hermann M Schätzl; Email: hschaetz@ucalgary.ca

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Introduction

Prions are unconventional pathogens devoid of a nucleotide genome. Nevertheless, a variety of prion strains have been characterized which can be explained by the existence of a quasi-species population of conformers. The epigenetic information for encoding a diversity of prion strains is therefore enciphered in the structure of the prion protein (PrP). Stable inheritance over generations is achieved by the high fidelity of the template-assisted refolding of the substrate PrP^{C} into the abnormal isoform PrP^{Sc}, which is both template and reaction product in prion conversion.[1,2] In general, genetic information on nucleotide genomes is encoded as "digital information" enciphered with a very limited number of bases. Obviously, this mechanism greatly contributes to stable inheritance, while the diversity of genes is achieved by permutation in the sequence patterns of the bases. On the other hand, variations in protein conformation as underlying prion strain diversity seem to be "analog media" rather than digital. How strain diversity and stable inheritance is achieved at the molecular level is presently not well understood, if at all. A better molecular understanding of the modalities of the PrP^{C}-PrP^{Sc} interaction and conversion reactions are prerequisites for this. We started to characterize this interaction in

prion-infected cultured cells and recently reported a PrP region which constitutes a potential interface in PrP^{C}-PrP^{Sc} interaction.[3] Here, we are going to present our overarching working hypothesis which goes beyond the published work and future directions of this project.

Does a PrP^{c}-PrP^{Sc} Interaction Site Define Epicenters of Structural Changes in PrP?

Our rationale for defining interfaces of the PrP^{C}-PrP^{Sc} interaction is based on the hypothesis that regions within the PrP^{C} substrate which strongly bind to the PrP^{Sc} template are the regions which also undergo initial regional structural changes. Subsequently, these intra-molecular structural changes propagate to adjacent regions until PrP^{C} is converted, functioning thereby as "epicenters" of structural changes. The viewpoint that substantial regional structural changes also occur in conversion-incompetent PrP is supported by production of PK-resistant short fragments in in-vitro conversion of conversion-incompetent N-terminally truncated PrP.[4,5] We hypothesized that such an epicenter would be a region whose regional structural changes as induced by template PrP^{Sc}, along with high-affinity adhesion, are independent of the global conversion or regional structural changes in other regions (**Fig. 1A, B**), and reasoned that such regions might also act as the interaction interfaces between conversion-incompetent mutant PrP and template PrP^{Sc} in dominant-negative inhibition (DNI).

DNI is a phenomenon where a conversion-incompetent PrP inhibits conversion of a co-existing conversion-competent PrP^{C} substrate, presumably by competing for the PrP^{Sc} template (**Fig. 1C**). DNI was initially used to test high-affinity binding of conversion-incompetent PrP^{C} to the postulated "factor X."[6] However, more recent in vitro conversion reactions demonstrated that DNI involves interaction of PrP substrate, inhibitory conversion-incompetent PrP and PrP^{Sc} template, even in the absence of any cellular components, suggesting that the process is independent of the postulated "factor X."[7,8] Of note, conversion incompetence is not synonymous with efficient DNI: some conversion-incompetent PrPs exert efficient DNI, whereas others do not,[6,9] as was also observed in our recently published work presented below.[3] We attributed the variations in DNI efficiency to their differential affinities for the PrP^{Sc} template, based on the functionality of their interaction interfaces (**Fig. 1C**, compare PrP 'B' and 'C'). Assuming that the affinity for PrP^{Sc} is modulated by resulting regional structural changes in or around the interaction interface, we considered this region as a possible epicenter region. An epicenter region can then be identified as an interaction interface for DNI by evaluating DNI

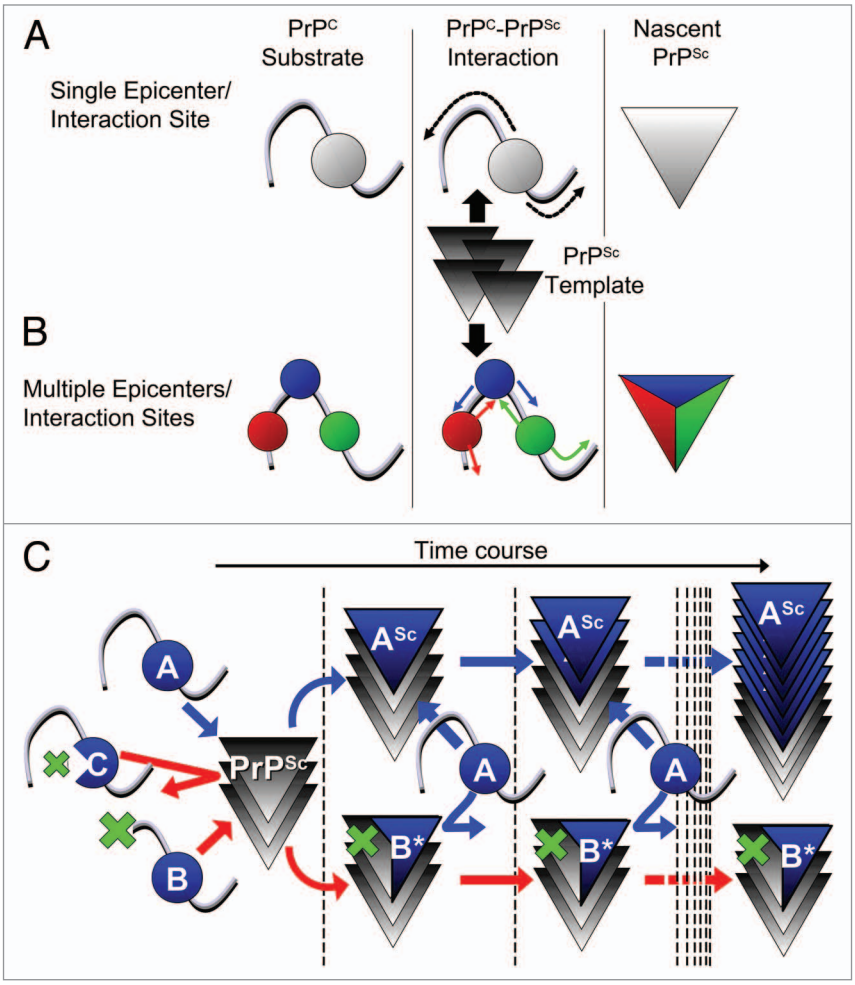

Figure 1. (**A, B**) Schemes illustrating differences between "single epicenter/interaction site model" and "multiple epicenters/interaction sites model." (**A**) Single epicenter/interaction interface model: PrP^{C} substrate interacts with PrP^{Sc} template at a specific region, irrespective of the strain type, and structural changes spread from this interaction interface to the entire molecule, acting as an epicenter of structural changes. (**B**) Multiple epicenter/interaction interface model: PrP^{C} substrate can interact with PrP^{Sc} template at more than one region and structural changes spread from each epicenter until the entire molecule is converted. (**C**) Scheme illustrating hypothetic mechanism of dominant-negative inhibition (DNI) when conversion -competent and -incompetent PrPC coexist. The interaction interface of PrP^{C} is represented by a "blue ball." The blue and red arrows indicate situations where the competition for PrP^{Sc} template was won by conversion-competent or conversion-incompetent PrP^{C}, respectively. At the beginning, molecules with an intact interaction interface can bind the PrP^{Sc} template irrespective of their conversion abilities, i.e., conversion-competent PrP, "A," or conversion-incompetent PrP with a defect outside the interface, "B," can bind, whereas conversion-incompetent PrP with a defect in the interface, "C," cannot even interact. After binding, "A" converts to a nascent PrP^{Sc} "A^{Sc}," while "B" undergoes regional structural changes to become "B*" for high-affinity binding. The PrP^{Sc} template bound by "B*" cannot function as the template anymore, consequently inhibiting conversion of "A."

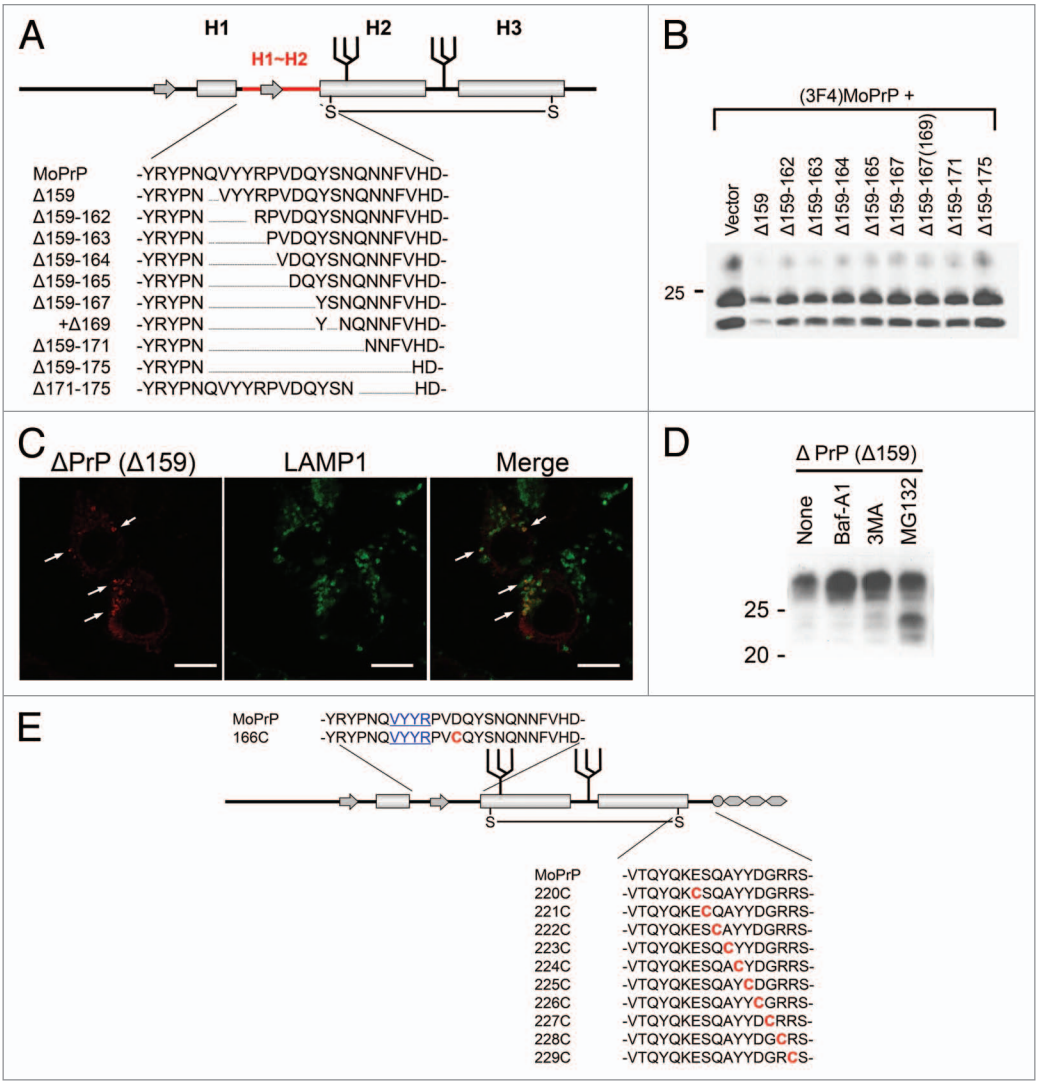

Figure 2. (**A**) Example of systematically-designed mutant PrPs with deletions in H1~H2, used to test in DNI assay whether the H1~H2 region is an interaction interface. (**B**) Representative immunoblot demonstrating inverse correlation between DNI efficiency and size of deletion in H1~H2. A conversion-competent and epitope-tagged wild-type PrP, (3F4)MoPrP, and a conversion-incompetent PrP mutant were co-transfected into 22L prion-infected N2a cells and PK-resistant PrP was detected. (**C**) Confocal image showing co-localization of PrPΔ159 and LAMP1. N2a cells transfected with PrPΔ159 were fixed, treated with 6M guanidine hydrochloride to remove excessive PrPΔ159 signal localized in ER, labeled with antibodies against PrP and LAMP1, and analyzed for co-localization. (**D**) Immunoblot showing that degradation of PrPΔ159 is inhibited by treatment with bafilomycin A1 (Baf-A1), autophagy inhibitor 3MA, or proteasome inhibitor MG132. (**E**) A schematic illustration of a series of mutant PrPs with two cysteine substitutions.

efficiencies of systematically-designed PrPs with mutations in or around the candidate region. Our model does not incorporate alternative mode of actions and we presently only have indirect experimental evidence that an interaction interface as defined in DNI is in fact equivalent to the interaction interface between PrP^{C} substrate and PrP^{Sc} template in prion conversion. It is also possible that DNI works "indirectly," either through an allosteric effect or by inhibiting polymerization in later steps. For the sake of clarity, such possibilities are not considered in **Figure 1**. We also expect that DNI is both strain and species specific as observed before when testing mutant PrPs with insertions in murine cell lines infected with different mouse prion strains.[10]

We did our analysis in persistently prion-infected cells as this provides authentic PrP^{Sc} and prion infectivity. Second the environment where the PrP^{C}-PrP^{Sc} conversion occurs is most similar to the in vivo situation, including pH and non-proteinaceous factors, and conditions are maintained constant as long as the same cell line is used. Third, PrP^{C} substrate and mutant PrP undergo similar post-translational maturation, e.g., glycosylation and GPI anchoring, and subcellular trafficking, although our later studies showed that glycosylation and trafficking can be different from wild-type PrP.

H1~H2 Region is Involved in PrP-PrP Interaction and Prion Conversion

Based on our hypothesis, we created a series of conversion-incompetent mutant PrPs with internal deletions of different

length in the region between the first (H1) and second (H2) α-helix (H1~H2) (**Fig. 2A**) and evaluated their DNI efficiencies. As a result, DNI efficiencies showed an inverse relation with the size of the deletion (**Fig. 2B**), suggesting that this region might be a possible interaction interface itself or a critical component of it. Even mutant PrPs which lack the entire part from the pre-octapeptide repeat region to H1 depended on H1~H2 for efficient DNI. On the other hand, deletion from the C-terminal end of H1~H2 highly affected DNI. Deleting five residues there (Δ171–175) resulted in a similar inefficient DNI as deleting the entire 17 residues (Δ159–175). A single deletion of residue 175 also significantly affected DNI efficiency. These findings imply that some cooperation between H1~H2 and the region C-terminal to it is significant for efficient DNI. Our analysis also lead to new findings in the cell biology of prion proteins. The ability to exert DNI requires physical interaction with PrP^{Sc} in a cellular compartment where conversion of PrP^{c} into PrP^{Sc} occurs. To reconcile the paradox of how an intracellular PrP can exert DNI, we showed that mutant PrPs are subject to both proteasomal and lysosomal/autophagic degradation pathways (**Fig. 2D**). Using autophagy pathways a fraction of mutant PrPs reaches the cellular locale of prion conversion (**Fig. 2C**), shedding light on the subcellular sites where prion conversion can occur and on PrP^{c}/PrP^{Sc} recycling pathways.

Is There Evidence for the Importance of H1~H2 Region as PrP-PrP Interaction Site from Other Studies?

Recently, Singh and colleagues studied in vitro synthesized mouse PrP fibrils by hydrogen/deuterium (H/D)-exchange analysis and suggested that a region encompassing residues 159 to 225 (they referred to as amyloid core) might first convert to the amyloid form and then structural changes in other parts including the region N-terminal to H1 follow.[11] Although experimental conditions for in vitro PrP fibril formation and PrP^{C}-PrP^{Sc} conversion in cultured cells are very different, this supports the viewpoint that the region which first interacts with the PrP^{Sc} template undergoes structural changes before structural changes in other regions occur. Interestingly, the amyloid core defined by these authors also started with residue 159 and covered the entire H1~H2 region. The involvement of the region C-terminal to H1~H2 for efficient DNI we observed might reflect the importance of this region in the conversion of the amyloid core. In addition, a substantial part of this postulated amyloid core region was found protected in H/D-exchange analysis of products from seeded–or unseeded protein misfolding chain amplification (PMCA)[12] and in PrP^{Sc} purified from prion-infected transgenic mice expressing PrP without a GPI anchor,[13] although with variations in the areas that were protected. The region corresponding to the amyloid core was also identified as 11~12 kDa fragments after PK digestion of PrP fibrils composed of full-length recombinant PrP[14] and similar fragments were seen in some types of sporadic Creutzfeldt-Jakob disease.[15] These findings suggest that this region has a strong propensity to undergo structural changes and to polymerize in the presence of PrP^{Sc} or PrP fibrils. Interestingly, all of those C-terminal derived fragments more or less contained H1~H2, indicating importance of H1~H2 in the conversion to fibrils or PrP^{Sc}. Mutations in H1~H2, specifically S170N and N174T, have been recently reported to enhance aggregation propensity of PrP, again corroborating the importance of this region for PrP-PrP interactions.[16,17]

Taken together, these data are in line with our findings, despite being produced with very distinct methodologies. This confirms our approach of using DNI of systematically designed mutant PrPs in prion-infected cultured cells for identifying putative PrP-PrP interaction interfaces.

Are There Other Interaction Sites or Epicenters?

There is experimental evidence that the region N-terminal to H1~H2 also has a strong propensity to aggregate, as demonstrated when testing fibril formation of a naturally occurring PrP mutation, Y145stop. Synthetic amyloid fibrils encompassing residues 107–143 enhanced fibril formation of full-length PrP[18] and this region might also contribute to the structural complexity of PrP fibrils and to strain barriers.[19,20] Protection of the region N-terminal to H1 in H/D-exchange has been reported for PrP fibrils,[21] PrP^{Sc}-seeded PMCA products,[12] and PrP^{Sc} purified from prion-infected mice.[13] Existence of multiple interfaces would introduce some "digital" characteristics to the PrP^{C}-PrP^{Sc} conversion reaction. On one side this would contribute to high fidelity in prion replication. On the other hand, some structural variability by differential predominance of structural changes or usage of epicenters would be facilitated, equivalent to genetic information. This might explain the presence of short fragments of ~7 kDa in certain types of prion diseases, e.g., Gerstmann-Straussler-Scheinker syndrome, variably-protease-sensitive prionopathy, or Nor98,[22] and provide a clue why mice expressing chimeric PrP are more susceptible to certain prion types and less susceptible to others than mice expressing wild-type PrP.[23-25]

We are presently looking for such additional interaction sites (**Fig. 1B**) and are studying the cooperation between H1~H2 and the region C-terminal to it. To do so, we created another series of mutant PrPs which have two cysteine substitutions (residue 166 and 220–229), providing an extra disulfide bond when in sufficient proximity (**Fig. 2E**). We expect that PrPs with two cysteine substitutions and a Δ159 deletion also will provide important answers whether DNI effects are mediated directly by competition for a PrP^{c}-PrP^{Sc} interaction site or indirectly via allosteric effects. In preliminary studies some of these mutant PrPs exerted efficient DNI when co-transfected into prion-infected cells. These results also suggest that DNI by ΔPrPs and conversion into PrP^{Sc} isoforms are closely related, very likely because of using the same interaction interfaces. Combining now these mutants with other deletions will allow us to study the functionality of additional PrP-PrP

interaction interfaces and to analyze how this impacts prion strain properties when using different strains.

Conclusions

Our approach can be used for characterizing PrP-PrP interaction sites in the context of prion-infected cells. Besides providing further insights into the molecular mechanisms of prion conversion, this strategy may help to elucidate how prion strain diversity is generated and maintained. Identification of interaction interfaces has also translational implications, as it might provide novel therapeutic targets. If a small chemical compound which binds to the interface region and inhibit its function as interaction interface is developed, it might represent an efficient anti-prion drug.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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