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Cellular factors implicated in prion replication

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ABSTRACT

Prions are the unconventional infectious agents responsible for prion diseases, which are composed mainly by the misfolded prion protein (PrP^{Sc}) that replicates by converting the host associated cellular prion protein (PrP^{C}). Several lines of evidence suggest that other cellular components participate in prion conversion, however, the identity or even the chemical nature of such factors are entirely unknown. In this article we study the conversion factor activity by complementation of a PMCA procedure employing purified PrP^{C} and PrP^{Sc}. Our results show that the conversion factor is present in all major organs of diverse mammalian species, and is predominantly located in the lipid raft fraction of the cytoplasmic membrane. On the other hand, it is not present in the lower organisms tested (yeast, bacteria and flies). Surprisingly, treatments that eliminate the major classes of chemical molecules do not affect conversion activity, suggesting that various different compounds may act as conversion factor in vitro. This conclusion is further supported by experiments showing that addition of various classes of molecules have a small, but detectable effect on enhancing prion replication in vitro. More research is needed to elucidate the identity of these factors, their detailed mechanism of action and whether or not they are essential component of the infectious particle.

1. Introduction

Compelling evidence indicate that prions are composed exclusively of the misfolded prion protein (termed PrP^{Sc}), which transmit the disease in the absence of nucleic acids by transferring its folding properties to the normal cellular prion protein (PrP^{C}) present in the host. The prion hypothesis remained highly controversial for decades until recent studies in which infectious PrP^{Sc} was successfully generated in a cell-free system [1–6]. Prion replication is hypothesized to occur when PrP^{Sc} in the infecting inoculum interacts specifically with host PrP^{C}, catalyzing its conversion to the misfolded form of the protein. A physical association between the two isoforms during the infectious process is suggested by the primary sequence specificity in prion transmission [7] and by the reported in vitro generation of PrP^{Sc} by mixing purified PrP^{C} with PrP^{Sc} [8,9]. However, the exact mechanism underlying the conversion is not known, particularly with respect to the putative role of other cellular factors in prion replication [10].

The existence of a host factor involved in prion replication was first suspected when transgenic mice expressing both human and mouse PrP^{C} were challenged with human prions. Surprisingly, mice co-expressing both proteins were resistant to prion replication while mice expressing only human PrP^{C} developed the disease following inoculation with human prions [7]. Interestingly, transgenic animals expressing a chimeric protein consisting of pieces of the human and the mouse gene were also susceptible to infection with human prions [11]. The interpretation of these results was that mouse PrP^{C} inhibited the conversion of human PrP^{C} by binding to an additional cellular protein implicated in prion replication. Further studies performed by the same group showed that the host factor, termed "protein X", was able to bind PrP^{C} through its C-terminal end [12] and this interaction can be precluded by some small molecule compounds [13]. More recent biochemical studies have provided additional evidence for the existence of a conversion factor in prion replication. We demonstrated previously that in vitro prion conversion does not occur under our experimental conditions when purified PrP^{C} and PrP^{Sc} are mixed and incubated [14]. The conversion activity was recovered when the bulk of cellular components were added back to the sample [14]. In a series of elegant studies, Supattapone and colleagues have shown that small, highly structured RNA, vertebrate RNA and, homopolymeric nucleic acids such as poly(A) and poly(dT) can take the place of the cellular conversion factor to enable PrP^{C} to PrP^{Sc} conversion by PMCA (Protein Misfolding Cyclic Amplification) and generation of infectious material [2,15–17]. Their findings revealed that RNA

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molecules are selectively incorporated into nuclease-resistant complexes with PrP during prion formation. Very recently, Wang et al., reported the de novo generation of PrP^{Sc} and infectivity when purified recombinant PrP was subjected to PMCA in the presence of RNA and lipids [4].

The aim of this work was to study the nature of the conversion factors implicated in prion replication by evaluating the ability of different cellular fractions to complement in vitro conversion of purified PrP^{C} and PrP^{Sc} in PMCA assays.

2. Materials and methods

2.1. PrP^{C} purification

PrP^{C} was purified from brain extracts using magnetic beads covered by 3F4 antibody (Covance). Magnetic beads-3F4 complex was prepared by incubating anti-mouse IgG covered Dynabeads M-280 (Invitrogen) with the antibody for 2 h at 4 °C. Glutaraldehyde was used in the process in order to avoid 3F4 release in the PrP^{C} elution process. Then immunoprecipitation was performed by using Syrian hamster (SHa) brain homogenates cleared by a short centrifugation process (2000×*g* for 2 min using an eppendorf 5810 R centrifuge). Samples were incubated overnight at 4 °C with shaking and then three sequential elutions of PrP^{C} in Conversion Buffer (1% Triton X-100 in PBS without Ca^{2+} and Mg^{2+}) containing 2.5 M NaCl. Collected PrP^{C} was pooled and concentrated using YM-10 microcon filters (Millipore) at 16 000×*g* and 4 °C until complete filtration of the sample. A PBS wash was later performed in order to remove salt excess. Finally, concentrated samples were collected by inverting the filter device in a new eppendorf tube and centrifuging for 2 min at 5000×*g*. The recovery yield was checked by Western blotting using 3F4 antibody. The purity of the preparation was checked by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) followed by silver staining and Western blots.

2.2. PrP^{Sc} purification

Brains from symptomatic Chinese hamsters (Cha) infected with the 263 K prion strain were homogenized at 10% in PBS. Samples were sonicated and centrifuged at 5000 rpm for 30 s using an eppendorf 5810 R centrifuge. Supernatants were mixed with 1/3 volume of PBS-Zwittergent 3-14 (Calbiochem), 10% sarkosyl and centrifuged for 2 h at 100 000×*g* in a Biosafe Optima MAX ultracentrifuge (Beckman–Coulter). Pellets were resuspended in PBS containing 10% NaCl and 0.1% Zwittergent 3–14 and sonicated. The resulting material was placed in a sucrose cushion (20% sucrose prepared in PBS plus 10% NaCl and 0.1% Zwittergent 3–14) and centrifuged at 100 000×*g* for 3 h. Pellets were resuspended in PBS containing 0.1% SB3–14 and the preparation was treated with proteinase K (PK) (100 μg/mL) for 2 h at 37 °C. PK treated samples were placed over a sucrose cushion and centrifuged at 100 000×*g* for 1.5 h. Finally, pellets were resuspended in PBS and purity was analyzed by silver staining. All centrifugation processes were performed at 4 °C.

2.3. Preparation of tissue homogenates

Tissues from different organs (liver, kidney, brain, heart and muscle from rabbit) were homogenized at 10% in CB (1% Triton X-100 and 150 mM NaCl in PBS plus Complete Protease Inhibitors Cocktail (Roche)). The same procedure was applied for lower organism tested. Homogenates were cleared by centrifuging the samples at 2000 rpm for 45 s at 4 °C.

2.4. Protein Misfolding Cyclic Amplification (PMCA)

The PMCA procedure has been extensively explained elsewhere [18–20]. Briefly, purified Sha-PrP^{C} complemented with several tissue homogenates or chemicals (Poly-A, bovine serum albumin, heparin or a mixture of fatty acids) were mixed with purified Cha-PrP^{Sc} and submitted to 96 cycles of PMCA. A cocktail of protease inhibitor was added to the sample (except when the effect of the proteases was evaluated) to avoid degradation of the substrate. Samples were loaded onto 0.2-mL PCR tubes. Tubes were positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 3000, Farmingdale, NY) and programmed to perform cycles of 30 min incubation at 37 °C followed by a 20 s pulse of sonication set at potency of 7.5 (75%). Samples were treated with 50 μg/mL of PK for 1 h at 37 °C and amplification of misfolded prions was assessed by Western blotting as explained below.

2.5. Western blotting

PK treated samples were fractionated by SDS–PAGE and transferred to Hybond Nitrocelulose membranes (Amersham) and probed using 3F4 or 6H4 antibodies (Prionics). Reaction was developed using ECL Plus system (Amersham).

2.6. Isolation of lipid raft fractions

Brain homogenates from rabbit were homogenized in CB and mixed with iodixanol (final concentration, 40%). The resulting mixture was deposited in the bottom of an ultracentrifugation tube and two layers of 35% and 30% iodixanol were added in order to generate a discontinuous gradient. Ultracentrifugation was performed at 200 000×*g* for 4 h. The fraction containing lipid rafts migrates at the interface between the top layer and the adjacent denser layer and can be easily collected with a pipette. In order to wash the samples from Triton X-100 and iodixanol, samples were mixed with nine volumes of cold PBS and further ultracentrifuged at 200 000×*g* for 2 h. Supernatant was carefully discarded and pellet containing the lipid raft was resuspended in CB. All procedures were performed at 4 °C. The presence of lipid rafts was analyzed for their PrP^{C} and Fyn content by Western blot with specific antibody: 6H4 for PrP^{C} (Prionics) and anti-Fyn (Santa Cruz).

2.7. Enzymatic treatment of brain homogenates

Brain homogenates were treated with benzonase (1.65 units/mL, 45 min at 37 °C), Heparinase II and Heparinase III (0.5 units/mL, 1 h at 37 °C), Chondroitinase ABC (0.5 units/mL, 1 h at 37 °C), and lipase A (5% v/v, 1 h at 37 °C). In the case of protein degradation by trypsin and PK, samples were incubated with the enzyme at a final concentration of 0.5 mg/mL (1.5 h at 37 °C). Before adding to the PMCA mixture, protease activity was stopped by heating the samples for 15 min at 85 °C. The resulting sample in each case was used to measure conversion activity in vitro. The efficiency of the procedures to eliminate nucleic acids and proteins was checked by electrophoresis.

3. Results

In order to develop a clean biochemical assay to evaluate for conversion factor activity, we worked with PrP proteins from Syrian and Chinese hamsters, two close hamster species in which there is only a small species barrier [21]. Despite the high sequence conservation between these species, only Syrian hamster (Sha) PrP is recognized with the widely used 3F4 antibody. In vitro conversion experiments showed that Sha-PrP^{C} was efficiently converted

by Cha-PrP^{Sc} (Fig. 1A). Indeed, the amplification efficiency was only slightly lower than the one obtained when Cha-PrP^{Sc} was propagated at expenses of Cha-PrP^{C}. The advantage of working with the heterologous mixture is that any signal observed with the 3F4 antibody corresponds to newly generated Sha-PrP^{Sc} and not to the inoculum added to trigger conversion.

To develop the conversion factor complementation assay, we partially purified Sha-PrP^{C} by affinity chromatography, as described in Methods. Analysis by silver staining and Western blot after polyacrilamide gel electrophoresis revealed that PrP^{C} is more than 70% pure (Fig. 1B). Incubation of this purified Sha-PrP^{C} with highly purified Cha-PrP^{Sc} (using a standard procedure that results in > 90% purity) produced no amplification by PMCA (Fig. 1C). Strikingly, a large amount of conversion product was observed when the same mixture was supplemented with 10% Cha normal brain homogenate (Fig. 1C). This result confirms our previous publication [14] and provides an excellent biochemical assay to test for conversion factor activity.

We first studied the species-specificity of the conversion factor by complementing the PMCA assay using purified Sha-PrP^{C} and Cha-PrP^{Sc} with brain homogenates from other mammal species. Brain homogenates from mice, *prnp* knock out mice and rabbits were able to complement prion conversion with similar efficiency as Cha tissue (Fig. 2A). This result indicates that the conversion factor is not species-specific and is likely present in the brain of various mammal species, including rabbit, an animal which is highly resistant to prion infection [22]. However, addition of similar quantities of total extracts from lower organisms, including bacteria, yeast and drosophila was unable to complement prion conversion (Fig. 2B), although a faint band with the yeast extract was detected in some experiments. These results indicate that the conversion factor was not present in these evolutionary lower organisms. Next, we studied whether the conversion factor was present in other major organs and tissues. PMCA complementation using 10% homogenates from liver, kidney, heart and muscles from rabbit produced a similar level of prion replication as the brain tissue

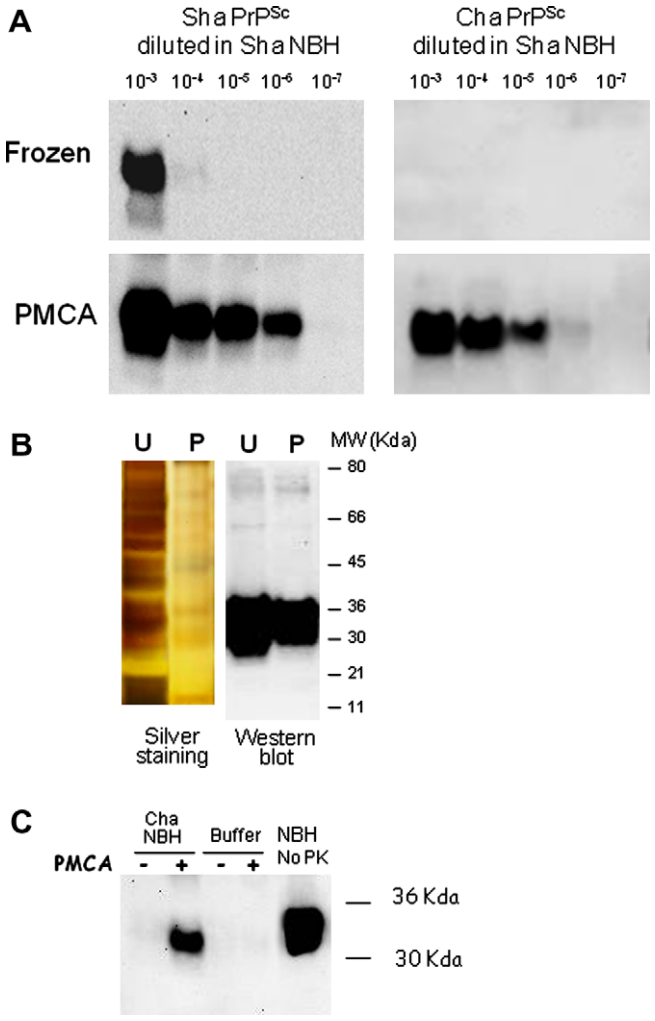

Fig. 1. PMCA complementation assay to study conversion factor activity. (A) Golden Syrian hamster normal brain homogenate (NBH) containing PrP^{C} was incubated with various dilutions of Sha or Cha scrapie sick brain homogenate. Samples were subjected to 96 PMCA cycles and either frozen (mixture without PMCA) or PMCA amplified material were analyzed by Western blot (after PK digestion) with the 3F4 antibody that detects Sha PrP, but not Cha PrP. (B) PrP^{C} was partially purified from Sha normal brain homogenate by affinity chromatography as described in methods. Both unpurified (U) and purified (P) samples were analyzed by silver staining (left panel) or Western blotting (right panel) using the 3F4 antibody. (C) Mixtures of purified Sha-PrP^{C} and Cha-PrP^{Sc} were subjected to PMCA amplification in the presence of conversion buffer or 10% Cha normal brain homogenate (NBH). PrP^{Sc} signal was evaluated after PK treatment by Western blot using 3F4 antibody.

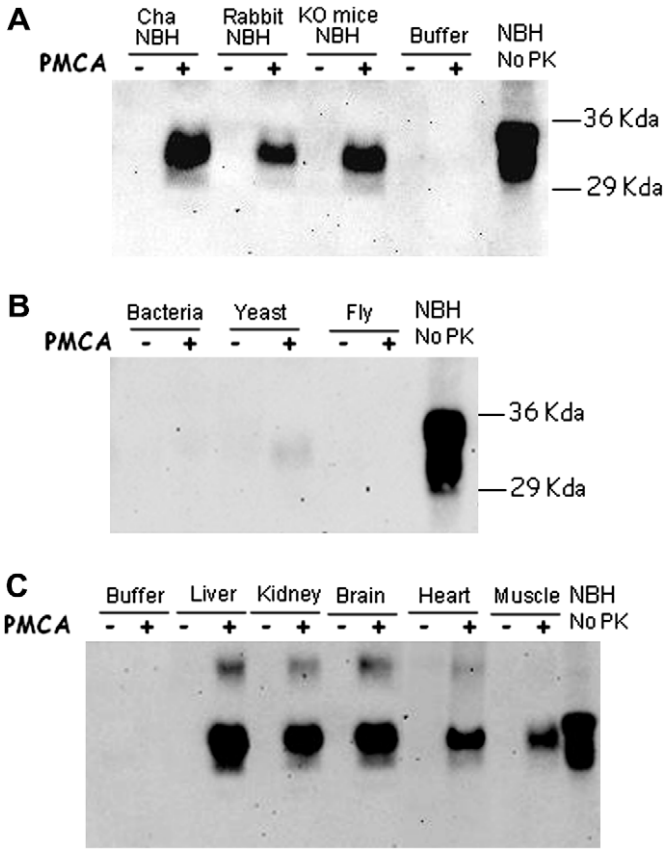

Fig. 2. Conversion factor activity in various tissue homogenates and extracts from distinct species. (A) To study species-specificity of conversion factor, PMCA of purified Sha-PrP^{C} and Cha-PrP^{Sc} was complemented with 10% brain homogenates from Cha, rabbit and PrP knockout mice. Buffer corresponds to the negative control in which no tissue homogenate (only buffer) was added to the sample. Mixtures were subjected to 96 PMCA cycles and formation of Sha-PrP^{Sc} was checked by Western blot after PK digestion. (B) A similar experiment as described in (A) was done using as complement 10% (w/v) extracts of bacteria (*Escherichia coli*), yeast (*Saccharomyces cerevisiae*) or fly (*Drosophila melanogaster*). Again the efficiency of PMCA complementation was checked by Western blot using 3F4 antibody. (C) The presence of conversion factor activity in various major organs (liver, kidney, brain, heart and muscles) was evaluated by using 10% tissue homogenates to complement PMCA using purified proteins. In the blots showed in this figure all samples were treated with PK to remove non-converted PrP^{C}, except in the migration control (NBH, No PK), which correspond to full-length Sha-PrP^{C}.

(Fig. 2C), indicating that the conversion factor activity is not exclusive of the brain, but is present in other major mammalian organs.

PrP is a cell surface protein located in specialized membrane micro-domains known as lipid rafts [23,24]. Since it is likely that prion conversion takes place with PrP^C bound to the membrane either on the cell surface or in endocytic vesicles [25], we reasoned that the conversion factor should be located in lipid rafts. To evaluate this hypothesis we purified lipid rafts from rabbit brain homogenate employing density gradient centrifugation, as described in Section 2. The efficiency of the lipid raft preparation methodology was checked using the lipid raft markers Fyn and PrP^C (Fig. 3A). Then each fraction of the gradient was tested for its ability to complement PMCA conversion of purified Sha-PrP^C triggered by purified Cha-PrP^{Sc}. As seen in Fig. 3B, the two fractions corresponding to the lipid raft preparation (fractions 1 and 2) showed the highest ability to complement prion conversion. This result is not dependent on the presence of PrP^C in the complementing material, since similar results were obtained when lipid rafts were purified from *prnp*−/− mice brain (data not shown). Some other fractions of the gradient showed a much lower, but detectable, conversion factor activity (e.g. fractions 3, 4, 6, 9 and 10). Considering that lipid rafts correspond to a very small fraction of the total brain, our findings suggest that lipid rafts might be an excellent starting point to attempt isolating the conversion factor by biochemical fractionation of this preparation.

To investigate in more detail the chemical nature of the conversion factor we subjected the brain homogenate samples to treatments that destroy different classes of molecules, including nucleic acids (benzonase, a promiscuous endonuclease that cleaves all forms of DNA and RNA), proteins (proteinase K and trypsin), lipids (lipase A) and proteoglycans (heparinase II and III and chondroitinase ABC). PMCA complementation assays using rabbit brain homogenates previously treated to destroy these different molecules did not produce any effect on conversion factor activity (Fig. 4A). These surprising results suggest that different classes of molecules can act as conversion factors and when one is removed the other one plays the role in vitro. Alternatively, it could be speculated that the conversion factor was not degraded by the treatments employed or belong to a class of molecules that was not affected by the enzymes used. No effect on prion replication was also observed when the brain homogenate was heated at either 85 °C or 100 °C. Indeed only a small decrease on PMCA efficiency was observed when the purified Cha-PrP^{Sc} and Sha-PrP^C were complemented with rabbit brain homogenate pre-heated at 100 °C during 30 min (Fig. 4B). These results further support the idea that the conversion factor is not a protein or any structure that can be heat-denatured. Finally to rule out that the conversion factor could be small ions (e.g. metals), amino acids, nucleotides or degradation products from macromolecules (e.g. small peptides), the brain homogenate was filtered using 10 kDa cut off filters. The filtrated material was unable to complement prion conversion (Fig. 4C), suggesting that the conversion factor is not a small chemical compound, such as salts, ions or other chemical molecules.

It has been suggested that the conversion factor might be a protein, RNA (or other polyanionic molecules), lipids or proteoglycans [4,15,16,26,27]. To assess the potential of this type of molecules to complement prion conversion in vitro, we carried out our assay in the presence of high concentrations of poly-A synthetic RNA, bovine serum albumin, heparin and a mixture of various fatty acids (Fig. 5). The results of these studies showed that although these molecules induce a small increase of PMCA amplification compared to the control experiment done in the absence of these molecules, the conversion efficiency is far lower than when complemented with entire brain homogenate. The interpretation of these results is that various classes of molecules may help in a rather unspecific way PrP^C to PrP^{Sc} conversion in vitro. It is possible that in vivo a mixture of various molecules may play the conversion factor role, although our negative results with extracts from lower organisms suggest that specific mammalian factors are necessary for high conversion efficiency.

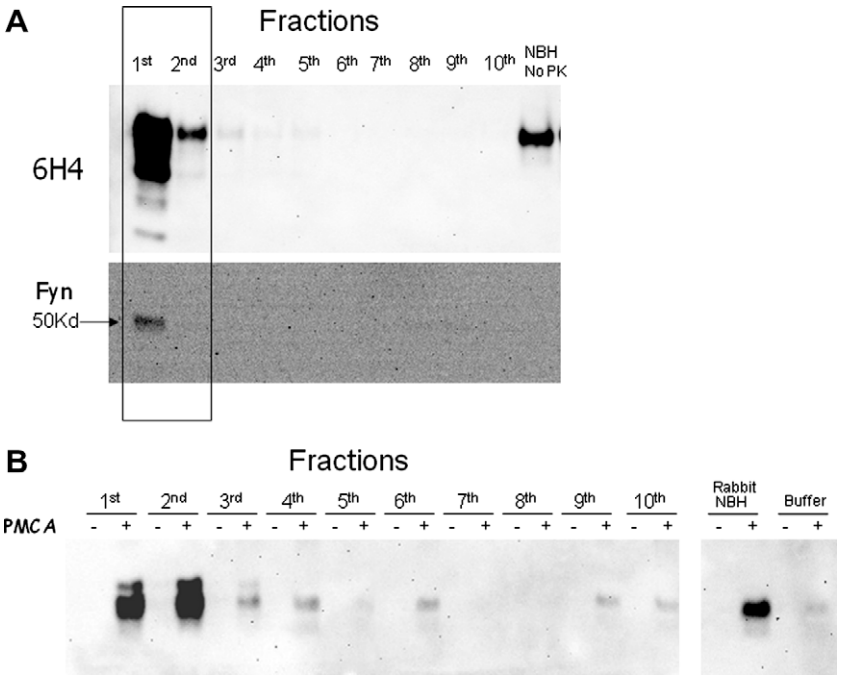

Fig. 3. The conversion factor is located on membrane lipid rafts. (A) Lipid rafts were purified from rabbit brain homogenate by density gradient centrifugation as described in methods. Different fractions from the gradient were loaded into SDS–PAGE and analyzed by Western blot using the anti-PrP antibody 3F4 and the anti-Fyn antibody. As expected the lipid raft markers appears on fractions 1 and 2 of the gradient (highlighted by a box). (B) Equal volume from each of the fractions was used to complement PMCA of purified Sha-PrP^C and Cha-PrP^{Sc}. Samples were subjected to 96 PMCA cycles and formation of new PrP^{Sc} was checked by Western blot using 3F4 antibody. As positive control samples were complemented with Rabbit normal brain homogenate (NBH) and as negative control, only buffer was added to the mixture of purified proteins.

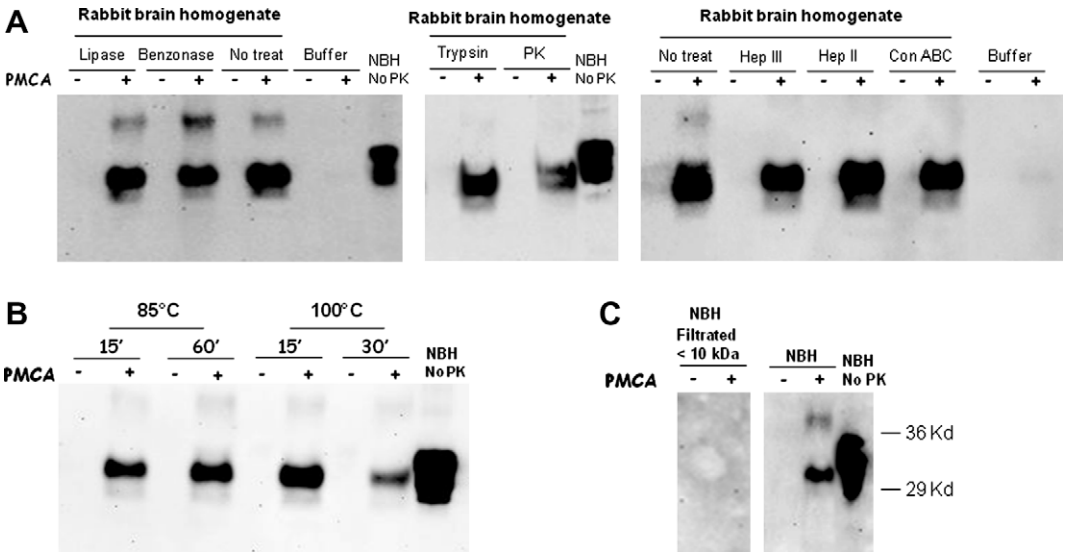

Fig. 4. Depletion of various classes of chemical molecules does not decrease conversion factor activity. (A) Rabbit brain homogenate was subjected to treatments to remove diverse classes of molecules, including lipids (lipase A), nucleic acids (benzonase), proteins (Trypsin and PK) and proteoglycans (Heparinase II and III and chondroitinase ABC). The efficiency of the removal of these molecules was checked by gel electrophoresis. In the case of the proteases, before addition of the treated mixture, the samples were heated at 85 °C to inactivate the proteases. The materials were used to complement PMCA reaction using purified Sha-PrP^{C} and Cha-PrP^{Sc}. Positive controls corresponded to complementation with non-treated samples of 10% rabbit brain homogenate (labeled as "no treat" in the figure). Negative controls corresponded to samples in which buffer was added to the mixture. All samples were treated with PK before Western blotting, except the migration control (NBH). (B) Samples of 10% rabbit brain homogenate were heated at either 85 °C or 100 °C for the indicated times. After cooling, samples were used to complement PMCA reaction of purified proteins. (C) Samples of 10% rabbit brain homogenate were filtered using a 10 kDa cut off filter. Filtrated material was used to complement PMCA of purified proteins. Samples were subjected to 96 PMCA cycles and formation of new PrP^{Sc} was checked by Western blot using 3F4 antibody. As positive control samples were complemented with Rabbit normal brain homogenate (NBH).

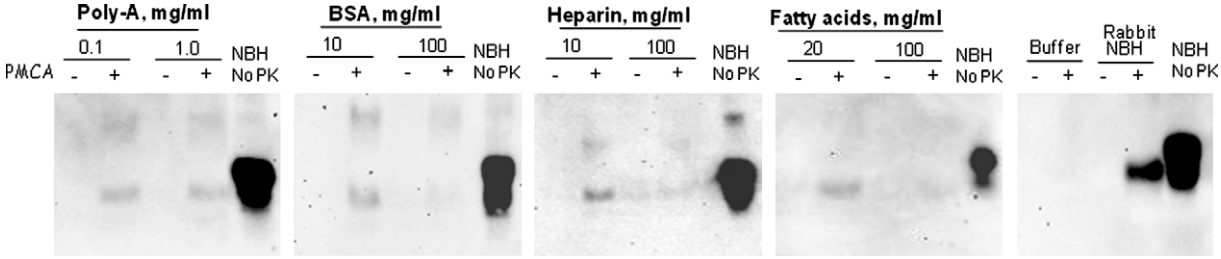

Fig. 5. Diverse classes of molecules can enhance prion conversion in vitro. The PMCA reaction of purified Sha-PrP^{C} and Cha-PrP^{Sc} was complemented by adding the indicated concentrations of synthetic poly-A RNA, bovine serum albumin (BSA), heparin or a mixture of fatty acids (Sigma) prepared in water (including methyl octanoate, nonanoate, decanoate, undecanoate, laurate, tridecanoate, myristate, pendecanoate, palmitate and heptadecanoate). Samples were subjected to 96 PMCA cycles and generation of Sha-PrP^{Sc} was evaluated by Western blots using 3F4 antibody. As before, the positive control corresponded to samples complemented with rabbit normal brain homogenate (NBH) and as negative control, only buffer was added to the mixture of purified proteins. All samples were treated with PK to remove non-converted PrP^{C}, except in the migration control (NBH, No PK), which correspond to full-length Sha-PrP^{C}.

4. Discussion

Transmission of TSEs is dependent on the auto-catalytic formation of PrP^{Sc} at expenses of the host PrP^{C}. In spite of substantial research on the nature of the prion infectious agent, the molecular and structural bases of prion replication are still largely unknown. One of the most important issues concerning the mechanism of prion replication is to determine the identity of the cellular co-factors involved in the process. Despite that the involvement of cellular co-factors in prion conversion is suspected since more than 15 years ago, the identity or even the chemical nature of these factors is completely unclear [10]. It is also unknown whether the co-factor is one specific molecule or several rather non-specific molecules that accidently participate in prion conversion.

The results described in this article provide further evidence for the existence of cellular factors involved in prion conversion. These conversion factors appear to be present in all major organs of mammals, but do not exist in lower organisms, including yeast, bacteria or fly. The conversion factor activity is enriched in the lipid raft fraction of cellular membranes and do not appear to be responsible for species barrier. Surprisingly, treatments that destroy all major type of chemical molecules do not affect conversion activity, neither does heating at high temperatures. One interpretation of these results is that various types of molecules can act as conversion factors in vitro, catalyzing PrP^{C} to PrP^{Sc} formation in the presence of PrP^{Sc} template. Indeed, our results indicate that synthetic poly-A RNA, fatty acids, proteins (BSA) and heparin can partially replace the conversion factor activity in our in vitro assay, confirming previously published results [4,15,16,26,27]. However, these molecules have far lower efficiency than the endogenous conversion factors present in mammalian tissues. Overall our findings indicate that although prion conversion can be slightly enhanced with various classes of chemical molecules, high efficiency prion replication requires factors that are specifically located in the lipid rafts of mammalian cells.

Elucidating the nature of the prion conversion factors would not only be important to understand the mechanism of prion replication, but would also provide novel targets for therapeutic intervention. Indeed, if the interaction of PrP with these additional co-factors is essential for PrP^{Sc} formation, compounds disrupting this interaction would likely prevent prion disease.

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