Prion-associated Increases in Src-family Kinases*

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Randal R. Nixon‡
From the Department of Pathology, Oregon Health & Sciences University, Portland, Oregon 97201

The prion diseases result from the generation and propagation of an abnormal conformer of the prion protein. It is unclear how this molecular event disrupts neuronal function and viability. Current evidence argues it is not due to loss of normal prion protein activity or direct toxic effects of the abnormal conformer. Both the normal and abnormal prion proteins are glycosylphosphatidylinositol-linked membrane proteins. Conversion to the abnormal isoform results in the formation and accumulation of prion protein aggregates. Because aggregation of glycosylphosphatidylinositol-linked proteins activates Src-family kinases, the activation status and levels of the Src-family kinases in prion disease were investigated. Elevations of Src-family kinases were found in a cell culture model and two separate animal models of prion disease. The elevations in Src kinases preceded the onset of symptoms and occurred concurrently with the appearance of detergent-insoluble prion protein. In addition, the total level of kinases phosphorylated at tyrosine residues associated with activation was increased. Similar alterations were not present in brain homogenates from presymptomatic animals early in the disease course, prion protein-ablated animals, or end-stage Tg2576 mice overexpressing mutant amyloid precursor protein. Identification of similar elevations in cell culture and animal model systems suggests the elevations are a specific response to the presence of the disease-associated conformer. Abnormal regulation of these signal transduction cascades may be a key element in the cellular pathology of the prion diseases.

The past decades have seen the identification and characterization of the controversial etiologic agent of the prion diseases; however, the mechanism underlying these diseases remains unknown. The current report presents the first evidence that prions are associated with increases in the levels of total and activated Src-family kinases.

The transmissible spongiform encephalopathies include Creutzfeldt-Jakob disease, new variant Creutzfeldt-Jakob disease, Gerstmann-Staussler-Scheinker disease, and fatal familial insomnia of humans, bovine spongiform encephalopathy, Gerstmann-Staussler-Scheinker disease, and fatal familial insomnia of humans, bovine spongiform encephalopathy, and new variant Creutzfeldt-Jakob disease, new variant Creutzfeldt-Jakob disease, new variant Creutzfeldt-Jakob disease, new variant Creutzfeldt-Jakob disease, and new variant Creutzfeldt-Jakob disease. These diseases are characterized by neuronal loss, astroglialis, vacuolar degeneration, and variable degrees of amyloid plaque formation (1). Ultrastructural examination has shown vacuolar degeneration results from synaptic deterioration, cytoskeletal disruption, membrane dissolution, and membrane fusion (2). Quantitative studies suggest a progression from synaptic loss to neuritic degeneration and neuronal death (3). A wealth of data indicate these diseases are due to prions (4). Prions are self-propagating proteinaceous infectious particles composed of an abnormal conformer of a normally expressed protein known as the prion protein (PrP).1 PrP typically exists as a normal cellular conformer (PrP♂), whereas a β-sheet enriched, disease-associated form is referred to as PrPscrapie (PrPSc). Recently, Legname et al. (5) generated infectious prions using recombinant prion protein, adding further support to this hypothesis. The normal function of PrP♂ is unknown; it may participate in synaptic structure (6), neurite formation (7), copper metabolism (8), or possibly signal transduction (9). PrPSc is produced by the conformational conversion of pre-existing PrP♂ to the abnormal isoform. PrPSc uniformly aggregates, is insoluble in detergents, and tends to be resistant to proteolytic degradation (4).

Formation of PrPSc is the initiating step in prion disease pathogenesis. Experimental inoculation of animals with prions results in the appearance of PrPSc before the development of symptoms and the colocalization of PrPSc with the pathologic lesions (10, 11). It has been suggested that loss of normal PrP♂ function or, alternatively, direct toxic effects of PrPSc are responsible for the development of disease. Loss of PrP♂ seems unlikely to be the principal biochemical lesion because prion protein-ablated mice (PrPo/o) fail to develop scrapie (12–14), and furthermore, one recent publication suggests total PrP♂ levels may increase during the disease process (15). The toxic impact of PrPSc also remains unclear. Conversion to PrPSc has been reported to alter PrP metal binding and antioxidant activity (16), lysosomal function (17), and membrane fluidity (18). In addition, PrPSc and a fragment of PrP spanning region 106–126 have been reported to be cytotoxic to cells in culture (19, 20). Nonetheless, inoculation of PrP♂/♂ mice with PrPSc results in neither the clinical nor pathologic changes of scrapie, whereas the neuropathologic changes of prion disease and PrPSc formation were restricted to regions of PrP-expressing tissues in brain transplantation studies (12, 21, 22). Together, these findings suggest that in situ conversion of endogenous PrP♂ to PrPSc per se may be essential to the pathologic process.

A number of features suggest an alternative mechanism linking PrPSc formation to the development of disease. We have shown previously (23) that PrP♂ and PrPSc are glycosylphospho-

1 The abbreviations used are: PrP, prion protein; PrP♂, PrP♂/♂, GPI, glycosylphosphatidylinositol; SFK, Src-family kinase; RML, Rocky Mountain Laboratory; ANOVA, analysis of variance; Aβ, amyloid β-peptide; PrP♂, PrP♂/♂, prion protein ablated; APP, amyloid precursor protein.
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RESULTS

Src-family tyrosine kinases share a common structural motif characterized by multiple conserved functional domains and a unique N-terminal region (26). In order to determine if phosphorylation or activation levels varied in scrapie-sick samples as compared with wild-type and PrPo/o controls, mice at 24 months of age were used. Levels of SRC-2 immunoreactive material in scrapie-sick mice were compared with wild-type and PrPo/o controls, utilizing an antibody against the conserved C terminus (SRC-2). These studies revealed a band that was clearly increased in the scrapie-sick mice as compared with the wild-type and PrPo/o mice (Fig. 1A). The apparent molecular mass of the immunoreactive band was ~60 kDa, consistent with that expected for the SFKs. Wild-type and PrPo/o control mice had similar levels of SFKs. Den-sitometric analysis of four separate sets of scrape-sick, wild-type, and PrPo/o mice revealed a 1.8-fold increase in SFKs in scrapie-sick mice as compared with wild-type controls. ANOVA with Bonferroni’s multiple comparison post-test confirmed a statistically significant increase in the scrape-sick Tg2866/MoPrP P101LPrP o/o mice (Fig. 1B; p = 0.0038), with no difference between wild-type controls and PrPo/o mice. Levels of SFKs were also examined in a scrape-sick RML-inoculated mouse and an asymptomatic un-inoculated control (Fig. 1C). Densitometric analysis (data not shown) revealed a 1.6-fold increase. Because only a single pair of brains using the RML model was available, statistical comparison could not be performed. Nonetheless, these studies revealed a remarkable similarity between the scrape-sick RML mouse and the transgenic animals.

During the course of the prion diseases, the brain undergoes a number of reactive and degenerative changes such as astrogliosis, vacuolation, and neuronal loss. In addition, the PrP 106–126 peptide has been shown to activate the SFKs Lyn and Syk in microglial cells (36). In order to clarify whether the observed SFK elevations in brain are due primarily to PrPSc rather than to a secondary nonspecific reactive process, levels
of SFKs were determined in N2a and ScN2a cells. Western blotting with the SRC-2 antibody revealed a marked increase in SFK immunoreactivity in ScN2a cells as compared with N2a cells (Fig. 2 A). Densitometric analysis confirmed a very highly statistically significant increase of SRC-2 immunoreactivity in the ScN2a cells (Fig. 2 B; unpaired t test, p < 0.0007). This indicates that the elevation of SFKs in the presence of PrPSc is not dependent on the presence of a non-neuronal cell population. Identification of elevated steady-state SFK levels in the scrapie-sick transgenic animals, RML-inoculated animals, and ScN2a cells strongly argues the increases are related to the presence of PrPSc. Elevations were not identified in 65-day-old presymptomatic Tg2866(MoPrP-P101L)PrPo/o mice (see Fig. 8A for the disease time course) or homogenates from end-stage Tg2576 mice overexpressing doubly mutated human App (Fig. 3 A) (32). Histologic examination demonstrated abundant cortical and hippocampal plaques (Fig. 3B), indicating the lack of SFK increase was not due to the absence of aggregated amyloid β-peptide (Aβ).

In the above-mentioned studies, equal amounts of sample were compared as determined by protein determinations and Ponceau S staining of the nitrocellulose membrane before immunoblotting. However, to further ensure that the observed differences in SFK levels were not the result of irregularities in sample preparation or loading, Western blotting for the transferrin receptor was performed. The transferrin receptor is a non-raft membrane protein frequently used as a control in studies of SFK activation due to aggregation of GPI-linked proteins. As shown in Fig. 4, equivalent levels of transferrin receptor were present in scrapie-sick, wild-type, and PrPo/o mice. Similarly, immunoblotting of cell lysates revealed that the ScN2a cells had equal or slightly lower levels of transferrin receptor as compared with N2a cells (Fig. 4). Thus, the elevations in SFK immunoreactivity seen with PrPSc infection were not due to disparities in sample loading and were not the result of a generalized increase in membrane protein content.

Confirmation that the elevated SRC-2 immunoreactive material in the scrapie-sick mice was indeed a SFK was obtained using a combination of immunoabsorption and Western blotting. Brain homogenates were first immunoabsorbed with the Src-specific monoclonal antibody pp60Src (34). Absorbed material was subsequently eluted and analyzed by Western blotting with either SRC-2 antibody as described above or N-16 (an antibody specific for the unique domain of Src). These studies revealed a solitary 60-kDa band in the scrapie-sick samples with both SRC-2 and N-16 (Fig. 5); no detectable immunoreactive material was found in the wild-type control samples. Absorbed material was subsequently eluted and analyzed by Western blotting with either SRC-2 antibody as described above or N-16 (an antibody specific for the unique domain of Src). These studies revealed a solitary 60-kDa band in the scrapie-sick samples with both SRC-2 and N-16 (Fig. 5); no detectable immunoreactive material was found in the wild-type control samples. Analysis of the immunoaosorbed brain homogenate supernatants found no residual Src (data not shown). As such, the lack of immunoprecipitable Src from the control homogenate is most probably due to nonspecific adsorptive losses. These results...
confirm the SRC-2 immunoreactive band identified in Fig. 1A contains Src and, furthermore, specifically identify Src as one of the SFKs elevated in scrapie-sick mice. In addition to Src, the Src-family kinases Fyn, Yes, and Lck are present in brain (26). Because the SRC-2 antibody recognizes multiple SFK members, studies were performed to determine the levels of these SFKs in scrapie infection. A composite image of typical results from Western blotting of mouse brain homogenates probed with antibodies specific for the individual SFKs is shown in Fig. 6A. Comparison of scrapie-sick and wild-type samples suggests elevations in all four SFKs, although there was considerable variability in the extent of increase. Visual inspection suggests the largest increases are in Src and Fyn, with lesser increases in Yes and Lck. Comparison of ScN2a and N2a cell lysates also shows elevations in multiple SFKs, but with a slightly different pattern (Fig. 6B). Src and Lck appear increased; however, unlike the transgenic animals, Fyn demonstrates a much more modest increase. Yes is marginally increased. Densitometric analysis of blots from multiple individual scrapie-sick and wild-type animals or homogenates of N2a and ScN2a cells is shown in Fig. 6, C and D, respectively. In scrapie-sick animals, Src is increased ~3-fold, whereas Yes, Fyn, and Lck are increased ~2-fold. Statistical analysis (ANOVA with Bonferroni post-test correction, p < 0.0001) confirms a highly significant elevation in Src (p < 0.001); the elevations in the remaining SFKs did not reach statistical significance. In ScN2a cells, Src and Lck are increased 2-fold and 3-fold, respectively, whereas Yes and Fyn are elevated by only ~30–50%. Statistical analysis (ANOVA with Bonferroni post-test correction, p < 0.0001) again found a highly significant difference in SFK levels with statistically significant elevations in Src (p < 0.05) and Lck (p < 0.001). These studies demonstrate increased steady-state expression of multiple Src-family kinases in both the animal and cell culture models of prion disease.

SFK enzymatic activity is regulated by phosphorylation of key tyrosine residues. Specifically, phosphorylation of Src tyrosine 416 or its equivalent in the other SFKs is associated with increased tyrosine kinase activity (26). The phosphotyrosine status of the SFKs in PrPSc-infected animals and cells was therefore determined using an antibody specific for this modification. A prominent band of ~60 kDa was identified in the scrapie-sick mice with little immunoreactive material in the wild-type or PrP0/0 control animals (Fig. 7A). Densitometric analysis found a 4.7-fold increase (Fig. 7B; ANOVA with Bonferroni post-test correction, p < 0.0001) in the scrapie-sick mice with little immunoreactive material in the wild-type or PrP0/0 control animals (Fig. 7A). Densitometric analysis found a 4.7-fold increase (Fig. 7B; ANOVA with Bonferroni post-test correction, p < 0.0001) again found a highly significant difference in SFK levels with statistically significant elevations in Src (p < 0.05) and Lck (p < 0.001). These studies demonstrate increased steady-state expression of multiple Src-family kinases in both the animal and cell culture models of prion disease.

The Tg2866(MoPrP P101L)/PrPo/o mice used in many of these studies offer the advantage of developing disease in a highly predictable manner. In our hands, these mice develop disease at 127.7 ± 4.5 days (Fig. 8A) as compared with the 145 days in the original report by Telling et al. (31) Given this degree of precision, transgenic mouse littersmates were sacrificed at 100, 118, and 126 days; the early time points corre-
spond to ~80% and 90% of the time through the disease time course. At each time point, the levels of total, detergent-soluble, and detergent-insoluble PrP, as well as total SFKs, were determined in brain homogenates and normalized to those from a wild-type control of 126 days of age. As shown in Fig. 8B, levels of total PrP, soluble PrP, and SFKs were the same in the 100-day-old transgenic animal and wild-type control. Trace amounts of detergent-insoluble PrP were detected at this time. By 118 days of age, levels of SFKs and detergent-insoluble PrP were clearly increased, although these animals were completely asymptomatic. Levels of soluble PrP remained equivalent to the control. With symptom onset at 126 days, the level of SFKs was increased ~2-fold (similar to the increase found by densitometric analysis for transgenic and inoculated animals above). Detergent-insoluble PrP continued to accumulate, whereas detergent-soluble PrP appeared to decrease.

**DISCUSSION**

Whereas the results presented in this study do not establish a direct mechanism, the following findings strongly support the hypothesis that the increase in SFKs is due to the presence of PrP\textsuperscript{Sc}. First, the absence of detectable SFK abnormalities in the PrP\textsuperscript{ScO} mice shows the increases are not due to the loss of PrP\textsuperscript{C}. Second, adult presymptomatic Tg2866/MoPrP P101L/PrP\textsuperscript{ScO} mice had no elevation in SFKs, indicating the elevations were not due to the 8-fold overexpression of the PrP transgene (31). Third, no increase in SFK levels was detected in response to amyloid plaques produced by APP transgenic animals overexpressing mutant human amyloid precursor protein by 5-fold (32). Fourth, the increase in SFKs and detergent-insoluble PrP occurred concurrently and preceded symptom onset. Fifth, similar increases in SFKs were detected in the well-established ScN2a cell culture system as well as two discrete animal models of prion disease, Tg2866/MoPrP P101L/PrP\textsuperscript{ScO} transgenic mice and RML-inoculated mice. In addition, the cell culture studies demonstrate that neuronal cells infected with PrP\textsuperscript{Sc} are sufficient to produce the SFK elevations but do not exclude contributions by other cell types. Additional studies will be needed to delineate the contributions of the individual cellular components to the overall elevations in SFKs.

The precise mechanism linking PrP\textsuperscript{Sc} formation to the activation and accumulation of the SFKs is unknown; however, a number of features suggest it is due to the raft-associated form of the abnormal protein. Specifically, we have demonstrated previously (23) that PrP\textsuperscript{Sc} is associated with membrane rafts. Recently, PrP\textsuperscript{C} and Src have been shown to colocalize to the same raft domain (29). In addition, because PrP\textsuperscript{Sc} has a markedly increased half-life (30), it is conceivable that formation of PrP\textsuperscript{Sc} aggregates leads not only to SFK activation but also to a decrease in the degradation of raft-associated proteins. In this
regard, it is interesting that CD9 (37), the insulin receptor (38), and the bradykinin receptor (18) are also increased in the prion diseases, and all have been reported in rafts (reviewed in Ref. 24). Of these, messenger RNA levels have only been investigated for the insulin receptor; no increase was identified (38), consistent with an alteration in protein turnover. The observation in the current study that the transferrin receptor was not increased is also consistent with this model because it is not a raft-associated protein. In addition, it is interesting to note that inoculation of mice with anti-PrP antibodies induces neuronal death (39), consistent with a model of PrPSc aggregation-induced pathology.

The mechanism of PrPSc aggregation is unknown. As stated earlier, peptides spanning residues 106–126 of PrP have been shown to activate SFKs in microglial cells. In addition, Gu et al. (40) reported that this peptide could induce PrP aggregation initiating cell death in vitro. NMR studies of PrPSc found that this domain existed in an unstructured form (41); however, more recent crystallographic data suggest that this region becomes structured and packed in PrPSc dimers with evidence for three-dimensional domain swapping and formation of intermolecular disulfide bonds (42) in the dimers. It is therefore conceivable that this domain is involved in mediating aggregation of PrPSc. Regardless of the mechanism of aggregation, it is likely that localization of PrPSc to the membrane rafts is via their GPI anchor.

Rafts are enriched in cholesterol, sphingolipids, and a variety of receptors and signaling molecules. Although the existence of rafts remains controversial, these domains have been implicated in a number of biologic events, including membrane fusion (24). Perhaps PrPSc alters normal raft function, resulting in unregulated membrane fusion as seen in the prion diseases by electron microscopy (2). Current evidence indicates that rafts are a mixed population of microdomains, each with a discrete composition; thus, not all rafts may contain PrPSc, and not all PrPSc-containing rafts may have the same complement of kinases. Such a distribution could explain the mixed pattern of elevations shown in Fig. 6. The impact of PrPSc on raft composition and protein turnover is currently being investigated in our laboratory. Significant alterations in raft function and/or homeostasis may indicate a new class of disorders, i.e. “raftopathies.”

Src-family kinases are ubiquitously expressed tyrosine kinases that regulate and may be regulated by numerous key cellular pathways (reviewed in Ref. 26). A partial list of functions modulated by these kinases includes cell survival, apo-
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For example, we hypothesize that PrPSc accumulates in rafts in underlying biochemical mechanisms involved in SFK activation. APP transgenic mice? This may reflect differences in the un- Parkinson’s and Lewy body disease. If the SFKs are involved formation. For example, abnormalities in mass action effects, whereas the elevated levels of mutation in the prion gene or wild-type animals inoculated with RML prions. Given the inherent abnormalities of signal- ing pathways in cultured tumor cells, in addition to the mixed cellular composition of the brain, it is not surprising that minor differences in the pattern of SFK accumulation in the in vivo and in vitro systems exist. Nonetheless, in both systems, the presence of PrPSc is associated with a significant increase in Src. This suggests that the Scn2a cell line not only accurately converts PrPc to PrPSc but also exhibits portions of the patho- logic cascade, even though the cells fail to develop the cyto- pathic features of disease.

In summary, the alterations in SFKs reported in the current study, the resistance of PrPc animals to development of disease, and the known biophysical properties of PrPSc are consistent with a model linking PrPSc formation with dysregulation of the Src-family kinases. PrP and Src are localized to membrane rafts. Conversion of PrPC to PrPSc generates a con- former prone to unregulated aggregation, and because PrPSc has a markedly prolonged half-life, it is likely that PrPSc aggregates exist for an extended period of time, resulting in aberrant SFK regulation and turnover. Such a disturbance could clearly have severe physiologic consequences and result in many of the pathologic lesions associated with the prion diseases.

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