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Effects of FIAsh/Tetracysteine (TC) tag on PrP proteolysis and PrPres formation by TC-scanning

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Abstract

The FIAsh/tetracysteine (FIAsh/TC) tag is a powerful tool for fluorescent labeling of proteins. However, even small tags such as FIAsh/TC could alter the behavior of the tagged proteins, especially if the insertion occurs at internal sites. Defining the influence of FIAsh/TC on nearby protein-protein interactions might aid in selecting appropriate positions for internal TC insertions and allow the exploitation of serial FIAsh/TC insertions (TC-scanning) as a probe to characterize sites of protein-protein interaction. To explore this application in the context of substrate-protease interactions, we analyzed the effect of FIAsh/TC insertions on proteolysis of cellular prion protein (PrP^{Sc}) in *in vitro* reactions and generation of the C1 metabolic fragment of PrP^{Sc} in live neuroblastoma cells. The influence of FIAsh/TC insertion was evaluated by TC-scanning across the cleavage sites of each protease. The results showed that FIAsh/TC inhibited protease cleavage only within limited ranges of the cleavage sites that varied from about 1 to 6 residues-wide depending on the protease, providing an estimate of the PrP residues interacting with each protease. TC-scanning was also used to probe a different type of protein-protein interaction, the conformational conversion of FIAsh-PrP^{Sc} to the prion disease-associated isoform, PrPres. PrP constructs with FIAsh/TC insertions at residues 90–96 but not 97–101 were converted to FIAsh-PrPres, identifying a boundary separating loosely versus compactly folded regions of PrPres. Our observations demonstrate that TC-scanning with the FIAsh/TC tag can be a versatile method for probing protein-protein interactions and folding processes.

Keywords

biarsenical; fluorescent probes; prion; protein interactions; protein structure

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Introduction

Prion protein (PrP) is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein that is expressed in various organs and is especially abundant in the nervous system. Although its physiological functions are still unclear, it is well-known that the amino acid sequences and three-dimensional structures of PrP are highly conserved among a wide range of mammalian species. PrP is the primary component of prions, which cause transmissible spongiform encephalopathies (TSE) including bovine spongiform encephalopathy (BSE) in cattle, Creutzfeldt-Jakob disease (CJD) in humans and scrapie in sheep. PrP normally exists in a conformation termed PrP^{sen} (or PrP^C) that is highly alpha-helical, sensitive to protease digestion, and soluble in non-denaturing detergents. During TSE infection, PrP^{sen} molecules undergo a conformational conversion and assemble into tightly-associated, beta-sheet rich, protease-resistant, detergent-insoluble aggregates termed PrP^{res}. These processes ultimately lead to neurodegeneration by mechanisms that remain unclear but may in part involve corruption of the normal function of PrP^{sen} via perturbation of its interactions with other molecules [1;2].

The search for improved insight into PrP^{sen} function under both normal and TSE disease-associated conditions has focused attention on the identification of molecules that bind to PrP. There is an ever growing list of proteins reported to interact with PrP [3–16]. One important class of such proteins includes cellular proteases responsible for processing PrP^{sen} and PrP^{res} [17–19]. PrP^{sen} undergoes a well-known cleavage called “ α -cleavage”, which occurs at residues 111/112 of human PrP^{sen} (110/111 in mouse PrP^{sen}) and generates two fragments, C1 and N1 [20–23]. The GPI-anchored, C-terminal C1 fragment remains cell-associated while the N-terminal fragment N1 is secreted [21;23]. There is some controversy over the specific protease(s) mediating N1/C1 cleavage [24–26] and the subcellular compartment where processing occurs [22;27;28], but members of the ADAM(a disintegrin and metalloprotease) family of matrix metalloproteases (ADAM8, ADAM10, and ADAM17) seem to account for part of this activity [25;29–34]. C1 cleavage is tolerant of a remarkable array of mutations (largely point mutations) near the cleavage site [35–39] with only more substantial modifications such as large deletions/sequence replacements inhibiting cleavage [35;39–43]. This has led some to conclude that cleavage depends more on the size rather than sequence of the cleavage region [39]. Sequence changes at the C1 cleavage site can enhance C1 formation induced by external stimuli or membrane environment in a species-specific manner [38]. There are conflicting reports regarding the role of ADAM10 in a C-terminal cleavage of PrP^{sen} near the GPI anchor that releases the ectodomain [24;25;44]. A separate cysteine protease-mediated cleavage very near the C-terminus generates a small cell-associated fragment called C3 [45]. A third cleavage process, termed “ β -cleavage”, involves cleavage around residue 96 and is mediated by reactive oxygen species (ROS) [37;46;47]. The products of β -cleavage are termed N2 and C2. In an unfortunate choice of terms, a “C2 cleavage” of PrP^{res} has also been described, but this species seems to largely correspond to truncation around residue 90 by various cellular proteases depending on the cell/tissue type in question [20;45;48–51]. TSE infection increases “total” C2 levels both in vivo and in cell culture models of infection in a manner that is inversely proportional to C1 levels [20;50]. In one cell model of TSE infection calpain-

dependent “C2 cleavage” of PrPres assists PrPres propagation, suggesting this cleavage may contribute to TSE pathogenesis [50].

Evidence has accumulated to suggest that the proteolytic processing of PrPsen is not merely a degradative process per se but rather is associated with important biological functions. Similar to other membrane-bound proteins such as Alzheimer’s-associated β -amyloid precursor protein (APP), proteolytic processing of PrP results in the generation of biologically active fragments that can inhibit A β 1–42 peptide aggregation and toxicity [26;52;53] and modulate cellular responses to various insults such as staurosporine or oxidative stress [37;53–56]. Moreover, since the C1 fragment does not convert to PrPres and is associated with enhanced resistance to TSE infection by acting as a dominant negative inhibitor of PrPres formation, α -cleavage has been suggested to have a protective anti-TSE function [57;58]. Collectively, the above observations highlight the need to develop tools to probe intermolecular interactions between PrP and other molecules, which would have broad applications across many fields.

A popular technique to probe intermolecular interactions is scanning mutagenesis, which involves creating a series of expression constructs with single amino acid insertions (e.g. alanine) at each position in the region of interest. Although it has been used successfully in many systems, some intermolecular interactions mediated by contacts spanning multiple residues may escape detection due to the relatively small size of the “probe”. More severe modifications such as sequence deletions provide information, however it can be difficult to identify what changes should be made if there are no clues (e.g. homology to known motifs). Thus, it would be beneficial to have an intermediate-scale probe large enough to perturb intermolecular interactions but sufficiently compact as to not impact overall folding of the target protein.

Biarsenical dye/tetracysteine (TC) tags are candidates for an intermediate scale probe. Biarsenical dyes (e.g. FIAsh) specifically bind to the tetracysteine motif (CCPGCC), thereby generating a fluorescent probe that affords biochemical and microscopic means of detecting the tagged protein. Importantly, biarsenical/TC tags are amenable to internal tagging of proteins provided the tag can be placed in appropriate sites that minimally affect the structure or function of the tagged protein. Although fusion to the N- or C-terminal end of proteins is advantageous in minimizing adverse effects of tags, as with fusion to a fluorescent protein, tags located at the termini can be cleaved off in cases where the proteins undergo proteolysis at the early phases of their life cycle. Internal tagging can circumvent this problem and enable tracking of proteolytic products of interest.

Despite the potential benefits, insertion of an internal tag into a protein is often difficult because of the above mentioned influences on structures or functions of the tagged protein. Effects on the structures could be minimized by placing a TC motif in unstructured regions of the protein or in a bipartite fashion over regions originally close to each other [59] if detailed structural information is available. However, even if the structures are maintained, the tag might interfere with protein-protein interactions and consequently the physiological function if it were near the interaction interface. Thus, internally-tagged proteins must be carefully designed based on those considerations. Experimental data about the effective

“bulkiness” of the biarsenical/TC tag might facilitate the design of studies using an internal TC-tag. If the tag does not interfere with physiological protein-protein interactions when located within or adjacent to the interaction interface, the main concern would be effects on the structure of the protein. On the other hand, if the tag interferes with the interaction within a certain range of the interface, it should be located away from the interface accordingly to avoid the interference. Although the small size of the biarsenical/TC tag is obvious, such data have not been reported.

In the present study, we report a systematic analysis of the effects of the FIAsh/TC tag on its surroundings by digesting “TC-scanning” series of partially-purified TC-PrPsen in vitro with trypsin or chymotrypsin. We then applied the TC-scanning method with FIAsh/TC to probe the regions of PrPsen involved in the C1 cleavage of PrPsen. We also utilized the bulkiness of FIAsh/TC as a novel probe for assessing the structure of PrPres. The data provide practical insight into the selection of candidate sites for internal TC tagging even without detailed structural information of a protein. Our studies also suggest that the FIAsh/TC tag might be used as a compact probe to map regions of interaction between proteins.

Results

Creating TC-scanning series

“TC-scanning” refers to the insertion of TC tags in each position over a given region of interest in a protein. As illustrated in Figure 1A, inhibition of the substrate-protease interaction by the biarsenical/TC tag is reported by the appearance of an uncleaved fragment (star) and decreased levels of the proteolytic fragment in question. The number of consecutive TC-PrPs that show inhibition of cleavage reflects a range within which the tag inhibits the substrate-protease interaction (inhibition range) and would correlate with the bulkiness of the tag.

For the regions to be scanned, we selected those that undergo physiological proteolysis by endogenous cellular proteases because such regions are usually unstructured and efficiently cleaved by proteases [60]. This would rule out the possibility of inhibition by regional structural features like alpha-helix or beta-sheet. Moreover, TC insertions in unstructured regions would be expected to minimally impact the overall folding of PrP, evidence for which has been obtained by other laboratories [61;62]. As reported previously, in neuroblastoma cells C-terminally-tagged TC-PrPsen (e.g. 230TC) undergoes proteolytic processing to produce C-terminal fragments called C1 and C3 [45] (Figure 1B and 1C). Another C-terminal cleavage process mediated by ADAM10 between Gly228/Arg229 would be predicted to generate a very short C-terminal GPI-anchored fragment similar to C3 [25]. The C1 cleavage sites of human PrPsen are His111 and Met112 [20], which correspond to His110 and Val111 of mouse PrP. The apparent molecular weight (MW) of the C3 fragment suggested this cleavage site is located near the GPI-attachment site, but the exact residue where cleavage occurs is unknown. An additional fragment of slightly higher apparent MW than C1, termed C2, was also observed. As shown below, this fragment seemed to correspond to a fragment that results from β -cleavage near residue 96 [37;46;47]. Therefore, to study the interactions between PrPsen and proteases that cleave near the C1

and C3 cleavage sites, two series of TC-scanning constructs encompassing residues 92 to 116 and 222 to 230 were made as specified below and transiently transfected into N2a neuroblastoma cells. We use a naming convention in which constructs are designated based on the number of the PrP residue C-terminal to the TC insertion (e.g. a construct with a TC between 89 and 90 becomes 90TC). All the constructs exhibited a normal glycosylation pattern (Figure 1D and data not shown). Given our labeling method (IDEAL-labeling) targets cell surface PrPsen^[45], this suggested the TC-PrPs underwent proper folding and trafficking.

Influence of FIAsh/TC on in vitro cleavage of PrP (102–113)TC by trypsin

To characterize the influence of FIAsh/TC on proteolytic cleavage at nearby sites, we initially investigated the effects of TC insertion on cleavage of the (102–113)TC-PrP series by trypsin, a protease with very well-defined properties. This region spans two cleavage sites located at Lys105 and Lys109. The series of fragments occurring in the absence of any TC-associated inhibition consisted of four types of fragments we term T1 to T4 as listed in Figure 2A. Note that T1 and T4 arise from missed trypsin cleavages at sites far-removed from the sites of TC insertion as proven in immunoprecipitation (IP) experiments described below. Given there is precedent for missed trypsin cleavages in non-denatured wild-type PrPsen^[63;64], it is highly unlikely these missed cleavages were associated with TC insertions in the unstructured 102–113 region of PrPsen. As expected, different banding patterns were observed depending on the site of TC insertion relative to the trypsin cleavage sites (Figure 2A). In the absence of cleavage inhibition, (102–105)TC-PrP were expected to generate a fragment of identical apparent MW, which we termed T1. However, a band of slightly increased apparent MW (termed T-I1) was observed starting at 104TC that persisted to some degree through 107TC (Figure 2A, lanes 1–6). Inhibition of cleavage at Lys105 should lead to the generation of a larger MW fragment containing additional residues Thr106–Lys109 (T-I1) and this is consistent with our results. This suggested that cleavage inhibition at Lys105 occurred starting at 104TC.

Insertions (106–109)TC were expected to produce a very short tryptic fragment we called T2 (Figure 2A). As discussed below, we unexpectedly found that short FIAsh/TC-tagged peptides (i.e. < ~10 residues, not including the TC motif itself) like T2 exhibit anomalous migration in SDS-PAGE, migrating as dim, very diffuse species of much higher apparent MW than predicted and in some cases migrating slower than full-length PrPsen. Indeed, diffuse high MW species were observed in (107–108)TC that were consistent with the expected behavior of the T2 fragment (Figure 2A, lanes 6–7, asterisks). In addition, the migration of these diffuse bands was unaffected by PNGase F treatment, showing that they lack glycans and thus cannot correspond to full-length PrPsen (Figure S1). Considering these observations in conjunction with the deduced identities of other bands in the (102–113)TC series, the data argue that the diffuse bands corresponded to the T2 fragment. The absence of T2 in 106TC and 109TC suggested inhibition of tryptic cleavage in these constructs (Figure 2A, lanes 5 and 8). Since the disappearance of T-I1 and generation of T2 are indicative of resumption of cleavage at Lys105, these findings suggest that trypsin cleavage at Lys105 was inhibited over a 4 residue-wide range.

Two fragments were possible for insertions C-terminal to Lys109 [(110–113)TC], T3 and T4, the latter associated with missed trypsin cleavages at several potential sites within the C-terminal folded domain of PrPsen. Trypsin digestion of (108–113)TC resulted in the generation of T3 and T4-like fragments (Figure 2A, lanes 7–12). However, closer examination of the digestion products of (109–113)TC on gels that were electrophoresed for a longer time period clearly showed that the bands in (109–111)TC were similar to each other but also of slightly higher apparent MW than those in (112–113)TC (Figure 2A, compare lanes 14–16 with lanes 17–18). This size difference mirrored the size shift observed between T1 and T-I1 in (102–103)TC and (104–107)TC and was consistent with resumption of cleavage at Lys109 as the TC insertion moved away from Lys109. Consequently, we deduced that the T3 and T4-like fragments in (108–111)TC were associated with cleavage inhibition at Lys105 and designated these fragments T-I2 and T-I3, respectively (Figure 2A). Altogether, these findings suggest that, as for cleavage at Lys105, there was also a 4 residue-wide inhibition range for TC insertions flanking Lys109. Similar results were obtained for TC-scanning studies of trypsin cleavage in the (222–230)TC series (~3 residue-wide inhibition range), indicating the inhibition range for trypsin was not dramatically influenced by sequence flanking the TC motif for the two unstructured regions of PrPsen we examined (data not shown).

Mapping FIAsh/TC-tagged PrP fragments by immunoprecipitation

We explored alternative approaches to provide independent evidence for our assignment of the various tryptic fragments shown in Figure 2A. We determined that identification of the bands by mass spectrometry was not feasible due to vastly insufficient yields of TX114-purified protein in our transient transfections. Immunoprecipitation (IP) has been used by other investigators to characterize proteolytic fragments of PrPsen [22;23;44]. Unfortunately, this was not possible for many fragments due to either the absence of antibody epitopes, in the case of small fragments, or the localization of the FIAsh/TC tag within antibody epitopes. However, we noted that antibodies were available with epitopes that could permit reasonable characterization of 3 tryptic fragments (T1, T3 and T4) produced in two representative constructs (102TC and 113TC) by IP. Positive control IPs on undigested samples using a panel of antibodies with epitopes spanning the deduced fragments showed that all antibodies immunoprecipitated full-length 102TC or 113TC (Figure 2B, “No Trypsin” gels, FL band).

The T1 fragment of 102TC was precipitated by 8B4, SAF32, and R30, whose epitopes span residues 37–103, but not R24, 7D9, or SAF61 [Figure 2B, 102TC (+Trypsin)]. Although trypsin digestion may remove Lys23–Lys24 at the extreme N-terminus of mature PrPsen, since R24 is a polyclonal antibody the most likely interpretation of these data is that the N-terminus of T1 corresponds to Tyr38 (Figure 2A). With the current localization of the 7D9 epitope to residues 105–125, the IP data alone cannot distinguish whether the C-terminus of T1 is Lys105 or Lys109. However, the small increase in apparent MW observed for the T-I1 fragment of (104–107)TC compared with the T1 fragment of (102–103)TC, when considered together with the IP data strongly support our interpretation that T1 corresponds to residues 37–105.

Both the T3 and T4 fragments of 113TC were precipitated by mAb132 (epitope residues 119–127) but not 6D11 [Figure 2B, 113TC (+Trypsin)], localizing the N-terminus to either Lys105 or Lys109. However, analogous to the localization of the C-terminus of T1, the small decrease in apparent MW observed between T-I3 and T4 (Figure 2A), together with the IP data, strongly argue that the N-termini of T3 and T4 are most likely Lys109. All other antibodies failed to precipitate T3. Since the nearest potential trypsin digestion sites C-terminal to Lys109 are Arg147 and Arg150 and the SAF61 monoclonal antibody epitope corresponds to residues 141–152, the C-terminus of T3 may correspond to residue Arg147 or Arg150 as depicted in Figure 2A. On the other hand, T4 was precipitated by SAF61, SAF70, SAF84, and R20, indicating this fragment contains multiple missed trypsin cleavages as can occur with digestions of native PrPsen^[63;64]. These missed cleavages might be attributed to the localization of the Lys or Arg residues within regions of secondary structure. Since the C-terminal region of mouse PrPsen that contains Arg228–Arg229 is unstructured^[65;66], and trypsin digestion can block binding of a different C-terminal monoclonal antibody R1 (epitope residues 224–230^[67]) (see below), it is plausible that the C-terminus of the T4 fragment corresponds to Arg228 or Arg229 (Figure 2A).

In “No Trypsin” control samples, certain antibodies also precipitated proteolytic fragments of PrPsen that were generated in N2a cells, providing mapping information to support our TC-scanning characterization of two other FIAsh/TC-tagged PrPsen fragments (C1 and C2) discussed in greater detail below. Briefly, as described above the C1 fragment is thought to correspond to cleavage at approximately residues 110 or 111 in mouse PrP^[20] while the C2 fragment is associated with β cleavage around residue 96^[37;46;47]. As expected, the FIAsh/TC-tagged fragment we designated C1 was present in 113TC but not 102TC and immunoprecipitated by all antibodies tested except 6D11, whose epitope (95–105) is N-terminal to the C1 cleavage site [Figure 2B, 102TC and 113TC, (No Trypsin) panels]. Also as expected, we observed a band consistent with the C2 fragment that was present in 102TC and 113TC and immunoprecipitated by all antibodies except those with epitopes N-terminal to (R24, 8B4, SAF32) or within (R30, 6D11) the predicted β cleavage site [Figure 2B, 102TC and 113TC, (No Trypsin) panels]. Collectively, the IP mapping experiments provided a very strong validation of our ability to identify the various FIAsh/TC-tagged proteolytic fragments reported in this study.

Influence of FIAsh/TC on in vitro cleavage of PrP (222–230)TC by chymotrypsin

To further investigate the influence of FIAsh/TC on proteolytic cleavage by a different protease, we analyzed another TC-scanning series from a structurally distinct region of PrP, namely from residues 222 to 230, using chymotrypsin (Figure 3A). We observed the proteolytic fragment profiles of (222–230)TC after chymotryptic digestion of TC-PrPs partially purified by TX114 extraction as described in the Experimental Section (Figure 2A). There are two chymotrypsin cleavage sites within (Tyr224 and Tyr225) and one near (Tyr217) the scan range. Therefore, three possible fragments, termed Ct1 to Ct3, were anticipated in the absence of inhibition depending on the position of the FIAsh/TC tag insertion (Figure 3A). However, we deduced that the observed fragment pattern was different. For example, although (226–230)TC were predicted to generate the same Ct3 fragment without any inhibition, the actual fragment sizes for (226–227)TC were larger than

for (228–230)TC (Figure 3A, compare lanes 5–6 and lanes 7–9). This change in fragment size occurred as the FIAsh/TC insertion approached the C-terminal side of the chymotrypsin cleavage sites at Tyr224 and Tyr225. Inhibition of cleavage at both Tyr224 and Tyr225 should give rise to a ~1 kDa larger inhibition-associated fragment we called Ct-I2, and this was consistent with the observed change in fragment size (Figure 3A). Cleavage inhibition only at Tyr225 would be expected to occur as FIAsh/TC insertions approached the C-terminus of PrP and allowed resumption of cleavage at Tyr224, giving rise to a smaller fragment called Ct-I3. Since Ct-I3 differs from Ct3 by only a single Tyr residue, we did not expect to resolve the difference between Ct3 and Ct-I3 by SDS-PAGE as acknowledged in Figure 3A. Nevertheless, since cleavage at Tyr224 resumed at 228TC it was likely that cleavage at Tyr225 would resume at 229TC as indicated in Figure 3A. The Ct-I2 fragment persisted through insertions up to 223TC (Figure 3A, lane 2), identifying a 5 residue-wide range of insertions that inhibited cleavage at both Tyr224 and Tyr225.

For FIAsh/TC insertions N-terminal to the chymotrypsin cleavage sites, there were diffuse fluorescent bands of ~20–37 kDa apparent molecular weight (MW) in (222–223)TC (Figure 3A, lanes 1–2 and 10–11, asterisks). We hypothesized that short FIAsh/TC-containing peptides like Ct1 and Ct-I1 might anomalously appear as slowly migrating, diffuse bands because of insufficient amounts of bound SDS as has been described for other polypeptides [68], whereas short GPI-anchored fragments would appear as more rapidly migrating, sharp bands because the lipid moiety bound more SDS molecules. This hypothesis was proven in experiments where proteolytic fragments of PrP(230TC) digested with phosphatidylinositol-specific phospholipase C (PI-PLC) migrated more slowly as diffuse bands (Figure 3B, lane 2, asterisks) while those without PI-PLC digestion formed sharp, rapidly migrating bands (Figure 3B, lane 1, arrow). The diffuse bands also exhibited an apparent reduction in fluorescence intensity relative to their rapidly-migrating counterpart, which might be attributed to their broader migration pattern. This dimming effect for the diffuse bands likely contributed to the lower band intensities observed in (222–223)TC, where our observations argue the short Ct1 and Ct-I1 bands were present (Figure 3A, lanes 1–2), and other samples where short TC-tagged peptides occurred such as the T2 fragment of (107–108)TC (Figure 2A, lanes 6–7). In the case of (222–223)TC, an additional factor was that FIAsh-PrPsen expression levels tended to be lower compared to other constructs in this series, perhaps due to their insertion within helix 3 [65;66]. Although our system may be unable to resolve a difference in Ct1 vs. Ct-I1, the appearance of the diffuse bands and lower intensity of Ct-I2 in 223TC suggested that cleavage had at least partially resumed at Tyr225 (Figure 3B, lane 2). Given that this trend continued as the TC insertion moved further N-terminally to 222TC (positive for Ct1/Ct-I1, negative for Ct-I2), the data suggested cleavage had fully resumed at least at Tyr225 (Figure 3B, lane 1). Therefore, our observations suggested that FIAsh/TC can inhibit chymotrypsin digestion when located within an approximately 6 residue-wide range across the cleavage site.

Influence of FIAsh/TC on C1 cleavage of PrP (92–116TC) in neuroblastoma cells

Next, we applied TC-scanning to probe proteolytic processing events on PrPsen occurring in neuroblastoma cells transiently-transfected with TC-PrP constructs. Since the C1 cleavage sites were previously identified as His110 and Val111, we initially used (102–116)TC

encompassing these sites (Figure 4A). However, it was readily apparent that the C1 species actually consisted of two fragments (Figure 4B, arrow and closed arrowhead). The lower of the two fragments was seen only in (106–116)TC (Figure 4B, closed arrowhead). Appearance of the upper fragment at 97TC in a TC-scanning series (92–102)TC suggested its N-terminus to be near 97TC (Figure 4C, lane 6 and lane 14, arrow). Interestingly, this site matched the N-terminal end of the C2 fragment derived from β -cleavage of PrPsen [47;54]. Although the mechanism for generation of the upper fragment in the present study is unknown, the data suggested that the upper fragment corresponded to the C2 fragment described by others. On the other hand, a drastic change in amount of the lower fragment across residue 109TC, close to the putative C1 cleavage sites, suggested this fragment was the C1 fragment (Figure 4B, graph). As presented above in Figure 2B, the interpretation of these bands as the C1 and C2 fragments was corroborated by mapping via immunoprecipitation with various anti-PrP antibodies.

Experiments using the 92–116TC series provided insight into how insertions at and C-terminal to the C1 cleavage site affected this processing event. However, the full range of TC insertions with inhibitory activity, especially insertions N-terminal to the C1 cleavage sites, could not be definitively mapped for two reasons. First, inhibition of C1 cleavage should result in the disappearance of the C1 fragment and accumulation of its precursor, full-length PrPsen (Figure 4B, “FL”). It was difficult to reliably compare full-length PrPsen levels between constructs because differences might be attributed to other factors such as varying expression efficiencies between samples. In addition, non-inhibitory TC tag insertions N-terminal to the C1 cleavage sites result in labeling of the N-terminal product of C1 cleavage, an 11–12 kDa fragment called N1, which others have shown is mostly secreted [23] and was not detected in our cell lysates (Figure 4B). To overcome these issues, we created a double-TC PrP series, each of which combines 230TC with another TC from 103 to 113 [e.g. PrP(113/230TC) has TCs at positions 113 and 230], to enable FAsH labeling of C1 in all the constructs (Figure 4D). C1 cleavage products that contain two TC tags located C-terminal to the C1 cleavage sites (termed “double-TC C1”) such as 112/230TC (Figure 4D, lane 9) exhibited a slightly higher apparent MW versus a C1 cleavage fragment containing a single TC tag (termed “single-TC C1”), as occurred for constructs with one TC insertion located N-terminal to the C1 cleavage sites [e.g. 103/230TC (Figure 4D, lane 2)]. Remarkably, C1 cleavage was not severely affected even for TC insertions located near the putative cleavage sites as judged by the similar levels of single-TC C1 amongst 103/230TC to 108/230TC (Figure 4D, lanes 2–5, closed arrowhead) and similar levels of double-TC C1 amongst 109/230TC to 113/230TC (Figure 4D, lanes 6–10, gray arrowhead). Double-TC C1 predominated over single-TC C1 in 109/230TC through 111/230TC despite the fact that in 109/230TC and 110/230TC one TC was located N-terminal to the putative cleavage sites and hence could permit simultaneous generation of single- and double-TC C1 (Figure 4D, lanes 6–8). These data suggested that, different from human PrPsen, C1 cleavage of mouse PrPsen predominantly occurs between the peptidyl bond of residues Leu108 and Lys109 and that there is a very small region of interaction between PrP and the protease mediating C1 cleavage.

Influence of FIAsh/TC on the conversion of PrPsen to PrPres

Cell-free digestion experiments with FIAsh-PrPsen in Figures 2 and 3 demonstrated that FIAsh/TC is sufficiently bulky to affect protein-protein interactions but only when it is located within or very near the interaction interfaces. This raised the possibility that FIAsh/TC could be exploited to detect compactly-folded or intimately-interacting regions of proteins. To investigate this idea and as an independent measure of the effects of FIAsh/TC on protein-protein interactions, we tested the effects of FIAsh/TC on the conversion of PrPsen to the PrPres isoform. This conversion process involves complex interactions between PrPsen and PrPres that result in refolding of PrPsen to a β -sheet-rich conformation and incorporation into the highly-ordered polymer of PrP molecules that constitute PrPres aggregates. Therefore, if FIAsh/TC interferes with either the intermolecular interaction between PrPsen and PrPres or intramolecular interactions between different regions in the refolding process, formation of PrPres would be impaired. The conformational conversion of PrPsen to PrPres can be detected by the acquisition of protease resistance of the C-terminal portion of PrP. Hence, this conversion assay serves as a sensitive probe for the influence of FIAsh/TC on intermolecular and intramolecular interactions.

The scan range was determined based on the N-terminal end of the protease-resistant core of PrPres; reportedly, this is around residue 81 for typical mouse-adapted sheep scrapie [69]. Since our cells were infected with the mouse-adapted scrapie strain, 22L, we tested the conversion efficiencies of (90–101)TC. When these constructs were transiently expressed in 22L scrapie-infected cells, only constructs with TC on the N-terminal side of residue 96 converted to the PrPres isoform as measured by chymotrypsin resistance (Figure 5). These data showed that for insertions within this region, FIAsh/TC was tolerated over a significant range and suggested that the most highly-ordered regions of PrP molecules in PrPres aggregates begins after residue 96.

Discussion

The fluorescent complex of FIAsh/TC has a unique conformation with a compact hairpin-like structure and no side chains of the constituent residues protruding from the complex [70;71]. This renders a very compact structure to FIAsh/TC, which could confer minimal susceptibility to enzymatic reactions mediated through side chains of amino acids. In a sense, FIAsh/TC might resemble a bulky “tight knot” on a peptide chain. In a previous study, we introduced the technique of IDEAL-labeling of cell surface TC-tagged proteins, focusing on insertions at just two separate locations in PrP (90TC and 230TC) that were strategically chosen as least likely to impact PrP structure and function [45]. The purpose of the current series of experiments was to assess the influence of this foreign structure on protein-protein interactions when it is located near the interaction interface and, by contrast, determine how close the FIAsh/TC tag must be to interfere with several types of interactions using PrP as a model protein.

We assessed the inhibitory effects of FIAsh/TC on cleavage by trypsin or chymotrypsin when it was located near the cleavage sites. As hypothesized, FIAsh/TC-dependent inhibition of cleavage was readily detected as alterations in proteolytic fragment patterns. The TC-scanning series allowed identification of the inhibition ranges for each protease. Our

data showed that the inhibition range for trypsin (~3–4 residues) was less than that for chymotrypsin (~6 residues). Insertion of FIAsh/TC near a cleavage site will change both the amino acid sequence and regional three-dimensional structure relative to the native protein. For instance, in the present study unstructured regions of WT PrPsen were interrupted by a hairpin FIAsh/TC complex in TC-PrPsen constructs. A proline in the P1' position is the most influential sequence modification capable of completely inhibiting trypsin and chymotrypsin digestion. Insertion of the TC motif results in a cysteine at this position. ExPASy PeptideCutter tool ^[72] analysis of the predicted effects of each TC insertion on all the trypsin and chymotrypsin cleavages described in this study indicated that only a single insertion (109TC) acting at a single site (trypsin digestion at residue Lys109) would prevent cleavage (reduced to 44% efficiency vs. 100% for wild-type sequence). Although such protein analysis tools are only a guide, this provided evidence that TC insertion was not expected to block trypsin and chymotrypsin cleavage for virtually every construct we tested, at least based on primary structure. The similar widths of inhibition ranges in different regions of PrP suggest that this regional inhibitory effect did not depend on the amino acid sequences flanking the cleavage sites, suggesting FIAsh/TC itself was responsible. We cannot specify the exact mechanisms of inhibition in this study, but they could involve either direct steric effects with FIAsh/TC acting as an obstacle to substrate-protease contact or indirect effects, such as FIAsh/TC inducing some local structural change analogous to what occurs with proline at the P1' position, or both. Regardless of the precise mechanism, this raises the possibility that FIAsh/TC might exert a similar inhibitory influence on other types of protein-protein interactions.

An important question arises as to what the width of inhibition range represents? It is well-known that chymotrypsin and trypsin require interaction between subsites and substrate peptides for efficient cleavage (e.g. subsites S2–S3' need to be occupied in the case of trypsin) ^[73;74]. These subsites and the counterpart residues on the substrate can be regarded as an interaction interface for the substrate-protease interaction. There was a striking similarity of the inhibition ranges to the range of residues involved in subsite-substrate interactions, suggesting that the widths of inhibition ranges might define the interaction interfaces. This has implications about the influence of FIAsh/TC on general protein-protein interactions, many of which involve analogous non-covalent bonding interactions to stabilize complexes. This leads to a more generalized hypothesis that the effects of FIAsh/TC might be so small that it interferes with protein-protein interactions only when it is located within the interaction interfaces and minimally affects them from outside of the interaction interfaces. This should encourage the application of FIAsh/TC-tagging to investigations of other protein-protein interaction systems.

As a practical application, our observations suggest that regions adjacent to protease cleavage sites may serve as good locations for internal TC-tagging. As protease cleavage sites are often loosely structured ^[60] and solvent-exposed for efficient cleavage, these regions are more likely to tolerate FIAsh/TC. There have been some examples of successful internal TC-tagging near protease cleavage sites: Alzheimer's amyloid precursor protein with a TC toward the beta-cleavage site ^[45]; PrP with TC near the GPI attachment site, where the GPI-anchoring process requires cleavage of a C-terminal signal peptide ^[45;75]; and HIV Gag poly protein precursor with TC near a cleavage site ^[76]. We expected that

insertions within regions of secondary structure such as α -helices might be problematic, and hence our studies focused largely on insertions in unstructured regions with the exceptions of 222TC and 223TC. If FIAsh/TC insertion within secondary structures is not tolerated, this could prevent the application of TC-scanning to probe regions of protein-protein interaction that involve such structures. However, unless FIAsh/TC interferes with the cleavage, regions adjacent to protease cleavage sites are viable locations for internal TC tags. This information may serve as a helpful guide to identify sites for TC insertion when detailed structural information of the protein is not available.

When we used TC-scanning to map the residues of PrP that interact with the protease responsible for C1 cleavage, surprisingly C1 cleavage was minimally affected by the presence of FIAsh/TC near the putative cleavage sites, especially on the N-terminal side. Although it was difficult to eliminate the possibility of partial inhibition by FIAsh/TC on the C-terminal side, there were no signs of inhibition such as a dramatic reduction in the amounts of the C1 fragment in constructs 111TC-116TC. These findings suggested that the effects of FIAsh/TC on C1 cleavage were rather small, and hence the C1-producing protease(s) in our system likely recognizes a very limited number of residues on PrP and/or has a flexible substrate binding site to accommodate the FIAsh/TC tag. Our observations are consistent with previous mutagenesis studies of the α -cleavage site as cleavage inhibition requires dramatic changes to this region by means of large deletions or sequence modifications [35;39–42]. This led one group to conclude that the recognition of the cleavage site is governed primarily by the size of the C1 cleavage region of PrP rather than primary sequence [39]. Crystal structures of ADAM family metalloprotease domains have shown that substrates bind to a small active site cleft where as many as six residues on the substrate form contacts with six enzyme subsites [77–79]. If ADAMs account for at least some of the C1 fragment produced in N2a cells, it is quite impressive that these proteases can somehow tolerate FIAsh/TC insertion at each position spanning the entire region flanking the cleavage site.

The contrasting results of C1, trypsin, and chymotrypsin cleavages clearly demonstrated that the influence of FIAsh/TC on nearby protease cleavage can vary between proteases, emphasizing the importance of the TC-scanning method to identify optimal insertion sites. It is unlikely that any exogenous tag will be completely innocuous under all circumstances. Nevertheless, our findings provide guidance on the application of FIAsh/TC tagging in conjunction with studies of the proteolytic processing of other proteins of broad interest such as APP or the various targets of matrix metalloproteases. An analysis of proteolytic processing of FIAsh/TC-APP has already been reported [45].

We also exploited FIAsh/TC to probe the looseness of PrP domains in PrPres aggregates because the fundamental molecular interactions that stabilize PrPres aggregates presumably occur in other protein-protein interactions (e.g. proteases with substrates) and in compactly-folded regions of proteins in that non-covalent bonds stabilize inter- or intramolecular structures. The observation that the FIAsh/TC tag did not inhibit conversion to FIAsh-PrPres at positions N-terminal to residue 96 suggests that this position delineates a more loosely-folded region of PrPres from a more compact/ordered region C-terminal to residue 96. However, this region does acquire protease resistance [80–82] and altered antibody

epitope exposure^[83] after conversion suggesting there is some structural change. This is consistent with hydrogen/deuterium exchange data of PrPres fibrils, which for the little exchange that does occur suggests slightly higher exchange for residues near the N-terminus of the PK-resistant core versus more C-terminal residues^[84]. Geissen et al. previously provided evidence that this N-terminal region of PrPres has some flexibility and tolerance for insertion of heterologous sequences by showing that insertion of a linear heptapeptide epitope before residue 95 did not interfere with conversion of the mutant PrP into PrPres^[85]. Although their findings are similar to those reported in the present study, their use of a linear-epitope insertion creates some ambiguity about the interpretation of the results. For instance, there is a possibility that the residues of the inserted sequence might have been capable of substituting for the native PrP residues during conversion to PrPres by adopting a similar conformation or forming similar molecular contacts with surrounding residues. By contrast, since the structure of FIAsh/TC is rather stable and compact, its influence is more predictable and provides more specific and direct information about the structural compactness of a protein at the insertion site. Altogether, our observations suggest that FIAsh/TC can be used as a localized conformational probe for the detection of tightly-folded regions of proteins.

It should be noted that we cannot predict how well our findings will translate to analyses of intermolecular interactions of other TC-tagged proteins. It is beyond our capability to test an exhaustive series of different proteins and in any case such a list would never account for the wide variation in behavior that can occur between proteins. Instead, we focused on extensive analyses of two different classes of protein-protein interaction with insertions covering two different regions of PrPsen, using three different enzymes associated with one class of protein-protein interaction (proteolysis). We believe this provides a solid basis on which to apply TC-scanning as a novel probe to characterize other protein-protein interactions.

Experimental Section

Cells and Reagents

FIAsh in-cell labeling kit was purchased from Invitrogen (Carlsbad, CA). Triton X-100, Triton X-114 (TX114), deoxycholate, dithiothreitol (DTT, cat# D9779-250MG), ethanedithiol (EDT), ammonium chloride, trypsin-TPCK, chymotrypsin, sodium acetate (3M solution, pH 5.2) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Pefabloc SC and Complete[®] protease inhibitors were from Roche Diagnostics Corporation (Indianapolis, IN). All the media, buffers [including Hank's Balanced Salt Solution (HBSS)], reagents and fetal bovine serum (FBS) for cell culture were purchased from Invitrogen (Carlsbad, CA). All the restriction enzymes and PNGase F were from New England Biolabs (Ipswich, MA). Phosphatidylinositol-specific phospholipase C (PI-PLC) was purchased from MP-Biomedicals LLC. (Solon, OH). E64 was purchased from Roche Diagnostics Corporation (Indianapolis, IN). PI-PLC was purchased from MP-Biomedicals LLC (Solon, OH). Antibody sources were as follows: 8B4 (Santa Cruz); R24, R30, R20 rabbit polyclonals^[48] (gifts from Byron Caughey); 6D11, 7D9 (Covance); SAF32, SAF61, SAF70, SAF84 (Cayman Chemicals); and mAb132^[86] (gift from Motohiro Horiuchi).

Neuro2a (N2a) cells, a mouse neuroblastoma cell line, and 22L-scrapie-infected N2a cells (22L/N2a) were reported elsewhere [87].

TC-PrP constructs

All the constructs of TC-scanning series were created with a site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) according to the manufacturer's instructions. A list of primers used for TC-scanning series is available as Supporting Information (Table S1). Each TC-scanning series construct was named after the position number of the residue immediately following the TC, e.g. 90TC has the TC between residues 89 and 90. The template was the mouse PrP open-reading frame (gift from Dr. Ryuichiro Atarashi, Nagasaki University, Nagasaki, Japan) previously cloned into the pCR cloning vector (Invitrogen, Carlsbad, CA) flanked by a *HindIII* site and an *XbaI* site on the 5' and 3' sides, respectively. After verification of the sequences, the mutated open-reading frame was excised with *HindIII* and *XbaI* and subcloned into an expression vector, pcDNA3.1(+) (Invitrogen, Carlsbad, CA). XL10-Gold cells were used for amplification of the plasmids and plasmids were purified with the QIAprep Spin Miniprep Kit (QIAGEN Inc., Valencia, CA).

Biarsenical Labeling

Cells were transfected with the designated constructs using the Effectene Transfection Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. The following day, transfected cells were labeled with FIAsh using the IDEAL-labeling protocol as described elsewhere [45]. Briefly, FIAsh was first pre-incubated with DTT and EDT in the dark at room temperature. Meanwhile, the cells were rinsed with OptiMEM I supplemented with FBS (1%) and ammonium chloride (20 mM) [OM(1%)]. Then, OM (1%) and DTT (2 M) were added to the pre-incubated FIAsh to final concentrations of 1–1.2 μ M FIAsh and 20 mM DTT and kept on ice until use. Cells were labeled (at room temperature) with the pre-chilled labeling medium for 3 minutes. After removal of the labeling medium, OM (1%) was added. After a 5–10 minute incubation at room temperature, OM(1%) was replaced with OptiMEM I containing FBS (10%) and the cells were placed in a CO₂ incubator. For consistency with other experiments, the point of 30 minutes after addition of labeling medium was regarded as 'Hour 0'. We recently found that a critical factor for specific labeling is the quality of the 2 M DTT. This is prepared by dissolving a whole bottle of DTT (250 mg) in sodium acetate (10 mM, pH 5.2) by gently rocking without vortexing. Prepare single-use aliquots and store at –20 °C.

Sample preparation/Fluorescent gel analysis

FIAsh-labeled cells were rinsed with HBSS and harvested either with Triton X-100 (0.5%)/deoxycholic acid (0.5%) in phosphate-buffered saline (TX100/DOC lysis buffer) or Triton X-114 (2%) in Tris-buffered saline (pH 7.5) (TX114 lysis buffer) depending on the subsequent sample preparation procedures. Since detailed protocols for sample preparations from each lysate were described previously [45], brief descriptions are presented below.

Whole-cell lysate samples were prepared by adding 1/4 volume of 5x sample buffer [5xSB; SDS(10%), glycerol (30%), Tris-HCl (250 mM, pH 7.1) and bromophenol blue (0.002%)] to TX100/DOC lysate and boiling for 5 minutes. When necessary, samples were

deglycosylated with PNGase F according to the manufacturer's instructions except that 1/20-volume of 5xSB was added to the lysates instead of the manufacturer's Denaturation buffer. For Triton X-114 extracted samples, lysates used for *in vitro* and C1 cleavage were harvested soon after labeling at Hour 0 (defined as 30 min after beginning the IDEAL-labeling procedure [45]).

For Triton X-114 extracted samples, lysates used for *in vitro* cleavage were harvested soon after labeling at Hour 0. Triton X-114 lysates were incubated at 37°C for 10 minutes for phase separation, centrifuged at 22,500 g for 10 minutes and the aqueous phase was discarded. The detergent phase was diluted again with 5 volumes of Triton X-114 (0.1%) wash buffer, cleared on ice, and subjected to a second phase-separation and centrifugation. After removing the aqueous phase, Triton X-114 (0.1%) wash buffer, methanol and chloroform were added for methanol/chloroform precipitation. After methanol/chloroform precipitation, the pelleted protein was dissolved in guanidine hydrochloride (20 µl; 6 M). Then it was diluted with of TX114 lysis buffer (200 µl), subjected to two cycles of phase-separation and removal of the aqueous phase, and precipitated with methanol/chloroform. Finally, the pelleted protein was dissolved in 1xSB and boiled for 5 minutes. For deglycosylated samples, the pelleted protein was dissolved in 0.25xSB with sonication, boiled for 5 minutes, deglycosylated with PNGase F according to the manufacturer's instructions, and then 1/4 volume of 5xSB was added prior to boiling.

Fluorescent gel analysis was described elsewhere [45]. Briefly, after harvest and sample preparation, the samples were electrophoresed on NuPAGE® 10% Bis-Tris precast gels with 2-(N-morpholino) ethanesulfonic acid running buffer (Invitrogen). After electrophoresis, the gel was scanned on a Typhoon scanner (GE Healthcare, Piscataway, NJ) with excitation at 488 nm and 520BP40 emission. Images were analyzed with ImageQuant-TL software (GE Healthcare).

Digestion with trypsin or chymotrypsin

For the proteolytic fragment profile analysis of TX114-extracted FIAsh-labeled TC-PrPs, the pellets of the second methanol/chloroform precipitation were reconstituted in TX100/DOC lysis buffer with sonication and then digested with proteases. When PI-PLC digestion was necessary, PI-PLC (0.3 µl) was added per 10 µl of lysate and placed at 37 °C for 30 minutes. For fragment profile analysis of whole-cell lysate samples, the TX100/DOC lysates were used directly. For protease digestion, 1/10-volume of trypsin stock solution (0.4 mg/ml) or 1/20-volume of chymotrypsin stock solution (1 mg/ml) was added to the lysates and incubated at 37 °C for 15–30 minutes. For samples without deglycosylation, 1/4-volume of 5xSB was added immediately after protease digestion and boiled for 5 minutes. When deglycosylation was needed, protease digestion was stopped by addition of 1/20-volumes each of 50x Complete® solution and 5xSB followed by boiling for 5 minutes. PNGase F digestion was performed according to the manufacturer's instructions. After deglycosylation, 1/4-volume of 5xSB was added and the sample boiled prior to SDS-PAGE.

Immunoprecipitations

Trypsin digestions of TX114-extracted FIAsh-labeled PrP(102TC) and PrP(113TC) were terminated by addition of 1/20th volumes of 50x Complete[®] solution and incubation on ice. Undigested whole cell lysates of FIAsh-labeled PrP(102TC) and PrP(113TC) in TX100/DOC lysis buffer served as “No Trypsin” positive controls for immunoprecipitation. All samples were adjusted to 0.5% SDS, denatured by boiling for 10 minutes, and deglycosylated with PNGase F as above. Following deglycosylation, the samples were diluted to 0.25 ml in either TX100/DOC lysis buffer or (for SAF84 only) detergent, lipid, protein complex buffer^[88], supplemented with anti-PrP antibody, and incubated overnight at 4°C. Protein-antibody complexes were captured using Protein G high-capacity agarose (Thermo Scientific). The beads were then washed three times with NaCl (0.5M)/N-lauroylsarcosine (1%)/Tris-HCl (50 mM, pH 7.0 at 4°C), once with high purity water, eluted by boiling in 5xSB without bromophenol blue, and analyzed by SDS-PAGE and fluorescence scanning.

Phosphotungstate (PTA) precipitation of PrPres

For analysis of FIAsh-labeled PrPres, 22L/N2a cells transiently-transfected with TC-scanning constructs were incubated in a CO₂-incubator for more than 8 hours after FIAsh labeling and then harvested with TX100/DOC lysis buffer. After removal of nuclear debris by centrifugation at 22,500 g for 1 minute, the lysate was subjected to PTA-precipitation as described elsewhere^[45]. Briefly, N-lauroylsarcosine (30%) was added to the lysates to make the final concentration 1.5–2%. The lysates were incubated at 37°C for more than 30 minutes with continuous agitation. Then 1/12-volume of PTA-stock solution [PTA(4%), magnesium chloride (170 mM), pH 7.4] was added to the lysate and incubated for 30 minutes at 37°C with continuous agitation. The lysate was centrifuged at 22,500 g for 30–40 minutes at 26°C. After complete removal of the supernatant, the pellet was sonicated in 0.25xSB(10 µl), boiled for 5 minutes, and deglycosylated with PNGase F according to the manufacturer’s instructions. After deglycosylation, 5xSB (4 µl) was added and the samples were boiled for 5 minutes prior to SDS-PAGE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Proteolytic cleavage products of PrPsen

A) Inhibition of protease cleavages by FIAsh/TC can be detected as alterations in fragment patterns of TC-scanning series. The left and right panels illustrate positions of TC in the TC-scanning series and fragment patterns observed on fluorescent gel analysis, respectively. (i) When there is no cleavage, only one fragment is observed irrespective of FIAsh/TC position. (ii) When the peptide chain is cleaved and the cleavage is not inhibited by FIAsh/TC, either the N-terminal or C-terminal fragment is detected as only the fragment with FIAsh/TC is visible. (iii) When cleavage is inhibited by FIAsh/TC at certain positions, a new fragment (star) appears which corresponds to the uncleaved fragment.

B) Illustration showing secondary structures and post-translational modifications of PrP and ranges of TC-scanning series created. Broad arrows represent short beta-strands and cylinders represent alpha-helices. Normal proteolytic processing of PrPsen by cellular

processes at the approximate sites indicated results in the generation of fragments termed C1, C2, and C3.

C) Fluorescent SDS-PAGE gel showing glycosylated (PNG, lane 1) and PNGase F-deglycosylated (PNG+, lane 2) FIAsh-labeled PrPsen (230TC). Deglycosylated full-length PrP, FL.

D) Fluorescent SDS-PAGE gel of whole cell lysates of FIAsh-labeled cells transiently-transfected with representative constructs from TC-scanning series. All constructs exhibit a glycoform profile typical of normal, cell-surface PrPsen. Bracket, FIAsh-PrPsen. Asterisks, autofluorescent molecules present in whole cell lysates.

Figure 2. In vitro cleavage of PrP(102–113)TC with trypsin

A) FIASH/TC can affect nearby trypsin cleavage in PrP(102–113)TC. Upper portion shows schematic of PrP illustrating position of (102–113)TC, potential trypsin cleavage sites, and cleavage products. The C-terminus of the T3 fragment may be R₁₄₇ or R₁₅₀. Lower portion shows the fragment pattern of trypsin-digested [Trypsin(+)], deglycosylated [PNG(+)]TX114-extracted samples (lanes 1–18). Lanes 19–24 show corresponding undigested controls [Trypsin(–)] for samples in lanes 13–18. The data are representative of three independent experiments. The lower panel for lanes 1–12 is from the same gel as the upper panel, but the levels are adjusted to emphasize the faint T-I1 (lane 6) and T-I3 (lane 7) bands. The right panel gels (lanes 13–18 and 19–24) are from an independent experiment where the gels were electrophoresed for a longer time period to more clearly show the details of the fragment pattern of (109–113)TC. The table shows the anticipated fragment pattern in the absence of inhibition by FIASH/TC and a plausible fragment pattern deduced from the results.

B) Mapping fragments of 102TC and 113TC by immunoprecipitation. Preparations of 102TC and 113TC were subjected to immunoprecipitation with the indicated antibodies after (+ Trypsin) trypsin digestion. None, control samples without immunoprecipitation.

A) FIAsh/TC can affect nearby chymotrypsin cleavage. The scan range of (222–230)TC is illustrated at the top along with predicted cleavage sites of chymotrypsin (Y₂₂₄ and Y₂₂₅) and a schematic of cleavage products. The fluorescent gel images show the fragment patterns of (222–230)TC after chymotrypsin digestion. Lanes 1–9 and 10–18 are two images of the same gel but intensity levels of the right hand image are adjusted to emphasize the Ct1/Ct-II bands in lanes 10–11. Asterisks indicate Ct1 and Ct-II bands. Circle, band corresponding to incomplete cleavage at Y₂₁₇, which shows the predicted ~5 kDa increase in apparent MW vs. Ct3 resulting from cleavage at F₁₇₄ combined with preservation of the PrP disulfide bond on the non-reducing gel (see schematic). Undigested controls are shown in lanes 19–27. FL, full-length PrPsen. C1, C1 fragment. All samples were deglycosylated with PNGase F. Data shown are representative of three independent experiments with the

exception that the band due to incomplete cleavage at Y₂₁₇ was not always present (i.e. the digest went to completion).

B) The diffuse bands are very short FAsH-labeled proteolytic fragments. The fluorescent gel, representative of two independent experiments, compares chymotrypsin-digested samples of PrP(230TC) that were subsequently digested with phosphatidylinositol-specific phospholipase C (PIPLC +) or not (PIPLC –). Asterisks indicate diffuse bands. Arrows indicates corresponding GPI-anchored fragment.

Figure 4. Influence of FIAsh/TC on C1 cleavage

A) A schematic illustration showing TC-scanning ranges of (102–116)TC and (92–102)TC in mouse PrP. The underlined residues in (102–116)TC correspond to the expected N-terminal residues of C1 fragments (i.e. putative C1 cleavage sites) based on comparison with the sites identified for human PrP.

B) C2 and C1 fragments in (102–113)TC and (106–116)TC. The left gel and the right gel show fragment patterns of (102–113)TC and (106–116)TC, respectively. The right gel, which was run longer than the left, clearly shows the band initially referred to as C1 consisted of two fragments, C2 and C1. FL, full-length PrP. The graph shows results of

quantification of C2 and C1 based on multiple independent experiments [$n=3$ for (102–105)TC and (114–116)TC, $n=6$ for TC(106–113)], showing mean $\pm$ SEM.

C) The N-terminus of C2 is near residue 96. The left panel shows the fragment pattern of whole-cell lysate (92–102)TC samples (representative of two independent experiments) and the right panel shows samples prepared from the detergent phase of TX114 lysates (TX114-Dt), which concentrated the labeled TC-PrPs to emphasize C2.

D) C1 cleavage is not severely affected even when TC flanks the putative cleavage sites.

The fluorescent gel shows fragment patterns of double-TC series (103/230–113/230)TC.

Lane 1 (230TC) indicates the apparent MW of single-TC C1. The result is representative of two independent experiments. Double-TCC 1, arrow. Double-TCC1, gray arrowhead.

Single-TC C1, black arrowhead. The schematic illustrates how double-TC and single-TC C1 are produced depending on the position of FIAsh/TC(star).

Figure 5. FlAsH/TC insertions at residues 97–101 inhibit conversion to PrPres

The fluorescent gel show sFlAsH/TC-PrPres production by the (90–101)TC series expressed in transiently-transfected 22L-scrapie-infected N2a cells. Constructs in lanes 1–6 were tested in six independent experiments. The 90 and (99–101) constructs (lanes 7 and 13–15) were tested in two independent experiments for this study. The gels shown are representative of data observed in the independent experiments for the respective constructs. FlAsH/TC-PrPres was detected by chymotrypsin digestion followed by precipitation with phosphotungstate.