Subunit Exchange of Polydisperse Proteins

MASS SPECTROMETRY REVEALS CONSEQUENCES OF αA-CRYSTALLIN TRUNCATION*

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The small heat shock protein, α-crystallin, plays a key role in maintaining lens transparency by chaperoning structurally compromised proteins. This is of particular importance in the human lens, where proteins are exposed to post-translational modifications over the lifetime of an individual. Here, we examine the structural and functional consequences of one particular modification of αα-crystallin involving the truncation of 5 C-terminal residues (αA1-168). Using novel mass spectrometry approaches and established biophysical techniques, we show that αA1-168 forms oligomeric assemblies with a lower average molecular mass than wild-type αα-crystallin (αAα). Also apparent from the mass spectra of both ααα1 and αA1-168 assemblies is the predominance of oligomers containing even numbers of subunits; interestingly, this preference is more marked for αA1-168. To examine the rate of exchange of subunits between assemblies, we mixed αα crystallin with either αAα or αA1-168 and monitored in a real-time mass spectrometry experiment the formation of heteroligomers. The results show that there is a significant decrease in the rate of exchange when αA1-168 is involved. These reduced exchange kinetics, however, have no effect upon chaperone efficiency, which is found to be closely similar for both ααα1 and αA1-168. Overall, therefore, our results allow us to conclude that, in contrast to mechanisms established for analogous proteins from plants, yeast, and bacteria, the rate of subunit exchange is not the critical parameter in determining efficient chaperone behavior for mammalian αα-crystallin.

The small heat shock proteins (sHSPs) are a ubiquitous class of molecular chaperone that prevent target proteins from aggregating and precipitating (1). sHSPs are so called due to their relatively low monomeric molecular mass (~12–42 kDa); however, a characteristic feature is their assembly into large multimeric species. The mammalian sHSP α-crystallin, a major structural protein of the mammalian eye lens, is a polydisperse protein that encompasses a broad molecular mass range (2, 3). Isolation of α-crystallin from the lens yields multimers with molecular masses ranging from ~300 kDa to greater than 1 MDa (4).

As a member of the sHSP family of proteins, it is thought that, aside from contributing to the refractive index of the lens, α-crystallin provides protection to other resident proteins that undergo unfolding events (5). Such destabilization may be initiated by any number of stresses during the lifetime of the lens, such as UV light exposure, oxidation, chemical modification, and post-translational modifications (6). The chaperone function of α-crystallin, and the other sHSPs, involves the formation of high molecular mass complexes with these structurally compromised substrates. In the lens, however, there is no known machinery to release and refold the trapped substrate proteins; thus, with age, the levels of free, water-soluble α-crystallin in the nucleus diminish dramatically (7). The chaperone function of α-crystallin, therefore, is a finite resource in the aging human lens. α-Crystallin is comprised of two highly homologous subunits, αA and αB, which, in the adult human lens, are found at an approximate ratio of 3:1 (8). Only the production of full-length αA and αB is under direct genetic control (9); however, a multitude of post-translational modifications to these proteins, including phosphorylation, deamidation, and truncation, have been reported in the healthy lens (4, 10–12). The impact of these modifications on the chaperone function of α-crystallin has been studied for many years with, in many cases, conflicting conclusions reported. For example, phosphorylation of α-crystallin has been reported variably to have no effect on chaperone activity (13, 14), to reduce chaperone activity (15), or to cause a decrease in oligomeric size (16). We have recently reported that an S45D phosphorylation mimic of recombinant αB-crystallin leads to changes in subunit organization within the oligomer and consequent loss of effective chaperone function (17).

Among the other major modifications reported for α-crystallin is truncation of residues from the C terminus. This process was first described in the bovine lens, where it was noted that a stepwise degradation of αA-crystallin at residues 169, 168, 151, and 101, and of αB-crystallin at residue 170, occurred post-synthetically (18). This study concluded that the degradation was unlikely to be the result of enzymatic processes as it was observed during prolonged dialysis of purified α-crystallin (18). Subsequently, in the human lens, C-terminal truncations have been reported for αα-crystallin at residues 101, 162, 168, and 172 (11, 12, 19–21), whereas no such degradation appears to occur in αB-crystallin.

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1 The abbreviations used are: sHSP(s), small heat shock protein(s); MS, mass spectrometry; SEC, size exclusion chromatography; MALDI, multianalyte laser light scattering; MS/MS, tandem mass spectrometry; WT, wild type.
Recently, total bovine lens extracts were shown to possess protease activity toward synthetic peptides corresponding to the C terminus of bovine and human αA-crystallin (22). The cleavage sites, on the carboxyl side of residues 168, 169, and 172, suggested that the reported in vivo truncation of αA-crystallin was due to the action of proteases present in lens extracts. Very recently, the calpain Lp82, which specifically removes the first 5 C-terminal residues of αA-crystallin (23, 24), has been identified in rat and calf lenses (25, 26). This protease, however, appears to be absent from the human lens (27), raising the question of an alternative protease or a non-enzymatic process being responsible for C-terminal degradation. Intriguingly, it has been shown that even in the presence of a complete protease inhibitor, R120G αA-crystallin, a mutant that causes a desmin-related myopathy, is intrinsically unstable in solution, with unfolding of the protein over time leading to aggregation and progressive truncation from the C terminus (28). The occurrence of similar non-enzymatic degradation cannot be ruled out in the case of αA-crystallin.

In the present study, we examined the higher order oligomeric structure of recombinant αA-crystallin by means of nano-electrospray mass spectrometry (MS). We have recently applied this technique to the examination of αB-crystallin, determining the relative populations of the various oligomers that constitute its polydisperse assembly and how they are affected by post-translational modification (17, 29). As αA1–168 is a degradation product found in most mammalian lenses (30), we have also expressed and characterized this truncated protein. Here, we report that αA1–168 differs from αAWT in its oligomeric structure. Significantly, removal of the 5 C-terminal residues had a profound effect upon the subunit exchange dynamics with wild-type protein.

**MATERIALS AND METHODS**

**Preparation of αA-Crystallins**—PCR technology was used for generation of αA1–168. DNA of αAWT was used as a template for the PCR amplification, and the following set of oligonucleotide primers was used: forward, 5′-GAATCTCATATGGATATCGCCATTCAGCAC-3′, and reverse, 5′-GATATCGATTGAGTTCAAGTCTC-3′.

Two restriction sites were added to the PCR product, an NdeI site at the 5′ and an XhoI site at the 3′. The expression vector pET21b (+) was linearized with NdeI and XhoI and subsequently digested with the gel-purified PCR product using the same cohesive ends. The ligation product was then transformed into Escherichia coli One Shot cells by standard methods. After amplification of the construct, plasmid DNA was extracted using the QiAprep Spin system. The DNA was digested with NdeI and XhoI and analyzed by gel electrophoresis. Clones containing the DNA insert of αA1–168 were sequenced to confirm the presence of the DNA insert. Plasmid DNA of αA1–168 was introduced into the BL 21(DE3) strain of E. coli by standard methods. The cells were grown in Luria-Bertani medium at 37 °C for 3 h following induction with isopropyl-l-thio-β-D-galactopyranoside at a final concentration of 0.5 mM. Cells were pelleted by centrifugation at 8000 × g for 10 min. The pellet was washed with fresh Luria-Bertani medium. The bacteria were disrupted at 37 °C for 15 min with lysis buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 8.0) containing lysozyme (100 μg/ml). Protein purification was by methods described previously (31).

**Size Exclusion Chromatography-Light Scattering**—The native proteins were analyzed by using size exclusion chromatography with online light scattering, absorbance, and refractive index detectors (32, 33). A Superose 6HR 10/30 column (Amersham Biosciences) was connected in line to a UV detector (Amersham Biosciences UV-900), a Dawn-EOS (Wyatt Technology Corp.) multiangle laser light scattering detector, and an Optilab DSP (Wyatt Technology Corp.) refractive index detector. Samples were loaded onto the column at a concentration of 1.5 mg/ml and eluted with 200 mM ammonium acetate, 1 mM dithiothreitol, pH 7.0.

**Mass Spectrometry Analysis**—Approximately 1 mg of the purified and concentrated protein (50 mg/ml) was buffer-exchanged by loading onto a Superose 6 PC 3.2/30 size exclusion column (Amersham Biosciences) and eluting at 0.07 ml/min with 200 mM ammonium acetate, at 5 °C. Fractions corresponding to the peak elution volume were pooled and analyzed directly.

Nanoelectrospray mass spectrometry experiments were performed on a Q-ToF 2 instrument (Micromass UK Ltd.) that has been modified for high mass operation (34). Conditions were carefully chosen to allow the ionization and detection of the αA-crystallins without disrupting the non-covalent interactions that maintain the quaternary structure. To dissociate the oligomers, ions of the selected m/z range were collided with argon atoms in the collision cell of the mass spectrometer. The asymmetric distribution of charge between the products allowed the unambiguous identification of the products. The experimental conditions employed for the recording of these MS and tandem MS spectra were the same as those described previously (29).

Subunit exchange analyses were performed at 43 °C using a thermocoupled nanoelectrospray probe, designed in-house (35). The proteins were buffer-exchanged into 200 mM ammonium acetate, and concentrations were standardized to 1.0 mg/ml by UV absorbance, assuming a molar extinction coefficient of 17,000 cm−1 M−1 for each protein in solution. Equal volumes of the proteins to be analyzed were mixed immediately prior to analysis, and acquisition of spectra was initiated within a 60-s equilibration period. Spectra were acquired as described above. Subunit exchange rates were calculated by measuring the decay of homooligomers as a function of time. This was achieved by monitoring the signal intensity over an m/z range 4 Thompsons wide, centered over the midpoint of the homo-oligomer peaks at the start of exchange. Regression analysis was performed using SigmaPlot software (SPSS Science).

**Chaperone Assay**—The chaperone efficiencies of αA1–168, αAWT, and a 1:1 mixture of the two proteins toward reduced α-lactalbumin were compared by using a modified assay (17). Prior to the assay, a 1:1 mixture of the proteins was incubated at 43 °C for 135 min to ensure complete subunit exchange. A-α-lactalbumin was then mixed with αA1–168, αAWT, and the 1:1 mixture at final concentrations of 0.080 and 0.032 mg/ml in the presence of 20 mM dithiothreitol. The final sample volumes were 500 μl, in 200 mM ammonium acetate. The samples, as well as a control of apo-α-lactalbumin, were placed in identical cuvettes in a heated (43 °C) multicell block. Their apparent absorbance at 360 nm was monitored in parallel over a 30-min period in a Cary 500 Scan spectrophotometer.

**RESULTS**

**Effect of C-terminal Truncation on Oligomeric Size of αA-Crystallin**—Two separate approaches were used to investigate the oligomeric size of αAWT and αA1–168: size exclusion chromatography/multiangle light scattering (SEC-MALS) and nanoelectrospray-MS under non-dissociating conditions. Figure 1A shows the SEC-MALS profiles of αAWT and the αA1–168 truncation mutant. Both proteins eluted at single symmetrical peaks, but αA1–168 eluted slightly later than αAWT. The calculated molar masses for species eluting at the top of each peak were 540 kDa for αAWT and 440 kDa for αA1–168. The slightly broader peak width of αA1–168 compared to αAWT suggested that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass. In agreement with the SEC-MALS data, αA1–168 also appeared to be slightly less polydisperse than αAWT, suggesting that it is of lower average molecular mass.

**Effect of C-terminal Truncation on Oligomeric Assembly of αA-Crystallin**—Neither the SEC-MALS nor the MS experiments described above were capable of distinguishing between the different species within a polydisperse sample. We have, however, recently developed a tandem mass spectrometry (MS/MS) approach that allows us to characterize the oligomers that...
constitute the overall assembly and to quantify their individual contributions (17, 29). MS/MS involves the selection of ions in a defined m/z window, prior to collision-induced dissociation with a neutral gas. In this case, the m/z window was chosen to select the peak corresponding to all oligomeric species, in which each carries an average of two positive charges per subunit (i.e. [(n)mer]^{2+}) (29). During the MS/MS process, monomers dissociate asymmetrically from the oligomers, giving rise to spectra in which highly charged monomers are observed at low m/z, whereas oligomers, stripped of up to three subunits, give rise to signals at high m/z values (29) (Fig. 2A). The region of the spectrum corresponding to oligomers from which two subunits have been dissociated in the MS/MS analysis of αAWT and αA1–168 is presented in Fig. 2B. These doubly stripped oligomers are sufficiently separated to enable the assignment of charge states to the peaks. Given that they originated from oligomers with two additional subunits, we are able to calculate the range and relative population of species in the sample (Fig. 2C). The major oligomers present in αWT ranged from 22 to 30 with a mean oligomeric size of 25.7 subunits. αA1–168, on the other hand, consisted of oligomers in the lower range of 20–27 with an accompanying lower mean size of 23.1 subunits. This is in good agreement with the SEC-MALS data, which find αWT and αA1–168 to be composed, on average, of 27 and 22 subunits, respectively. Moreover, both proteins exhibited a preference to form oligomers containing an even number of monomers, a characteristic we have previously observed in recombinant αA-crystallin (17). Interestingly, the level of this “dimeric preference” appears to be slightly greater in the case of αA1–168.

Effect of C-terminal Truncation on Subunit Dynamics of αA-Crystallin—Previous measurements of subunit exchange in α-crystallin have been performed using fluorescence resonance energy transfer after prelabeling the protein with donor and receptor molecules (36, 37). This technique requires that labeling be performed for extensive periods at 22 and 37 °C prior to the initiation of subunit exchange. The use of MS in the analysis of subunit exchange has the advantage of being able to use unmodified protein without prior incubation at elevated temperature. A study of the exchange kinetics of monodisperse plant sHSPs demonstrated that MS is a powerful approach for the real-time analysis of transient species and their relative populations during the subunit exchange of multimeric protein complexes (38). The application of this technique to the α-crystallins required a different approach due to the spectral complexity that arises from their polydispersity. Here, we have developed a technique in which the mass of the major overlapping peak in the doubly stripped oligomer region is monitored. In this way, it is possible to follow gross changes in the subunit composition of two oligomers over time. This is due to the fact that the major overlapping peak for a homo-oligomer in this region occurs at an m/z value equal to the mass of the monomer, i.e. where all oligomers carry one charge per subunit (29). Thus, at the first time point after preparing an equimolar mixture of αAWT and αBWT, two distinct peaks at 19790 and 20160 m/z were observed, which correspond to the monomeric mass of the two proteins, respectively (Fig. 3A). Over time, these peaks broadened and converged to a midpoint corresponding to fully exchanged hetero-oligomers of αA and αB: a single peak centered around 19 975 m/z (the average mass of the two subunits) after ~30 min of exchange at 45 °C. This confirms that, on average, the hetero-oligomers formed contain an approximately equal number of the two different subunits.

In subunit exchange experiments in which αA1–168 was mixed with either αAWT or αBWT, very different exchange dynamics were observed. Fig. 3B shows spectra acquired during the same time period for subunit exchange of αA1–168 with αAWT. Again, at the start of the experiment, peaks for each protein were present at m/z values equivalent to their monomer masses. However, unlike exchange between the full-length proteins, after 30 min, little subunit exchange had occurred. Full exchange was not achieved until ~80 min after mixing (not shown). Similarly, exchange between αA1–168 and αBWT (Fig. 3C) was also found to be much slower than for both full-length proteins. By plotting the decrease in intensity of the various homo-oligomers, we obtained graphs that allowed us to determine the rates of subunit exchange (Fig. 4). First order rate constants of 8.9 × 10^{-2} min^{-1}, 3.4 × 10^{-2} min^{-1}, and 4.3 × 10^{-2} min^{-1} were calculated for the exchange of αAWT:αPT, αA1–168:αAWT, and αA1–168:αBWT, respectively. Thus, a 3-fold decrease in exchange rate when αA1–168 is involved demonstrates that truncation of the αA-crystallin C terminus dramatically compromises the dynamics of this protein.

Spectra recorded during the subunit exchange process, in the region corresponding to the monomers dissociated from the oligomers during the tandem MS process, were also examined (Fig. 5). The spectrum acquired after 2 min of exchange showed a clear preponderance of charge states arising from αAWT monomers. Since these protein subunits are released as a result of collisional activation of the oligomers, this observation suggests that under these gas-phase conditions, αAWT is a less stable oligomer than αBWT. Over the course of the experiment, however, a marked shift in the relative levels of dissociated species was observed such that after 24 min, αBWT monomers accounted for 75% of the monomer population (Fig. 5). Thus, as the homo-oligomers mixed to form equilibrated hetero-oligomers, the lability of αBWT relative to αAWT increased greater than 2-fold. The same trend was not observed in the case of the αA1–168:αAWT mixtures (data not shown), suggesting that αAWT and αBWT may occupy quite distinct regions in the quaternary arrangement of total α-crystallin oligomers.
Effect of C-terminal Truncation on Chaperone Function of αA-Crystallin—As this truncation is found in vivo, and we have shown that it does form hetero-oligomers with the full-length α-crystallins, albeit on a slower time scale, a pertinent factor is whether these hetero-oligomers have altered chaperone function as compared with the wild-type proteins. The chaperone efficacy of αA1–168 versus αAWT has been compared previously, with no difference between the two proteins reported (39). We repeated these experiments in phosphate-buffered saline, and our results agree with this observation (data not shown). For completeness, however, we also performed chaperone assays in ammonium acetate, the same buffer as that used for the subunit exchange MS. These assays compared the ability of αAWT, αA1–168, or a 1:1 equilibrated mixture of the two to prevent the aggregation of reduced α-lactalbumin at 43 °C (Fig. 6). The results show that a 1:10 w/w ratio of chaperone to substrate was sufficient in all cases to provide almost complete protection against aggregation over a 30-min period. Upon reducing this ratio to 1:25 w/w, partial protection against aggregation was still conferred to the substrate as compared with the control, and no significant difference in chaperone efficacy was observed between αAWT, αA1–168, or the 1:1 equilibrated mixture. Taken together with the subunit exchange data (Figs. 4 and 5), this is an important finding that demonstrates that chaperone activity is not directly related to subunit dynamics.

**DISCUSSION**

We have examined the effects of truncation of the 5 C-terminal residues on the oligomerization, subunit exchange, and chaperone-like function of recombinant αA-crystallin. It has been reported previously that the oligomeric state of αAWT is not affected by this truncation; however, we found αA1–168 to have an average molecular mass of ~80 kDa less than αAWT. Moreover, the modified protein displayed a slight reduction in polydispersity and an increased preference to form oligomers with an even number of subunits as compared with wild type. It is thought that the C-terminal extensions of the mammalian sHSPs may be responsible for their relative polydispersity as compared with those of plant, yeast, and bacterial origin (40). In fact, consistent with this thesis, it has been reported that deletion of 11 or more C-terminal residues results in the formation of an octameric species of αA-crystallin (39). Clearly, αA1–168 exhibited no such homogeneity, but the decrease in size and polydispersity does support the C termini as being determinants of polydispersity. We have shown previously that perturbation of the N terminus of β-crystallin, by phosphorylation at Ser-45, leads to an increase in polydispersity and a decrease in dimeric preference (17). This is in contrast to the observation here that C-terminal perturbation causes a decrease in polydispersity and an increase in dimeric preference. Taking these results together, we propose that the N termini of αA-crystallin...
the α-crystallins are responsible for dimeric interactions, whereas the C termini regulate global quaternary structure and variability.

Truncation was found to not only affect oligomeric organization but also to have a profound impact on the dynamics of the crystallin assemblies. Subunit exchange experiments revealed that although a mixture of αA_WT and αB_WT reached equilibrium within a 30-min time period, when the exchange involved αA1-168, there was a marked decrease in the rate of reaction. Thus, the C terminus of αA-crystallin plays an important role in subunit exchange dynamics. The mechanisms involved in this process are not clear, but NMR studies have shown that αA- and αB-crystallin possess highly flexible and solvent-accessible C-terminal extensions of 8 and 10 amino acid residues, respectively (41). These tails may act as “keys” in opening up an exchange pathway, their hydrophilicity encouraging the detachment of suboligomeric species, which are then free to interact with different oligomers. This suggests that the retarded rate of exchange observed upon removal of the last few residues of these tails (SAPSS in the case of αA_WT) is due to an increase in the activation energy of the dissociation process.

Subunit exchange of the sHSPs is a first order process (36–38), and consequently, dissociation of the oligomers into suboligomeric species is rate-determining. This means that the reduced subunit exchange dynamics of αA1-168 relative to αA_WT lead to a decrease in the concentration of these suboligomeric species in solution. The assays performed here and previously (39), however, do not reveal a difference in the chaperone efficiency among αA_WT, αA1-168, or indeed a mixture of the two. This implies that there is no correlation between the concentration of free suboligomeric species and chaperone activity, and therefore, for these proteins, it is the intact oligomers that are the functional chaperoning units.

This conclusion contrasts with that reached for the non-mammalian sHSPs, in which it has been proposed that dimers are the active units involved in chaperone activity (42, 43). It may, however, also explain the observation that, unlike plant (35) and yeast (42) sHSPs, the α-crystallins do not dissociate at heat-shock temperatures (44). These observations highlight that despite the many similarities between members of the sHSP family, there are important differences too. The dissimilarity displayed in the chaperone mechanism between the non-mammalian sHSPs and the α-crystallins may well be due to the environmental conditions to which the host organism is exposed. Mammals have a highly evolved body temperature regulatory system in which temperatures more than a few
other hand, bacteria and plants in particular are exposed to climatic conditions involving huge fluctuations in ambient and thus cellular temperature. It is perhaps due to this that the latter have sHSPs that can respond, through dissociative mechanisms, to extremes of temperature, whereas this function is not required for the mammalian sHSPs.

The primary function of subunit exchange in α-crystallin may in fact be to obtain the optimum ratio and arrangement of αA to αB subunits (or with the other sHSPs outside of the lens) for the specific cellular conditions and requirements. Evidence for this arises from the observation that during the exchange reaction between the wild-type proteins, monomers of αA_WT became less likely to be dissociated from the hetero-oligomers than αB_WT. This agrees with the suggestion that αA_WT subunits are incorporated predominantly in the core of these mixed assemblies (45, 46), and thus, that favored quaternary arrangements of these hetero-oligomers exist. The dynamics that we observe through subunit exchange may in fact not be required for the proper function of a specific oligomer but rather are necessary for the formation of the most efficient hetero-oligomer for a specific cellular task.

The αA_{1-168} truncation has been found exclusively in the water-insoluble fraction of the human lens (11, 19), suggesting that it may compromise the function of α-crystallin due to a reduction in solubility, brought about by the removal of the hydrophilic C terminus. Furthermore, higher levels of C-terminally truncated α-crystallin have been found in cataractous as compared with normal human lenses (47), implicating this degradation product in age-related nuclear cataract. Although αA_{1-168} appears to have similar chaperone function to αA_WT in vitro, we have demonstrated in this study that the subunit dynamics of the oligomers are severely impaired by this truncation, and it is this that may have profound consequences in the crowded milieu of the cell interior.

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