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Viral capsid assembly as a model for protein aggregation diseases: Active processes catalyzed by cellular assembly machines comprising novel drug targets

Rita Marreiros[a], Andreas Müller-Schiffmann[a], Verian Bader[a], Suganya Selvarajah[b], Debendranath Dey[b], Vishwanath R. Lingappa[b], Carsten Korth[a,*]

[a] Department Neuropathology, Heinrich Heine University Düsseldorf Medical School, Moorenstrasse 5, 40225 Düsseldorf, Germany
[b] Prosetta Biosciences, Inc, San Francisco, CA 94107, USA

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ABSTRACT

Viruses can be conceptualized as self-replicating multiprotein assemblies, containing coding nucleic acids. Viruses have evolved to exploit host cellular components including enzymes to ensure their replicative life cycle. New findings indicate that also viral capsid proteins recruit host factors to accelerate their assembly. These assembly machines are RNA-containing multiprotein complexes whose composition is governed by allosteric sites. In the event of viral infection, the assembly machines are recruited to support the virus over the host and are modified to achieve that goal. Stress granules and processing bodies may represent collections of such assembly machines, readily visible by microscopy but biochemically labile and difficult to isolate by fractionation.

We hypothesize that the assembly of protein multimers such as encountered in neurodegenerative or other protein conformational diseases, is also catalyzed by assembly machines. In the case of viral infection, the assembly machines have been modified by the virus to meet the virus' need for rapid capsid assembly rather than host homeostasis. In the case of the neurodegenerative diseases, it is the monomers and/or low n oligomers of the so-called aggregated proteins that are substrates of assembly machines. Examples for substrates are amyloid β peptide (Aβ) and tau in Alzheimer's disease, α-synuclein in Parkinson's disease, prions in the prion diseases, Disrupted-in-schizophrenia 1 (DISC1) in subsets of chronic mental illnesses, and others. A likely continuum between virus capsid assembly and cell-to-cell transmissibility of aggregated proteins is remarkable. Protein aggregation diseases may represent dysfunction and dysregulation of these assembly machines analogous to the aberrations induced by viral infection in which cellular homeostasis is pathologically reprogrammed. In this view, as for viral infection, reset of assembly machines to normal homeostasis should be the goal of protein aggregation therapeutics.

A key basis for the commonality between viral and neurodegenerative disease aggregation is a broader definition of *assembly* as more than just simple aggregation, particularly suited for the crowded cytoplasm. The assembly machines are collections of proteins that catalytically accelerate an assembly reaction that would occur spontaneously but too slowly to be relevant *in vivo*. Being an enzyme complex with a functional allosteric site, appropriated for a non-physiological purpose (e.g. viral infection or conformational disease), these assembly machines present a superior pharmacological target because inhibition of their active site will amplify an effect on their substrate reaction. Here, we present this hypothesis based on recent proof-of-principle studies against Aβ assembly relevant in Alzheimer's disease.

Proteins have evolved to execute multiple functions, amongst them the prominent function of catalysis, i.e. the acceleration of a chemical reaction without changing its final equilibrium. Biochemical reactions occur spontaneously if given enough time, but their kinetic facilitation by enzymes greatly accelerates evolvability of biological processes themselves which is why, ultimately, they were selected by evolution (Kirschner and Gerhart, 1998).

Proteins are generated in the cell through translating mRNA according to the genetic code on multiprotein assemblies containing RNAs, called ribosomes, followed by posttranslational

* Corresponding author. Tel.: +49 211 8116153; fax: +49 211 8117804.
E-mail address: ckorth@hhu.de (C. Korth).

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modifications by virtue of a remarkable diversity of more or less complex energy-requiring processes (Xue and Barna, 2012). Complementing Anfinsen's paradigm of sequence-encoded spontaneous (Anfinsen, 1973), and unassisted folding of a protein notwithstanding, two critical dimensions of this process require enzymic catalysis to be fully explained. The first is how these processes proceed on the timescale observed in living cells, and the second is how they can occur in the crowded environment within the cell, where interacting proteins cannot find themselves as readily as it would occur for purified proteins in isolation (Fulton, 1982; Alberts, 1998; Hartl et al., 2011).

Full functionality of a protein is achieved after a series of posttranslational modifications that occur simultaneously with or after protein biogenesis. These can include covalent modifications like glycosylation, sumoylation and others, but also folding into different conformers or multimerization. For posttranslational modifications such as glycosylation, the host glycosyl transferases that catalyze the addition of carbohydrate moieties to defined amino acids within a recognition motif (Dennis et al., 1999) are known. However, host factors regulating the folding of a protein into different conformations (Lingappa et al., 2002) or multimers (Fink, 1999) are less well understood. One reason for this could be that the enzymatic activity for protein multimerization is not performed by a single protein molecule as for glycosylation but by a transient and labile complex of proteins that are difficult to detect as such. Put another way, if the ribosome, itself a multiprotein complex RNA-containing covalent assembly machine, were as unstable as hypothesized here for non-covalent assembly machines, we might still not understand how proteins are made.

In the course of our studies we have noted a striking similarity between new findings in virus capsid assembly (Gay and Neuman, 2013; Lingappa et al., 2013a,b) and endogenous protein aggregation. This leads us to speculate that much of what has been conventionally viewed as spontaneous protein *aggregation* may in fact be catalyzed protein *assembly*. By analogy to viral capsid formation, long viewed as self-assembly, and only more recently recognized as the result of host catalysis through the action of labile multiprotein complexes, perhaps diseases of protein aggregation are initiated by action of aberrant assembly machines and their disordered or dysregulated assembly intermediate products. The novel notion that viral capsid assembly is an active process executed by host factors that may be similar, overlapping or completely different from host factors promoting assembly of other endogenous host proteins, including those involved key events in neurodegenerative diseases, has consequences for drug discovery strategies – it may greatly accelerate drug discovery by directing research in a completely new direction from the current dominant focus. What we should be asking then, is what are the catalysts that bring this about, how are their functions different from the catalysis that occurs under physiological, homeostatic conditions, and how can that dysfunctional non-homeostatsis catalysis be normalized.

1. Virus capsid assembly

Viruses can be considered as dynamic molecular assemblies, containing a nucleic acid genome that is enclosed in a protein capsid shell. In many cases a lipid envelope is also observed. Typically, the envelope also contains the protein(s) that bind to cell surface receptors to target the virus. Some viral families (e.g. the *Picornaviridae* and *Enteroviridae*) have no envelope: for them, the capsid *is* the virus.

Through evolutionary selection pressure favoring the fastest generation cycles, virus components have evolved to manipulate host cellular machinery for rapid and optimal replication (Dimmock et al., 2007; Prasad and Schmid, 2012). By virtue of their fast replication cycles they have, in effect, discovered all cellular "niches" that can be exploited to their advantages. That diverse viral families appear to have chosen distinctive pathways of host-catalyzed capsid assembly is remarkable (Lingappa et al., 2013b): it suggests that such reprogramming of assembly machines is highly profitable for viruses and therefore may be an inherent weakness of metazoan biology–the price we pay for such complex organ systems such as the central nervous system (CNS) in constant need of repair and attention, and therefore at constant risk of subversion. And since protein machines are likely themselves built by protein machines, the potential for "prion-like" epigenetic propagation of dysfunctional assembly may exist.

Viral replication and propagation is a process of alternating cycles with viral invasion of suitable cells, release of virus genome into the cytosol where encoded viral components are replicated and, finally, assembly of new infectious particles before their release from the host cell, subsequently possibly infecting other host cells and re-initiating the replication cycle (Mateu, 2013b). The viral capsid consists of a protein shell that serves as a protection of the virus genome and encodes invasion and release signals.

Several different stages in virus morphogenesis can be distinguished such as capsid assembly, nucleic acid packaging, and virus particle maturation. Here, the focus will be on capsid assembly. The size and shape of capsid structure is in general regular like symmetric oligomers made by assembling of capsid protein subunits (Klug, 1999; Prasad and Schmid, 2012). The individual protein subunits are asymmetrical, but they are assembled to form symmetrical structures. Different viruses can build their capsids with a different number of capsid protein subunits. The most abundant type of capsids throughout virus families are helical and icosahedral capsids. Theoretically, the structure of helical capsid is extremely simple, since the number of capsid protein subunits required is extendable, depending only the length of nucleic acid genome that needs to be encapsulated. On the other hand, the small icosahedral capsids are made exactly with 60 capsid protein subunits that limit the size of nucleic acid genome that can be enclosed. The larger icosahedral capsids can be made using 60 multiples of subunits of a capsid protein (Amos and Finch, 2004; Carter and Saunders, 2007). The intracellular compartments where this process occurs are denominated "viral factories" (Novoa et al., 2005) (see below).

So far, capsid assembly has mainly been viewed as a spontaneously occurring process of self-assembly (Zlotnick, 2005), dependent only on the presence of capsid protein subunits themselves (Johnson et al., 2005; Mateu, 2013a; Prevelige, 1998). Thermodynamically, viral capsid assembly is described in two states, the dissociated state where monomers of the capsid protein are found, and the associated state where the capsid is formed (Fig. 1a). Energetically, this assembly process follows a sigmoidal curve with a lag phase where the concentration of the proteins that compose the capsid determines the speed of the reaction rate. Off-pathway reactions can occur leading to aberrant capsid formation where the quaternary structure is not native (Endres and Zlotnick, 2002; Zlotnick, 2003). In the self-assembly of complex icosahedral capsids, different thermodynamic kinetic models have been proposed consisting of a lag phase, an equilibrium phase, and an elongation phase with the formation of new virus capsid nuclei, that initiate viral capsid assembly (Endres and Zlotnick, 2002; Zlotnick, 2005). *In vitro* experiments are useful to understand the self-assembly of capsids, but do not take into account the interaction with scaffolding proteins or viral nucleic acids that are present *in vivo*. More specifically, because something can happen spontaneously does not mean that *in vivo* it is not accelerated by catalysis.

Recently, findings comprising various viral families (Klein et al., 2004, 2011; Lingappa et al., 2006, 2013a,b; Zimmerman et al., 2002) suggest that the cellular pathway to capsid assembly may be

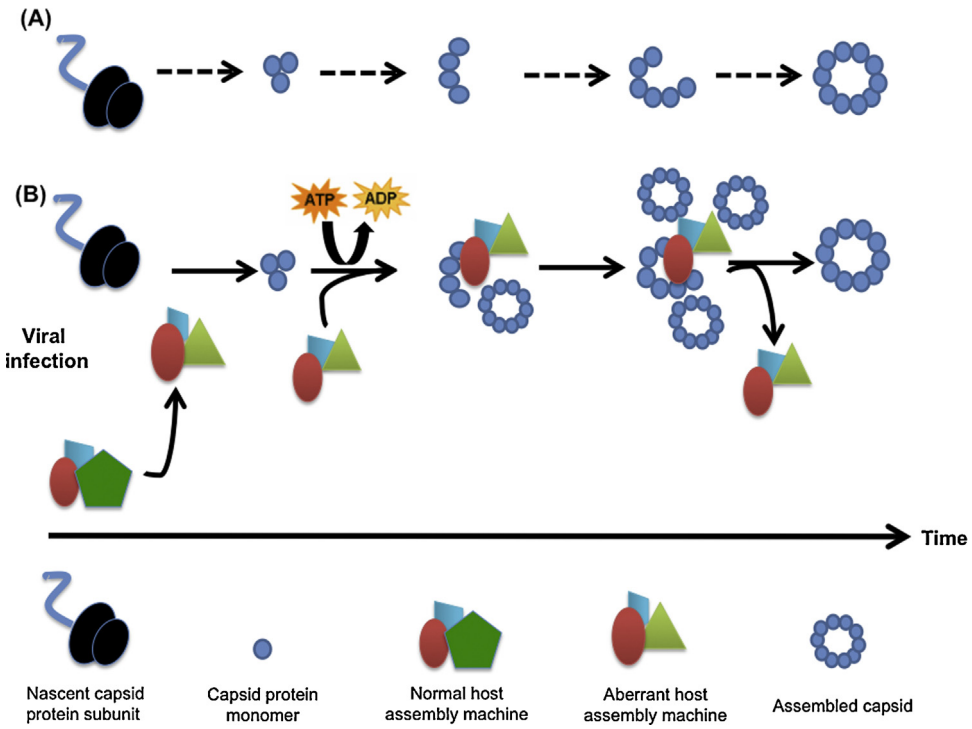

Fig. 1. Representative scheme of the strategies for the assembly of virus capsids. (A) Unassisted capsid self-assembly. Note that the lines are dashed because capsids would form by a progressive accretion of monomers rather than via discrete assembly intermediates. (B) Capsid assembly is catalyzed by host proteins serving as aberrant assembly machines modified from their normal homeostatic assembly-related functions, and is an energy-dependent process occurring via discrete assembly intermediates, perhaps with the participation of different assembly machines for distinct steps.

substantially different from the foundational self-assembly models that conform to the minimum requirements of thermodynamics. Indeed, without such catalytic "add ons" viral propagation might not be possible, given the obstacles not only of cytoplasmic crowding but also of innate immune mechanisms. Specifically, it has been suggested that capsid assembly is accelerated by transient formation of virus-recruited multiprotein complexes with enzymatic activity that serve as "assembly machines" to accelerate capsid formation (Lingappa et al., 2013a,b). This catalyzed viral capsid assembly was demonstrated to be an energy-dependent process, and host protein factors seem to play a major role (Lingappa et al., 2013a,b). These novel assembly machines, perhaps together with more classical notions of molecular chaperones and/or other scaffold proteins, would have an essential role in directing newly synthesized capsid protein subunits through discrete assembly intermediates culminating in capsid formation (Fig. 1b). The catalytic nature of this reaction was deducted from the absence of co-factors in the final capsid and the process and its energy-dependence (Lingappa et al., 1994). A detailed investigation of this new pathway of viral capsid assembly will be highly relevant to develop novel pharmaceutical targets for antiviral drugs (Gay and Neuman, 2013; Lingappa et al., 2013a) (see below).

2. Protein conformational disorders

A hallmark of neurodegenerative disorders is the accumulation of disease-specific proteins in the brain (Taylor et al., 2002). Remarkably, the same proteins accumulate in sporadic and in inherited forms of neurodegenerative diseases (Prusiner, 2001). These disorders are therefore seen as protein conformational diseases where multimerization of a specific protein is massively out of balance, with the ultimate consequence of fatal neuronal loss.

Over the last thirty years, specific proteins identified as aggregated or insoluble in biochemical purification procedures have been identified in these diseases (Table 1), like Aβ or tau for Alzheimer's disease (AD), α-synuclein for Parkinson's disease (PD), superoxide dismutase 1 in amyotrophic lateral sclerosis, huntingtin in Huntington's disease (for review see, for example (Prusiner, 2001), or Disrupted-in-schizophrenia 1 (DISC1) in subsets of chronic mental illnesses (Korth, 2012; Leliveld et al., 2008; Ottis et al., 2011).

The classical prion diseases like Creutzfeldt-Jakob disease in humans or scrapie in sheep are caused by an alternatively folded, disease-associated protein, termed PrP^{Sc}, that induces conversion of "normally" folded substrate conformers, termed PrP^{C}, into the disease-associated conformer thereby initiating a chain-reaction type of replication of the alternatively, disease-associated conformation which triggers a neurotoxic cascade (Prusiner, 1998). Initially thought to be unique to the specific, extracellular prions, this concept of cell-to-cell transmission is now seen also in other protein aggregates, including yeast prions, Aβ and cytosolic proteins such as α-synuclein, tau, polyglutamine diseases, or DISC1 assemblies (Auli et al., 2014; Bader et al., 2012; Clavaguera et al., 2009; Desplats et al., 2009; Domert et al., 2014; Grad et al., 2011; Korth, 2012; Lee et al., 2010; Li et al., 2008; Munch et al., 2011; Ottis et al., 2011; Ren et al., 2009).

Thus, compelling *in vitro* and *in vivo* evidence demonstrates that prion replication of protein aggregates is a general biological feature. Even though the misfolding pathway of most of these proteins (Table 1) can be modeled in cell-free *in vitro* systems with exclusively synthetic or purified protein present, an efficient replication *in vivo* in the context of a crowded, complex cytosol is likely to involve assembly-assisting molecules or multimolecular complexes. These are potentially energy-dependent as many steps in cellular protein folding from protein biogenesis to protein assembly are such energy-dependent, active processes, perhaps not unlike that described for capsid assembly above.

Spontaneous folding as originally conceived by Anfinsen (1973) seems to be mainly caused by solvophobic interactions of polar water molecules with hydrophobic side chains of proteins. In the

Table 1
Protein confromational diseases associated with protein misfolding and amyloid aggregation.

Disease	Protein involved	Cellular localization of aggregated protein	Possible aggregation caused
Prion diseases	PrP	Extracellular	Mutations in prion protein gene, spontaneous conversion[a]
AD	Aβ	Extracellular	Mutations in APP gene and/or APP cleavage enzymes in prion protein gene, increase in Aβ generation[a]
	Tau protein	Intracellular	Post-translational modifications of tau protein[a]
PD	α-Synuclein	Intraneuronal	Mutations or of α-synuclein or other genes, toxic effects on dopaminergic neurons[a]
Amyotrophic lateral sclerosis	Superoxide dismutase 1	Intracellular	Mutation in Cu/Zn superoxide dismutase[a]
Huntington's disease	Huntingtin	Intracellular	Mutation in huntingtin gene with CAG expansion[a]
Tauopathies, Frontotemporal dementias	Tau, TDP-43	Intracellular	Mutations in Tau[a], TDP-43
Familial amyloidotic polyneuropathy	Transthyretin	Extracellular	Mutations in Transthyretin[a]

[a] Associated with gene mutations, or other alterations, the amyloid can also arise following disruption in the clearance mechanisms of the cell.

resulting dimensional structure of a well folded protein, the majority of hydrophobic side chains are buried in the internal core, while the hydrophilic residues are exposed to polar water molecules (Dyson and P.E., 2005; Uversky et al., 2000). However, even in cell-free *in vitro* systems, evidence against the Anfinsen dogma has been demonstrated: proteins that normally function in globular states can also adopt intermediate conformations (Waudby et al., 2013). Some proteins that have a correct folded structure, can also adopt a subsequently unfolded structure, and as a consequence display dynamic structural fluctuations (Vendruscolo and Dobson, 2013). Upon cellular stress, incorrect protein folding may occur, resulting in aggregation, loss of function or association with other cellular components (gain of function), leading to dramatic changes in the biological functions and homeostasis of the cell (Dobson, 2004; Sgarbossa, 2012).

In order to ensure that cellular integrity is maintained, the chaperone machinery plays a key role in securing correct protein folding. This is executed by reversible binding to unfolded and misfolded proteins, accelerating formation of a correct conformation of a non-native protein in an energy-dependent manner. Proteins that are not correctly folded may also be removed from the cell by the ubiquitin-proteasome system or autophagy, depending on its size (Hartl and Hayer-Hartl, 2002; Lee et al., 2013). Misfolded proteins that escape from the cellular cleaning mechanism tend to aggregate in insoluble clusters, in the extreme cases of neurodegenerative disease packaged into fibrils or amyloid (Knowles et al., 2014; Stefani and Dobson, 2003).

The amyloid fold of a protein is a particular fold because it is one of the tightest ways a protein can be packaged (Greenwald and Riek, 2010). Patterns of amyloid fibrils observed in studies using X-ray diffraction demonstrated that the core of each protofilament adopts a cross-β-structure, in which β-strands form effectively continuous hydrogen-bonded β-sheets run along the length of the fibril (Jimenez et al., 2002; Sunde et al., 1997). *In vivo*, amyloid fibrils can also be characterized by the binding affinity to Congo red dye, showing green birefringence when viewed in polarization microscopy (Glenner et al., 1972; Sipe et al., 2010; Wolman and Bubis, 1965).

The process where proteins are converted from functional, globular and soluble folds to the amyloid fold is a complex phenomenon where multiple precursor species are present (Apetri et al., 2006). In this process, nucleated polymerization is a crucial step for the formation of aggregates from soluble precursor species (Nelson et al., 2005). In the first phase of this process, called lag phase, nucleation occurs through interaction between monomers that expose their amyloid-forming segments at the same time and at sufficient concentration for bonding and templating of the fibril pattern (Eisenberg and Jucker, 2012). Nucleation is considered a rare event that is considered to be a slow process and not frequent for intracellular proteins. Once the aggregate nucleus is formed, a rapid growth phase occurs and the single molecules will end up forming β-sheet rich structures in a thermodynamically favorable process, called elongation phase (Eisenberg and Jucker, 2012; Jarrett and Lansbury, 1993). Subsequently, the amyloid process reaches a plateau, the saturation phase, wherein the total quantity of protein is in equilibrium (Nelson et al., 2005). The susceptibility of proteins to form amyloid structures is correlated to their chemical properties such as net charge, secondary structure, hydrophobicity and aromatic interactions (Chiti et al., 2003).

Remarkably, not all amyloid proteins are toxic, but some even execute physiological functions (for review see Greenwald and Riek, 2010). In bacteria, fungi and yeast, amyloid species can function in formation of biofilms, helping in host invasion, in the modulation of fungal adhesion and promotion of an antiviral innate immune response (Chapman et al., 2002; Fowler et al., 2007; Hou et al., 2011). In mammals, these protein species are involved in skin pigmentation and some hormones adopt the amyloid structure when stored in secretory granules (Maji et al., 2009).

Amyloidogenic HetS protein has a function in fungal self/non-self recognition (Seuring et al., 2012; Wasmer et al., 2008), and in the invertebrate Aplysia, CPEB amyloid plays a role in regulating synaptic strength (Si et al., 2010, 2003).

Even though the presence of amyloid deposits may be the most striking feature in neurodegenerative diseases, the primary pathogenic agents in these conditions seem to be oligomeric species rather than the fibrils that correlate with clinical symptoms (Lue et al., 1999; McLean et al., 1999; Naslund et al., 2000). These oligomers may fulfill physiological functions and, in a disease context, are observed in the transition phase between protein assembly and aggregation with neurotoxic properties (Cremades et al., 2012; Haass and Selkoe, 2007; Koffie et al., 2009). Oligomer toxicity could in part be caused by their inherently misfolded nature, since the oligomer surfaces display chemical groups that are non-accessible under normal physiological conditions in a normal cellular environment (Cheon et al., 2007). Several different types of oligomeric species can be detected during the aggregation process depending on the nature of the cells where they are formed and correlating with a distinct cellular pathology (Campioni et al., 2010) (Table 1). Fibril formation has also been considered to be part of a cellular defense mechanism against oligomer toxicity (Ross and Poirier, 2004). At any stage during the assembly of protein monomeric educts to low-n oligomers and larger multimers or amyloid could assembly machines assist. For replication of mammalian prions (PrP^{Sc}), early on the existence of cellular cofactors assisting in prion conversion was deduced based on genetic experiments (Kaneko et al., 1997; Telling et al., 1995). It is likely that these are specific assembly machines. It is remarkable that the concept of prion replication has now been extended to many other protein mutlimers

characteristic in particular neurodegenerative diseases (Prusiner, 2013). It is clear that a cellular, catalyzed assembly will greatly accelerate cell-to-cell transmission of an otherwise slowly occurring conversion as demonstrated numerous times in cell-free *in vitro* systems.

The following section will review the evidence for a sharing of host factors or assembly machines between viral (capsid) assembly and endogenous multimer assembly, respectively.

3. Evidence for the use of similar cellular factors for virus assembly and protein assembly

Similarities between viral factories and the cellular localization of protein aggregates in protein conformational diseases support the notion that viruses and endogenous protein assemblies use similar, overlapping or sometimes even same cellular machinery for molecular assembly (Wileman, 2007). Disturbance of the cell by hijacking the cellular machinery for aberrant purposes by either viruses or protein conformational disease creates a proteostasis problem which then results in a specific cellular phenotype of protein inclusions. We now introduce a selection of examples for this.

3.1. Involvement of cellular clearance systems

Cellular structures involved in the removal of protein aggregates localize not only in the cytoplasm but also in the nucleus. These structures are called nuclear domain 10 (ND10) bodies and can contain ubiquitinated proteins and molecular chaperones (Fu et al., 2005). When the degradative capacity of the cell is exceeded or a defect in the mechanisms of clearance of the cell occurs, misfolded proteins are deposited near the microtubule organization center (MTOC) and/or in ND10 bodies and form aggregates (Wileman, 2007). In cells infected with viruses, similar structures are formed by material containing full viruses and virus assembly intermediates designated "viral factories" (Novoa et al., 2005; Wileman, 2006). In both cases, protein aggregates and viral factories are localized near the MTOC, recruiting cellular chaperones, ubiquitin and mitochondria (Wileman, 2006).

Mitochondria are invariably recruited during the viral assembly indicating energy-dependent processes involved in viral replication/assembly. Studies with African swine fever virus (Cobbold et al., 2000) and with Human Immunodeficiency Virus (HIV) Type 1 (Tritel and Resh, 2001) showed that virus assembly is an ATP-dependent process. Similarly, mitochondria are also recruited to the cellular locations of protein aggregation to provide ATP for correcting folding where the chaperone machinery is also of paramount importance (Hartl and Hayer-Hartl, 2002). Expression and colocalization of the chaperone machinery seems to be present during the entire virus assembly process, and, for example, for vaccinia virus, the inhibition of HSP90 chaperone negatively affects virus assembly (Hung et al., 2002). Finally, cell-free systems that reconstitute viral capsid formation *de novo* have revealed its ATP-dependence, suggestive of energy-dependent catalysis (Lingappa et al., 1994, 1997, 2013a) (see below).

The autophagy system is another subcellular structure that is important for the removal of pathogens from the cytoplasm of the cell, in a process termed xenophagy (Gomes and Dikic, 2014; Kirkegaard et al., 2004). Viruses submerged by autophagosomes can be released from cells after fusion of lysosomes with the plasma membrane, just as the clearance of soluble and aggregated forms of protein in protein conformational disorders (Ravikumar and Rubinsztein, 2006; Wileman, 2006). In poliovirus, for example, the suppression of Atg12 and Atg18 genes, both required for autophagy, leads to decreased poliovirus production (Jackson et al., 2005). Similar links exist to other viruses: in HIV type 1, autophagosome formation is a platform for viral assembly (Cheng et al., 2014). During cellular infection with hepatitis B virus, the autophagy machinery from the host is activated and when autophagy is insufficient, production of hepatitis B virus particles is decreased (Li et al., 2011).

Interestingly, it has been observed for a long time that viral infection of cells can induce subcellular, inclusion-like structures that resemble protein aggregates (reviewed in Moshe and Gorovits, 2012). In fact, historically and in the absence of molecular diagnostics, the morphology of inclusions was an important diagnostic phenotype.

For example, Negri bodies (NBs), cytoplasmic inclusions formed during rabies virus infection (Jackson et al., 2001; Kristensson et al., 1996) resemble aggresomes seen in some neurodegenerative disorders. NBs have a functional role in the Rabies virus transcription and replication. The organization of NBs can follow a mechanism similar to cytoplasmic inclusions formed by a mutant form of huntingtin protein in Huntington's disease. These agressomes are composed by a dense core of huntingtin aggregates surrounded by a ring that consists of proteins that are successively recruited from the exterior surface of the dense core (Matsumoto et al., 2006). The NBs can also have a concentric organization due to the association of Toll-like receptors and viral proteins, forming a ring into which proteins can be inserted. Although the complete composition of NBs is still unknown, these structures also accumulate HSP70 and ubiquitinated proteins similar to aggresomes from protein conformational diseases (Menager et al., 2009). However, NBs are not localized at the MTOC where the "viral factories" are preferentially localized, likely because of the lack of involvement of microtubule network in the formation of these structures (Lahaye et al., 2009). Possibly, NBs are a consequence of a cytoplasmic sequestration within a cellular defense mechanism against viral infection involving components of aggresome pathway such as some host factors involved in stress response (Lahaye et al., 2009).

In protein conformational disorders, higher order oligomers and aggregates that are inaccessible to the narrow proteasome opening have a higher dependency of autophagy for the degradation process. Previous studies using knockout mice for *Atg* genes, essential gene for autophagy, revealed that autophagy is a critical process for neruonal health. It was demonstrated contingent with loss of *Atg5* and *Atg7* genes, an age dependent accumulation of intraneuronal aggregates with polyubiquitination, inclusion formation and neurodegeneration occurred (Hara et al., 2006; Komatsu et al., 2006). A change in this process can be one of the key factors involved in the increase of cell aggregates evident in misfolding diseases.

3.2. Direct or indirect interactions of viral proteins with hallmark proteins of protein conformational disease

The similarity of the cellular localization of viral factories and that of endogenous protein aggregates has led to the hypothesis that viral infections increase the risk of protein conformational diseases (De Chiara et al., 2012; Zhou et al., 2013). Likewise, alterations in the CNS related to aging, the increase in oxidative stress and the impairment in energy production can lead to an increased susceptibility of the CNS to infections agents (Mattson, 2004). Taking this into account, external insults, including the toxicity of viral proteins or proteins aggregates, can lead to a vicious circle of mutually increasing the vulnerability of aged neurons.

So far, direct evidence for a mechanistic involvement of such a mutual increase in vulnerability of viral infections and protein conformational diseases is rather sporadic or anecdotal. Chronic exposition to Herpes simplex virus type-1 (HSV-1) has been associated to an increase in the risk of AD by herpes viruses particles specifically recruiting cell membranes from the host containing

amyloid precursor protein (APP) (Bearer, 2004). This process can affect the location and proteolysis of APP, and consequently leads to an increase in toxic Aβ formation, resulting in a neuronal dysfunction associated with the progression of AD (Satpute-Krishnan et al., 2003).

The presence of viral DNA in post-mortem brains of AD patients, mainly in patients that carry the allele of the gene encoding apolipoprotein E, a risk factor for this disease, is one of the facts that supports this hypothesis (Itzhaki et al., 1997). Another study identified an increased phosphorylation of several residues of tau protein due to a HSV-1 infection (Wozniak et al., 2009). Wozniak et al. also demonstrated a higher association between HSV-1 and Aβ plaques in temporal and frontal cortices of AD patient's brains, compared with normal aged brains. Some genome wide association studies have also correlated the brain susceptibility to HSV-1 infection with a genetic risk of AD (Porcellini et al., 2010).

Infection by HIV seems also to be correlated with an increased deposition of Aβ plaques in the brains (Andras and Toborek, 2013). However, the mechanism that triggers this is not fully understood and could be due to the increase of Aβ synthesis (Aksenov et al., 2010), decreased Aβ degradation by the inhibition of neprilysin, an enzyme responsible for Aβ degradation (Rempel & Pulliam, 2005), changing the transport mechanisms across the blood brain barrier (Andras et al., 2010), or a combination of all. The improvement and general well-being of AD patients after antiviral treatment remains to be established (Zhou et al., 2013).

Influenza virus has been implicated in PD when typical symptoms developed in the aftermath of the acute infection. This disease was coincident with encephalitis lethargica causing postencephalitic Parkinsonism, apparently occurring at the same time of influenza A pandemic (1916–1926) (Maurizi, 1985; Poskanzer and Robert, 1963). In addition, it was subsequently shown that individuals born during the influenza pandemic had a two or three-fold increased risk of developing PD compared to individuals born before or after the pandemic (De Chiara et al., 2012). Immunohistochemical and biochemical analyses were performed in different brain regions of patients with postencephalitic parkinsonism and neurofibrillary tangles with hyperphosphorylation of 3R and 4R variant of tau protein were detected. Interestingly, Lewy bodies or other α-synuclein deposits, characteristic features of idiopathic PD, however, were not detected, thus presenting a possibly atypical pathological variant of parkinsonism (Buee-Scherrer et al., 1997; Jellinger, 2009).

Jang et al. demonstrated that in mice infected with A/Vietnam/123/04 influenza virus, viruses can be detected in the CNS. A loss of dopaminergic neurons in substantia nigra, the region most affected in PD, a persistent activation of microglia, an increase in phosphorylation, and aggregation of α-synuclein was detectable—all features of pathological alterations observable in PD patients (Jang et al., 2009). Rohn et al. demonstrated the presence of influenza A virus in macrophages of the substantia nigra of PD patients (Rohn and Catlin, 2011). There is however no direct correlation between presence of the virus and PD symptoms or typical pathology which leaves a mechanistic understanding difficult.

These scattered findings but not systematical investigations show that viral infections of CNS could be causative agents or at least co-factors of some of brain protein conformational diseases.

4. Targeting assembly host factors for pharmacotherapy

Thus, in summary, it is conceivable that not only virus capsids are formed aided by redirected host factors to serve as enzymes of capsid assembly, but that protein aggregation in neurodegenerative diseases is ultimately the result of aberrant catalysis by dyregulated/misdirected assembly machinery. These cellular factors may be transiently assembled and biochemically labile multimeric complexes themselves which is why they may be difficult to detect.

These ideas led us to hypothesize that compounds acting to block the aberrant virally modified assembly machines might prove to be valuable starting points for drug discovery relevant to degenerative diseases not related to viral infections. Thus, for example, the promising compounds or their chemical analogues acting against neurotropic rabies capsid assembly (Lingappa et al., 2013a,b) might also exhibit activity in protein conformational disease, like AD. The assumption would be that the lead drugs would target allosteric sites on similar assembly machines (Tsai et al., 2009).

According to the amyloid cascade hypothesis (Hardy and Selkoe, 2002), AD is triggered by increased production or reduced clearance of the aggregation prone Aβ peptide, in particular $A\beta_{42}$. Especially the formation of synaptotoxic low-n oligomers (dimers and trimers) of Aβ has been correlated with disease progression (Mc Donald et al., 2010). Synaptotoxicity of these oligomers has been demonstrated for naturally-derived, secreted, as well as for synthetic oligomers, with cell-derived Aβ dimers and trimers being invariably up to 100× more toxic than their fully synthetic counterparts (Jin et al., 2011; Reed et al., 2011). Bulk preparation of synthetic Aβ oligomers is generally carried out in non-physiological buffer systems without any cellular co-factors and using high micromolar concentrations of the peptide which leads to a highly heterogenous assembly of unfolded Aβ monomers. Within the cell, the processing of APP as well as N- and C-terminal modifications of the resulting Aβ peptide is tightly controlled by cellular factors (Muller-Schiffmann et al., 2011). Oligomerization of Aβ supported by cellular assembly machines may yield superior biological activity since there is evidence that very low picomolar concentrations of Aβ oligomers positively modulate synaptic plasticity (Puzzo et al., 2008).

If mechanisms of protein assembly between capsids and proteins in neurodegenerative diseases were shared, drugs identified for interfering with viral capsid assembly (Lingappa et al., 2013a,b) should also interfere with assembly of proteins aggregating in neurodegenerative diseases.

In order to test this hypothesis we used the 7PA2 cell line that secretes high amounts of synaptotoxic low-n oligomeric Aβ species (Podlisny et al., 1995). These cells express full length APP including the familial Indiana mutation that increases the amount of cleaved, aggregation-prone $A\beta_{42}$ peptide. These cells have frequently been used to screen drugs with anti-oligomerization potential (Muller-Schiffmann et al., 2010; Walsh et al., 2002, 2005; Yamin et al., 2009). We analyzed a subset of structurally related antiviral compounds that had been demonstrated to interfere with the cellular assembly machine of rabies virus (Lingappa et al., 2013a,b).

Here, 1 μM of the compounds was used to treat 7PA2 cells as described before (Muller-Schiffmann et al., 2010). After immunoprecipitation, the secreted Aβ species were visualized on Western Blot using the 4G8 antibody (Covance) (Fig. 2). In the control lane, SDS-stable dimeric and trimeric Aβ species are clearly visible. The monomeric Aβ is absent due to the lack of monomer stabilizing serum (Podlisny et al., 1995). Non-toxic concentrations of the antiviral compounds used in this assay had different effects on oligomer formation. Compound C strongly reduced the overall formation of low-n Aβ oligomers without changing APP expression levels indicating an effect on a very early step of Aβ oligomer assembly, whereas compound B led to a conversion of oligomers back into monomers. In contrast, compound A was without effect. Thus, all of these compounds may interfere with Aβ oligomerization but at different assembly steps and with qualitatively different

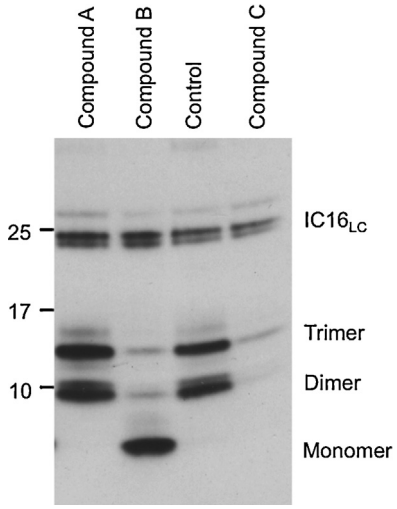

Fig. 2. Assembly-inhibiting antiviral compounds have distinct effects on oligomerization of Aβ peptides. Western Blot of Aβ species (monomers to trimers) derived from supernatants of 7PA2 cells that were treated for 7 days with 1 μM of different antiviral compounds derived from a capsid assembly assay (Lingappa et al., 2013a). Aβ was immunoprecipitated by IC16 that recognizes an epitope in the N-terminus of Aβ (aa 2-8) and visualized by the 4G8 antibody (epitope 17–24 of Aβ). Signals in the range of 25 kDa belong to the light chain of the IC16 antibody that was used for the IP. Compared to the mock treated control, the compounds differentially affected oligomer formation.

activity, indicating that different components and allosteric sites of the assembly machine were targeted.

In summary, surprising similarities in the cellular biology of virus capsid assembly and endogenous protein assembly suggest that cellular host factors, i.e. assembly machines, assist in and accelerate protein multimerization. Through their rapid generation cycles compared to their host cells, viruses have identified and exploited cell-resident macromolecules provided by host proteins used them to their advantage, i.e. fast and stable virus replication including capsid assembly. Similarly, same assembly machines may also accelerate endogenous protein multimerization, either aberrant in protein conformational diseases, or beneficial as in the case of functional protein multimers. The catalytic nature of these assembly machines makes them ideal targets for drug discovery since blocking assembly machinery will slow down assembly and the ensuing functional or toxic effects at substoichiometric concentrations. As proof-of-principle, we present candidate compounds, identified by virtue of their activity against a host assembly machine subverted by a neurotrophic virus (rabies), that inhibit various assembly steps of Aβ, the key molecule in AD.

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