The proteosomal degradation of fusion proteins cannot be predicted from the proteosome susceptibility of their individual components

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Abstract

It is assumed that the proteosome-processing characteristics of fusion constructs can be predicted from the sum of the proteosome sensitivity of their components. In the present study, we observed that a fusion construct consisting of proteosome-degradable proteins does not necessarily result in a proteosome-degradable chimera. Conversely, fusion of proteosome-resistant proteins may result in a proteosome-degradable composite. We previously demonstrated that conserved influenza proteins can be unified into a single fusion antigen that is protective, and that vaccination with combinations of proteosome-resistant and proteosome-degradable antigens resulted in an augmented T-cell response. In the present study we constructed proteosome-degradable mutants of conserved influenza proteins NP, M1, NS1, and M2. These were then fused into multipartite proteins in different positions. The stability and degradation profiles of these fusion constructs were demonstrated to depend on the relative position of the individual proteins within the chimeric molecule. Combining unstable sequences of either NP and M1 or NS1 and M2 resulted in either rapidly proteosome degraded or proteosome-resistant bipartite fusion mutants. However, further unification of the proteosome-degradable forms into a single four-partite fusion molecule resulted in relatively stable chimeric proteins. Conversely, the addition of proteosome-resistant wild-type M2 to proteosome-resistant NP–M1–NS1 fusion protein lead to the decreased stability of the resulting four-partite multigene products, which in one case was clearly proteosome dependent. Additionally, a highly destabilized form of M1 failed to destabilize the wild-type NP. Collectively, we did not observe any additive effect leading to proteosomal degradation/nondegradation of a multigene construct.

Keywords: proteosome degradation and resistance; chimeric proteins

DNA vaccines containing multigene fusion constructs employing several genes important for broader and possibly more potent immunity have been tested against

HIV-1, but rarely against influenza virus (Kmieciak et al. 2001; Shinoda et al. 2004; Scriba et al. 2005; Blazevec et al. 2006; Stoloff and Caparros-Wanderley 2007). However, influenza virus encodes several conserved proteins that are attractive targets for CTL-based vaccine strategies. Influenza nucleoprotein (NP), and to a lesser extent matrix proteins M1 and M2, are known to induce a significant degree of cross-protection (Chen et al. 1998, 1999; Epstein et al. 2002; Fiers et al. 2004; De Filette et al. 2005, 2006; Epstein et al. 2005; Tompkins et al. 2007).

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We previously reported that DNA vaccination with a combination of plasmids encoding NP, M1, and nonstructural (NS1) proteins provides protection both in mice and avian models (Zhirkov et al. 2007). We have also demonstrated that plasmids encoding fusion constructs that include wild-type (wt) NP–M1–NS1 and NP–M1–M2–NS1 are protective in mice (Ilyinskii et al. 2008a).

Viral proteins produced by an infected cell are degraded by proteasomes that ultimately produce overlapping peptides that then associate with MHC class I heavy and light chains. These complexes move via the secretory pathway to the cell surface where they are recognized by CD8⁺ T cells (Yewdell and Haeryfar 2005). CTL activity is vital in the containment of viral replication in infected individuals. Several groups attempted artificial targeting of viral genes of interest for proteasome degradation in order to enhance their Th1 immunogenicity in DNA immunization models, specifically the ability of modified viral antigens (Ag) to induce the CTL response (Townsend et al. 1988; Restifo et al. 1995; Wu and Kipps 1997; Wong et al. 2004; Altstein et al. 2006). However, vaccination regimens based on utilization of highly proteasome-degradable forms of antigens alone is no longer considered to be a promising strategy due to disappointing results and new insights into the mechanisms of antigen presentation and Th1/Th2 immunogenicity (Wong et al. 2004; Jensen 2007; Prlic et al. 2007).

We have recently demonstrated that concurrent and/or sequential utilization of proteasome-resistant and proteasome-degradable forms of two conserved influenza proteins, M1 and NS1, leads to an augmented CTL response (Ilyinskii et al. 2008b). We also proposed a technology to create proteasome-degradable variants of proteins that are normally proteasome resistant (Ilyinskii et al. 2008b).

Since the concurrent utilization of proteasome-degradable and nondegradable forms of M1 and NS1 lead to CTL augmentation, and given that multigene fusion proteins based on proteasome-resistant wild-type sequences of NP, M1, M2, and NS1 were shown to possess significant protective potential, we aimed to create a fusion protein that consists of several proteasome-degradable forms of influenza proteins. The rationale was to generate such unstable forms for several proteins and assemble them into multi-epitope molecules that could ultimately be incorporated into an effective influenza immunization regimen.

Results

Generation of proteasome-degradable influenza NP forms

We constructed a series of influenza NP mutants that we hypothesized would be less proteasome resistant than wild-type NP (Fig. 1A). Deletion mutants included

Figure 1. Influenza NP mutants and their metabolic testing. (A) Structure of NP mutants. Boundaries of introduced deletions are shown (N-terminal truncations designated by broken lines). (▼) Equals the insertion of tandem DD amino acids. NP-2DD and NP-10DD have two and 10 DD insertions, correspondingly. (B–G) Metabolic stability and proteasomal degradation of influenza NP mutants. Experiments were done as described in Materials and Methods. (B) Wild-type NP (lanes 2–7) and NP-2DD (lanes 8–13). Lane 1, mock-transfected cells. Times of chase: 0 h (lanes 2,7,8,13), 1 h (lanes 3,9), 2 h (lanes 4,10), 4 h (lanes 5,11), and 6 h (lanes 6,12). (C) NP-10DD mutant. Amount of loaded total protein is 10-fold greater compared with those shown in panels B and D–F. Lanes 1–3, anti-FLAG antibody used for detection, lanes 4–6, anti-HA tag antibody used. Times of chase: 0 h (lanes 1,4), 3.5 h (lanes 2,5), and 7 h (lanes 3,6). (D) NPdel1 mutant. Times of chase: 0 h (lane 1) and 8 h (lanes 2,3). MG132 was added to the sample in lane 3. (E) NPdel2 (lanes 1–5) and NPdel3 (lanes 6,7). Times of chase: 0 h (lanes 1,6), 1 h (lane 2), 2 h (lane 3), 4 h (lanes 4,5), and 24 h (lane 7). MG132 was added to the sample in lane 5. (F) NPdel12 (lanes 1–3) and NPdel23 (lanes 4–6). Times of chase: 0 h (lanes 1,4), 3.5 h (lanes 2,5), and 7 h (lanes 3,6). (G) NPdel12 mutant. Times of chase: 0 h (lanes 1–3), 3.5 h (lanes 4,5), and 7 h (lanes 6,7). Amount of protein loaded in lane 1 is two times less than in all other lanes in this panel. MG132 was added to the samples in lanes 5 and 7.

NPdel1 (amino acids 1–193), NPdel2 (164–329), NPdel3 (299–499), NPdel12 (1–329), and NP del23 (164–499); insertion mutants NP-2DD and NP-10DD had either two or 10 DD insertions in the positions shown (Fig.

1A). These mutants were engineered to bear either long truncations that should severely affect 3D structure of the protein or multiple DD insertions in hydrophobic amino acid stretches designed to destroy the predicted NP α -helical structures (Ye et al. 2006). We then tested the metabolic stability of these mutant forms. As expected, wild-type influenza NP protein was shown to be very stable (Antón et al. 1999; Altstein et al. 2006) and manifested little or no degradation in vitro over an extended period of time (Fig. 1B). Similarly, the NP-2DD mutant failed to produce faster degradation kinetics than the wild-type NP protein (Fig. 1B). While the NP-10DD mutant was notably less stable than the NPwt, it was also significantly less efficiently expressed (especially when assessed using an antibody to the C-terminal HA-tag; see Fig. 1C). Therefore, insertion mutagenesis as a means of destabilizing influenza NP was abandoned given that it resulted in severe expression defects.

NP deletion mutants resulted in varying degrees of protein degradation. Two constructs, NPdel1 (comprising the N-terminal region) and NPdel2 (comprising the central region of NP), exhibited faster degradation kinetics than the wild-type protein (Fig. 1D–E). At the same time, the NPdel3 mutant (comprising the C-terminal region) was as stable as the NPwt. Furthermore, the degradation of both NPdel1 and NPdel2 was suppressed by the proteasome inhibitor MG132, proving that it is indeed proteasome dependent (Fig. 1D, lane 2 vs. 3; Fig. 1E, lane 4 vs. 5). Two additional NP mutants were also tested: NPdel12 (C-terminal truncation) being notably less stable than the NPwt while NPdel23 (N-terminal truncation) was completely stable (Fig. 1F). The NPdel12 that was degraded in a proteasome-dependent manner (Fig. 1G) was selected for future utilization in multigene constructs.

Generation of proteasome-degradable influenza M2 forms

In a further development of the suggested approach, we constructed a number of deletion/truncation mutants of M2, another highly conserved influenza protein. We aimed not only to create a proteasome-degradable form of M2, but also to inactivate this protein, which, while able to provide moderate protection against viral infection on its own (Slepishkin et al. 1995; De Filette et al. 2005, 2006), was also shown to possess possible negative effects on the vaccination (Ilyinskii et al. 2007). Two deletion mutants of the highly conserved influenza minor matrix (M2) protein were initially designed (Fig. 2A). M2 in its wild-type form was shown to be particularly resistant to proteasome degradation, similar to other conserved influenza proteins (Fig. 2A, lanes 1–3). However, expression of its deletion forms was not detectable (not shown). Inhibition of proteasomal degradation enabled

Figure 2. Influenza M2 mutants and their metabolic testing. (A) Structure of M2 mutants with deletion boundaries shown. (B) Metabolic testing of M2wt (lanes 1–3) and M2del1 (lanes 4–6). Chase times: 0 h (lanes 1,4), 4 h (lanes 5,6), and 16 h (lanes 2,3). Lanes 3,6 MG132 added.

the detection of the M2del1 mutant (comprising M2 ectodomain and transmembrane regions, amino acids 1–45). This testified to the extremely rapid proteasome-dependent degradation of this mutant form (Fig. 2B).

Proteasome-degradable forms of influenza M1 and NS1

Collections of influenza M1- and NS1-expressing mutants and the rationale for their construction have been described earlier (Ilyinskii et al. 2008b). In the current study we utilized those forms of mutated M1 and NS1 that were shown by us to be both highly expressed and proteasome degradable. These are the M1pm1 mutants with 8 DD insertions in their predicted α -helical regions, the M1del2 mutant comprising amino acids 55–252 of the wild-type protein, and two deletion mutants of NS1 comprising amino acids 1–132 and 141–230, designated as NS1del2 and NS1del4 (Figs. 3A, 4A; see Ilyinskii et al. 2008b for details).

The degradable form of M1 fails to promote proteasome-dependent degradation of fused NPwt

Since M1pm1 was both strongly expressed and efficiently degraded in a proteasome-dependent manner (Ilyinskii et al. 2008b), we attempted to use it as a tool to augment proteasome-driven degradation of influenza NP, known to be the most instrumental protein antigen in inducing cross-protection against influenza infection in vivo. The M1pm1 sequence was attached to NPwt on either end and the metabolic characteristics of the resulting fusion proteins were assessed (Fig. 3). No degradation of M1pm1–NPwt was detected when using antibodies against sequences tagged to N or C termini of the fusion protein (Fig. 3B–C). While some degradation of NPwt–M1pm1 was detected using an antibody against C-terminal HA-tag (Fig. 3B), this

Figure 3. M1pm1 and NPwt fusion constructs and their metabolic stability. (A) Structure of fusion proteins. Amino acid residue positions in wild-type proteins are italicized. Sizes of full-length fusion proteins are shown in bold. (▼) Equals the insertion of tandem DD amino acids. (B,C) M1pm1–NPwt (lanes 1–4) and NPwt–M1pm1 (lanes 5–8) stability assessed with (B) anti-HA antibody (C-terminal-tag), (C) with anti-FLAG antibody (N-terminal tag). Chase times: 0 h (lanes 1,2,5,6), 3.5 h (lanes 3,7), and 7 h (lanes 4,8). Lanes 1 and 5 contain 1/3 of total protein loaded to other lanes. Positions of molecular weight markers are shown.

reflected partial degradation of M1-derived sequences while NPwt sequence was not affected as demonstrated when the anti-N-terminal antibody was used in the same setting (Fig. 3C). It is likely that the lower molecular weight band seen in Figure 3C, lanes 6–8, represents a product of the incomplete degradation of NPwt–M1pm1 that fails to affect NPwt sequence. Therefore, attempts to use NPwt were abandoned and NPdel12 was utilized in all subsequent multigene fusion constructs.

Design and testing of fusion multipartite constructs comprising proteasome-degradable mutants of two conserved influenza proteins

We decided to unify those NP, M1, NS1, and M2 forms that were shown to be degraded in a proteasome-dependent manner and to test the metabolic stability of such multipartite fusion forms. First, we fused NPdel12 with either M1pm1 or M1del2 in both orientations (Fig. 4A). Separately, we also created four multipartite constructs from proteasome-degradable NS1 and M2 mutants using NS1del2, NS1del4, and M2del1 sequences in different orders (Fig. 4A). All the NP–M1 mutant forms were proteasome degradable, but to different extents. Moreover, expression of these forms varied significantly, with NPdel12–M2del2 being the most stable (Fig. 4B; less than threefold decrease within 8 h), notably exceeding the stability of the fusion protein in which the same sequences were used in the reverse order (Fig. 4D, lanes 7–12, nearly 10-fold decrease within 8 h). While all other

novel NP–M1 mutant forms assayed up to this point were degradable, M1pm1–NPdel12 was expressed at the highest level (two times more than the others) and was therefore selected for the next round of studies. In addition, among

Figure 4. Metabolic testing of bipartite constructs bearing either NP–M1 or NS1–M2 sequences. (A) Schematic structures of fusion mutants constructed from either proteasome-degradable NP and M1 mutants or NS1 and M2 mutants. Amino acid numbers corresponding to wild-type sequences are italicized; sizes of fusion proteins are shown in bold. (B–F) Metabolic stability and proteasome-dependent degradation of NP–M1 and NS1–M2 fusion mutants. (B) NPdel12–M1del2. Chase times: 0 h (lanes 1–3), 4 h (lanes 4,5), and 8 h (lanes 6–8). Lane 1 contains 1/3 of total protein loaded to all other lanes. MG132 was added to samples in lanes 5 and 8. (C) NPdel12–M1pm1. Chase times: 0 h (lanes 1,2), 4 h (lanes 3,4), and 8 h (lanes 5,6). Lane 1 contains 1/3 of total protein loaded to lanes 2–6. MG132 was added to samples in lanes 4 and 6. (D) M1pm1–NPdel12 (lanes 1–6) and M1del2–NPdel12 (lanes 7–12). Chase times: 0 h (lanes 1,2,7,8), 4 h (lanes 3,4,9,10), and 8 h (lanes 5,6,11,12). Lane 1 contains 1/3 of total protein loaded to lanes 2–6; lane 7 contains 1/3 of total protein loaded to lanes 8–12. MG132 was added to samples in lanes 4,6,10,12. (E) NS1del2–M2del1–NS1del4 (lanes 1,2), NS1del4–M2del1–NS1del2 (lanes 3,4), NS1del4–NS1del2–M2del1 (lanes 5,6), NS1del2–NS1del4–M2del1 (lanes 7,8). Times of chase: 0 h (lanes 1,3,5,7) and 8 h (lanes 2,4,6,8). (F) Proteasome-dependent degradation of NS1del2–M2del1–NS1del4. Times of chase: 0 h (lanes 1–3), 5 h (lanes 4,5), and 8 h (lanes 6,7). Lane 1 contains 1/4 of total protein loaded to lanes 3–7, lane 2 contains 1/2 of total protein loaded to lanes 3–7. MG132 was added to samples in lanes 5 and 7.

NS1–M2 constructs only NS1del2–M2del1–NS1del4 demonstrated noticeable degradation (Fig. 4E, lanes 1,2, a two-fold decrease within 8 h), that was also proteasome dependent (Fig. 4F). This effect was less pronounced than in any fusion protein in the NP–M1 series.

Design and testing of fusion constructs comprising proteasome-degradable mutants of four conserved influenza proteins

Sequences comprising M1pm1–NPdel12 and NS1del2–M2del1–NS1del4 were fused together in both orientations and their metabolic stability assessed (Fig. 5). Very little degradation (2%–5% within 7.5 h) was detected for both NS1–M2–M1–NPmgd and M1–NP–NS1–M2mgd, when the antibody directed against their C termini was used (Fig. 5B, lanes 1–8). Moderate degradation of M1–NP–NS1–M2mgd (3.5-fold within 7.5 h) was detected when the antibody to the N terminus was used. This probably reflected degradation of the M1pm1-derived sequence similar to that observed earlier with the M1pm1–NPwt fusion construct (Fig. 3). This effect was partially (40%) suppressed by MG132 (Fig. 5C, lanes 4–6), thus indicating that degradation of this chimeric protein is only partially mediated by proteasome.

Figure 5. Four-partite constructs and their metabolic testing. (A) Schematic sequences of NS1del2–M2del1–NS1del4–M1pm1–NPdel12 (NS1–M2–M1–NPmgd) and M1pm1–NPdel12–NS1del2–M2del1–NS1del4 (M1–NP–NS1–M2mgd). (B) Metabolic stability of NS1–M2–M1–NPmgd (lanes 1–4 and 9–11) and M1–NP–NS1–M2mgd (lanes 5–8 and 12–14). Chase times: 0 h (lanes 1,2,5,6,9,12), 3 h (lanes 3,7,10,13), and 7.5 h (lanes 4,8,11,14). Anti-HA-tag antibody (lanes 1–8) and anti-FLAG-tag antibody (9–14) were used for protein detection. Lanes 1 and 5 contain 1/3 of total protein loaded to lanes 2–4 and 6–8. (C) Proteasome-dependent degradation of NS1–M2–M1–NPmgd (lanes 1–3) and M1–NP–NS1–M2mgd (lanes 4–6). Chase times: 0 h (lanes 1,4) and 7.5 h (lanes 2,3,5,6). MG132 was added to samples in lanes 3 and 6. Anti-FLAG antibody was used for protein detection.

Destabilization of multipartite fusion protein comprising proteasome-resistant wild-type sequences of conserved influenza proteins by a proteasome-resistant wild-type M2 sequence

We have previously reported the construction and protective capacities of multipartite fusion proteins comprising wild-type sequences of influenza NP, M1, and NS1 genes with or without an M2 sequence (Ilyinskii et al. 2008a).

When the proteosomal stability of three such multipartite fusion proteins, NP–M1–NS1, NP–M1–NS1–M2, and NP–M1–M2–NS1 was studied, we noted an unexpected effect exhibited by addition of a wild-type M2 sequence (Fig. 6) to NP–M1–NS1. Similar to our previous study of NP–M1–M2–NS1 (Ilyinskii et al. 2008a) both four-partite fusion proteins showed lower expression levels than the three-partite NP–M1–NS1 fusion protein, whether assayed by antibody to the N-terminal or C-terminal tag (Fig. 6B). Moreover, it was clear that while NP–M1–NS1 is metabolically stable during the 8-h period (Fig. 6B; lanes 3,4,9,10), the levels of both NP–M1–NS1–M2 and NP–M1–M2–NS1 decrease up to 50% over the same period (Fig. 6B; compare lanes 1 and 2, 5 and 6, 7 and 8, 11 and 12). We further investigated if this effect is proteasome dependent. It was clear that the proteasome inhibitor MG132 was able to completely abort the degradation of NP–M1–M2–NS1 that in the absence of the inhibitor reached 70%–90%, depending on the antibody used (Fig. 6C,D; lanes 11 and 12 vs. lanes 13 and 14). The intensity of NP–M1–M2–NS1 bands from MG132-treated samples developed with either antibody showed no statistical difference when compared to the zero time point (Fig. 6C,D; lanes 9 and 10), while the MG132 effect on NP–M1–NS1–M2 degradation (50%–70% intensity decrease) was significantly less pronounced and observed only when anti-N-terminal FLAG-tag antibodies were used for the visualization of fusion proteins (Fig. 6C,D; lanes 4 and 5 vs. lanes 6 and 7; 20% stabilization observed in Fig. 6C and none in 6D).

Discussion

There are two lines of evidence that provide a rationale for the current work. First, attempts to create recombinant antiviral vaccines based on a combination of multiple conserved viral proteins are considered to be a superior strategy compared to vaccination with a single antigenic epitope or a single protein. It has been repeatedly demonstrated that DNA vaccination with alternative combinations of conserved influenza proteins provides protective benefits in different animal models (Chen et al. 1998, 1999; Epstein et al. 2000, 2002; Jimenez et al. 2007; Zhirmov et al. 2007).

Figure 6. Metabolic testing of NP-M1-NS1, NP-M1-NS1-M2, and NP-M1-M2-NS1 multipartite proteins. (A) Structure of fusion proteins. (B) Metabolic stability of NP-M1-NS1-M2 (lanes 1,2,7,8), NP-M1-NS1 (lanes 3,4,9,10), and NP-M1-M2-NS1 (lanes 5,6,11,12). Chase times: 0 h (lanes 1,3,5,7,9,11) and 8 h (lanes 2,4,6,8,10,12). Emetine was added to samples in lanes 2,4,6,8,10,12. Anti-FLAG-tag (lanes 1–6) and anti-HA-tag (lanes 7–12) were used for protein detection. (C,D) Proteasome-dependent degradation of NP-M1-NS1-M2 (lanes 1–7) and NP-M1-M2-NS1 (lanes 8–14). Chase times: 0 h (lanes 1–3 and 8–10) or 8 h (lanes 4–7 and 11–14). Lanes 1 and 8 contain 1/3 of total protein loaded to lanes 2–7 and 9–14, correspondingly. MG132 was added to samples in lanes 6,7,13,14. Duplicated samples were independent and similarly treated. (C) Anti-FLAG-tag antibody was used for protein detection; (D) anti-HA-tag was used.

Second, our recent studies have demonstrated that vaccination regimens based on simultaneous and/or consecutive utilization of both proteasome-resistant and proteasome-degradable forms of an antigen provide increased CTL activity compared to vaccination with any of these forms alone (Ilyinskii et al. 2008b). Taken together, these data suggest that generating two forms of multigenic fusion constructs encoding NP, M1, M2, and NS1 influenza proteins, both proteasome degradable and proteasome resistant, has the potential to benefit anti-influenza vaccine product development.

Our hypothesis was that combining proteasome-resistant wild-type influenza proteins into one chimeric protein would result in a proteasome-resistant fusion construct. Similarly, a fusion construct should be proteasome degradable if it consists of proteasome-degradable component proteins. Therefore, we created proteasome degradable forms for each of the four conserved influenza proteins that are important for broad-spectrum vaccine development. We then proceeded to combine wild-type proteasome-resistant or mutated proteasome-degradable forms into fusion proteins and tested these constructs for proteasome sensitivity. Contrary to our initial hypothesis, we found no direct correlation between the proteasome susceptibility of the fusion constructs and their component parts. Thus, the degradation

or resistance of the fusion product could not be directly predicted from the properties of its elements.

Wild-type proteins of influenza have been demonstrated by us and others to be extremely resistant to proteosomal degradation (Antón et al. 1999; Altstein et al. 2006; Ilyinskii et al. 2008b). A well-accepted approach to increase the proteasome degradation of a protein is its gene-engineered ubiquitination or fusion with ornithine decarboxylase (ODC), one of the most rapidly degraded proteins in eukaryotic cells (Townsend et al. 1988; Wu and Kipps 1997; Zhang et al. 2004; Starodubova et al. 2006). It was shown to lead to an enhanced proteosomal degradation of HIV RT and made such fusions attractive as CD8⁺ T cell-inducing gene vaccines (Starodubova et al. 2006). However, similar attempts to enhance proteosomal degradation of influenza NP by its covalent fusion with ODC were unsuccessful (Altstein et al. 2006).

We have been able to produce rapidly degradable forms of M1 and NS1 either by introducing DD insertions into hydrophobic amino acid stretches of M1 or by designing deletion mutants that collectively overlap ~95% of protein NS1. A deletion mutant of M2 that bears its most immunologically important region was also shown to be rapidly degraded, while the wild-type protein was extremely proteasome resistant (Fig. 2). We utilized an NP deletion mutant that comprised ~70% of its sequence since the C-terminal region of this protein was demonstrated to be highly instrumental in maintaining its stability (Fig. 1). It is likely, although not certain, that this “stabilization determinant” is the same NP multimerization region that was recently identified (Ye et al. 2006). Attachment of NPwt to rapidly degradable M1pm1 sequence did not increase degradation of the NP part of such a bipartite protein (Fig. 3).

Unification of degradable forms of NP, M1, NS1, and M2 into multigene fusion proteins did not necessarily result in generation of rapidly degradable molecules, which were usually highly expressed in vitro. The metabolic stability of these constructs was also notably affected by the respective position of various domains within the molecule with several bipartite proteins demonstrating differing degrees of stability depending on the order of identical sequence components. Moreover, unification of two degradable bi-gene chimeric proteins into a single four-partite fusion product did not result in generation of a rapidly degradable product (Fig. 5). It is possible that several degradable proteins may provide mutual stabilization when unified in a single molecule. It is less likely, but not inconceivable, that its large size may play a role in the slowing down of intracellular proteasome-dependent processing or even contributing toward proteasome “clogging,” which has been described for a number of abnormal proteins with a high molecular weight. Moreover, domains of other proteins fused to

NPdel12 may compensate for the loss of its C-terminal oligomerization domain (Ye et al. 2006), resulting in formation of stable oligomers by chimeric proteins. In line with this, it was recently demonstrated that the central domain of M1 (comprising amino acids 88–165) is capable of mediating NP–M1 binding and also M1 self-association (Noton et al. 2007). Although several mutations that were introduced into M1 during the present study are located within this region, it is conceivable that they did not interfere with the hence unidentified signals that are essential for NP–M1 and M1–M1 binding. Moreover, a known multifunctional signal directs M1 interaction with other proteins, RNA molecules, and subcellular structures, and is located at positions 101–105 (Elster et al. 1997; Akarsu et al. 2003; Garcia-Robles et al. 2005), and may have also contributed to the proteosomal resistance of the multipartite chimeras described in this study.

However, a completely different effect resulted from the addition of a wild-type M2 gene sequence into a highly expressed and stable fusion protein comprising full sequences of influenza genes NP, M1, and NS1 (Fig. 6). First, the expression of such four-partite fusion genes was partially suppressed, and second, their metabolic stability was decreased. Moreover, when the M2 sequence was located in the middle of the fusion molecule (as in the construct NP–M1–M2–NS1) its degradation was proteosome dependent, while degradation of the fusion molecule containing wild-type M2 at its C terminus was non-proteosome-dependent (Fig. 6). One may speculate that oligomerization of chimeric protein is impeded when the M2 sequence, possessing a very rigid spiral-forming structure (Ilyinskii et al. 2007), is located in the center of the multipartite molecule. It is well known that distortion of a protein's native conformation often causes its degradation by the proteosome (Goldberg, 2007), and it seems likely that in some cases introduction of a particular sequence may affect the folding of a resulting chimeric protein, especially if it contains long stretches of unmodified functional domains, i.e., a wild-type sequence. Interestingly, we were able to demonstrate that proteosome-sensitive NP–M1–M2–NS1 has a significant protective effect in a DNA vaccination model despite its relatively low expression level (Ilyinskii et al. 2008a), while similarly assayed stable M1–NP–NS1–M2mgd described in this study did not possess any noticeable protection when tested in the same model (not shown).

It is necessary to point out that the proteosome inhibitor that we employed in the current study, MG132, while widely used in similar approaches (Goldberg, 2007) is also known to inhibit certain lysosomal cysteine proteases as well as the calpains. However, in no case was the turnover of the short-lived proteins we generated shown to be catalyzed by a non-proteosomal pathway (Lee and Goldberg, 1998) except for protein degradation by cas-

pases during apoptosis, which has no relation to the subject discussed herein.

The three main mechanisms of gene evolution are duplication, recombination, and sequence divergence. In particular, recombination results in two mutually reciprocal phenomena, gene fusion and fission, whereupon genes coding for two separate gene products in one organism get merged in the course of evolution into one gene or, respectively, genes coding for large multidomain proteins get split into smaller genes coding for individual domains (Kummerfeld and Teichmann 2005). Susceptibility to proteosome processing may constitute yet another evolutionary mechanism of selection for multidomain proteins based on their proteosome stability, which would be more or less physiologically favorable than the proteosome degradation profile of the fused proteins. Our data may also reveal an underlying reason for the failure of some biotechnology projects. Indeed, if fusing degradation-resistant proteins onto a chimeric protein results in a highly proteosome-degradable product, its manufacturing will be severely compromised.

Materials and Methods

Cells and plasmids

All experiments were performed using 293 human embryo kidney (HEK) cells. The full-length genes encoding influenza A/WSN/33 (H1N1) NP, M1, NS1, and M2 proteins were chemically synthesized (GenBank sequence accession numbers V01084, L25818, J02150, and L25818), inserted into the pcDNA3 vector (Invitrogen) and their sequence verified. All N-terminal deletions have preserved initiating methionine. An HA-tag (YPYDVPDYA) was added at the C terminus of all fusion constructs to enable their efficient immunological detection. All NP mutants and multigene mutants also contained N-terminal FLAG-tag (DYKDDDDK) in addition to HA-tag. All deletion and point mutants were constructed by PCR-directed site-specific mutagenesis of the wild-type gene. All sequences were then cloned into the pCAGGS expression vector (Niwa et al. 1991) and assayed for metabolic stability and proteosome-dependent degradation.

Transfection and protein expression

Two hundred ninety-three HEK cells were transfected at 60%–80% confluency in 35-mm plates with Lipofectamine 2000 (Invitrogen) for 4 h or overnight (1 µg of total plasmid DNA per 3 µL LF2000). Control cells were transfected with the same amount of empty vector pCAGGS. Forty hours after the end of transfection, 12 µM emetine was added and samples were taken (2× wash in PBS, –80°C) at the indicated time points. All constructs were chased for various time periods falling within 0–24 h after emetine addition with or without 10 µM of proteosome inhibitor MG132 (Sigma), which, when utilized, was always used for the whole duration of chase. Cells were then homogenized on ice in lysis buffer (about 150 µL/35-mm dish) 150 mM NaCl, 50 mM Tris–HCl (pH 7.4),

1 mM EDTA, 1% Triton X-100 with protease inhibitors. Samples were adjusted for equal total protein and run on SDS-PAGE, transferred to nitrocellulose, stained with Ponceau S, assessed by densitometry to verify equal protein loading, and then immunoblotted with monoclonal anti-FLAG (Sigma) or monoclonal anti-HA (6E2) (Cell Signaling) antibodies. Protein band intensity was assessed using Quantity One software, version 4.6 (Bio-Rad Laboratories) with the normalization done using an antibody against β -actin (AC-15) (Sigma), which in neither experiment has shown >20% deviation between the samples.

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