Ubiquitous Autofragmentation of Fluorescent Proteins Creates Abundant Defective Ribosomal Products (DRiPs) for Immunosurveillance*

Jiajie Wei, James S. Gibbs, Heather D. Hickman, Stephanie S. Cush, Jack R. Bennink, and Jonathan W. Yewdell

From the Laboratory of Viral Diseases, NIAID, National Institutes of Health, Bethesda, Maryland 20892

Background: Fragments are rapidly generated from GFP.

Results: Fragmentation occurs in all fluorescent proteins examined, requires chromophore rearrangement, and is a major source of MHC-I ligands.

Conclusion: Fragmentation, a by-product of chromophore rearrangement, creates DRiPs.

Significance: Fluorescent protein fragments are generated at high levels, likely influencing experimental outcomes. GFP fragments are the first natural DRiPs biochemically characterized relevant for immunosurveillance.

Green fluorescent protein (GFP) and other fluorescent proteins are essential tools for biological research. When fused to peptides or proteins as a reporter, GFP enables localization and quantitation of gene products in otherwise unmanipulated live cells or organisms. We previously reported that a sizable fraction of nascent GFP is post-translationally converted into a 20-kDa Triton X-100-insoluble proteasome substrate (Qian, S. B., Princiotta, M. F., Bennink, J. R., and Yewdell, J. W. (2006) J. Biol. Chem. 281, 392–400; Dolan, B. P., Li, L., Veltri, C. A., Ireland, C. M., Bennink, J. R., and Yewdell, J. W. (2011) J. Immunol. 186, 2065–2072). Here, we show that a similarly sized fragment is generated by all GFP and red fluorescent protein family members we examined. We demonstrate that fragmentation is a by-product of GFP chromophore rearrangement. A non-rearranging GFP mutant fails to fragment and generates diminished levels of Kb-SIINFEKL complexes when SIINFEKL is genetically transcribed first-order degradation kinetics, with an average half-life of ~24 h (2–4). DRiPs are a subset of nascent proteins that are ubiquitous and abundant fragmentation must be considered when interpreting experiments using these extremely useful probes.

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and its many genetic spectral variants, including eGFP,2 which exhibits improved folding at 37 °C (1), are used in a stunning variety of biological applications. The GFP chromophore is formed post-translationally by the autocatalytic cyclization of three amino acid residues at positions 65–67 followed by a dehydration reaction and a dehydrogenation requiring molecular oxygen. GFP paved the way for discovering red-shifted fluorescent proteins from coral reef organisms, expanding the biological color palette.

GFP and other fluorescent proteins are widely considered to be highly stable in cells and are often treated as neutral reporter molecules for myriad biological studies, including those involving major histocompatibility complex (MHC) class I-based immunosurveillance. MHC class I molecules bind peptides of 8–12 residues in the endoplasmic reticulum and follow the standard secretory pathway to the cell surface, where the complex can be recognized by CD8+ T cells. CD8+ T cell lysis of target cells and release of cytokines play an important role in immune recognition of viruses, other intracellular pathogens, tumor cells, transplanted tissues, and autoimmune targets.

Class I peptides derive largely from proteins synthesized by the cells’ own ribosomes. There are two general classes of substrates that provide peptides as follows: retirees and defective ribosomal products (DRiPs). Retirees are proteins degraded via the normal process of protein aging. Proteins generally demonstrate first-order degradation kinetics, with an average half-life of ~24 h (2–4). DRiPs are a subset of nascent proteins that are

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1 To whom correspondence should be addressed: Laboratory of Viral Diseases, NIAID, 33 North Dr., Bethesda, MD 20892. Tel.: 301-402-4602; E-mail: JYEWDELL@niaid.nih.gov.

2 The abbreviations used are: eGFP, enhanced green fluorescent protein; MHC, major histocompatibility complex; RFP, red fluorescent protein; DRiP, defective ribosomal product; PSI, protein synthesis inhibitor; NP, nucleoprotein; rVW, recombinant vaccinia virus; VV, vaccinia virus; BFP, blue fluorescent protein; m.o.i., multiplicity of infection; hpi, hour post-infection.
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degraded with more rapid kinetics than the corresponding retiree pool. We conjured DRiPs to explain the extremely rapid generation of peptides from otherwise highly stable viral proteins. Originally, we defined DRiPs as prematurely terminated or misfolded full-length proteins produced from bona fide mRNA (which includes subunits of multi-protein assemblies that are produced in excess) (5). As the model matured over the years, we expanded DRiPs to include both defective gene products resulting from errors in transcription and translation and the translation products of “immunoribosomes,” ribosomes specialized for generating antigenic peptides for immunosurveillance (6–9).

From the inception of the DRIP model, it was clear that retirees contribute to the peptide pool (10), and our recent data show that peptide generation from retirees can be highly efficient (11). DRiPs appear to account for the bulk of peptides, particularly viral peptides, where DRiPs peptides appear to dominate the presented repertoire (12). Furthermore, based on recent evidence of co-translational ubiquitylation and degradation (13, 14), the fraction of rapidly degraded proteins synthesized by cultured cells appears to be far closer to 30–40%, as originally reported (15, 16), than “at most, only a few percent,” quoting Vabulas and Hartl (17).

Although kinetic studies robustly support the importance of DRiPs to antigen presentation (7, 18, 19), there has been little evidence regarding their biochemical properties. Here, we investigate the generality of fragment generation from fluorescent proteins and extend our previous finding that a large fraction of nascent GFP is recovered as a 20-kDa fragment (20–22) by defining its mechanism of generation and its contribution to antigen processing.

Experimental Procedures

Cells, Viruses, and Antibodies—L-Kb and HeLa cells were maintained in DMEM with 7.5% FBS in a 9% CO2 incubator. rVVs expressing influenza nucleoprotein (NP) fused to SIINFEKL peptide and eGFP (NPSGFP) and expressing β-gal were previously described (18). GSGF<sup>+</sup> and GSGGF<sup>–</sup> mutations were introduced into the NPSGFP coding sequence by replacing the small Ncol-NotI restriction fragment with similarly digested two-step splice overlap extension PCR products by ligation. Primer pairs used in the first step PCR to mutate TYG to GSG<sup>+</sup> were oJW006F (5‘-GTCGCCACCATGTTGAGCAA-3’), oJW010R (5‘-GACCCCGGACCCCCAGGGTGTCACAGGGGTG-3’), oJW009F (5‘-GACCCACCGG-GCTCCGCGGTGTCAGCCGCTACC-3’), and oJW011R (5‘-ATTCCAGCGCCGCTTTACTTGTACAGCTC-3’). PCR product was then excised with SalI and NotI restriction enzymes and ligated with similarly digested pSC11SBAKN.

We introduced GSGF<sup>+</sup> and GSGGF<sup>–</sup> by replacing the small Sall-NotI restriction fragment with similarly digested two-step PCR products by ligation. Primers used in the first step PCR to mutate TYG to GSG<sup>+</sup> were oJW001F, oJW005R (5‘-ATTCCACGCGCCGCTTTACTTGTACAGCTC-3’), oJW009F, and oJW010R. oJW001F and oJW005R were then used in the second step PCR. Similarly, GSGGF<sup>–</sup> was constructed using oJW001F, oJW005R, oJW013F, and oJW014R. rVVs were generated, propagated, and titered as described previously (23).

Cells were infected at a multiplicity of infection at 37 °C in a balanced salt solution containing 0.1% bovine serum albumin. Cells were then incubated at 37 °C in complete growth medium for the remainder of the assay. Anti-GFP C-terminal antibody was from Roche Applied Sciences. Anti-GFP N-terminal antibody was from GenScript. Rabbit polyclonal anti-RFP antibody was from Abcam. Mouse monoclonal anti-β-actin antibody was from Sigma. Mouse monoclonal anti-NP antibody HB-65 and mouse monoclonal anti-E3L antibody TW2.3 were produced in-house. Infrared secondary antibodies were from LI-COR. The AlexaFluor 647-conjugated 25D1.16 monoclonal antibody was previously described (24). AlexaFluor 647 goat anti-mouse IgG(H+L) was from Life Technologies, Inc.

Clones and Transfections—Erik L. Snapp (Albert Einstein College of Medicine, Bronx, NY) generously provided plasmids expressing moxsynGFP, mGFP A206K, Emerald, and Venus. We transfected cells with plasmids using Lipofectamine LTX according to the manufacturer’s instructions (Life Technologies, Inc., Carlsbad, CA).

Immunoblotting—Detergent fractionation procedures have been described in detail (25). To prepare whole cell lysates, harvested cells were washed in PBS and then resuspended to a concentration of 10<sup>4</sup> cells/ml in SDS extraction buffer (1% SDS, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 15 units/ml DNase) containing protease inhibitor mixture (one cOmplete protease inhibitor mixture tablet from Roche Applied Science per 10 ml). Cells were incubated at 99 °C for 10 min. Cell lysates were mixed with 4× NuPAGE buffer (Life Technologies, Inc.), resolved by 4–12% NuPAGE gels (Life Technologies, Inc.), and blotted onto nitrocellulose membranes. Immunoblots were incubated with the indicated primary antibodies and respective infrared secondary antibodies and analyzed using the Odyssey Imaging System (LI-COB).

Antigen Presentation Assay—Cells were harvested at the indicated time points and washed with PBS. Kb-SIINFEKL levels on the cell surface were determined after incubating cells at 4 °C for 30 min with the AlexaFluor 647-conjugated 25D1.16 monoclonal antibody. To determine the protein level of vaccinia viral protein E3L, half of the harvested cells were fixed in 3.2% paraformaldehyde at room temperature for 30 min, washed in PBS, incubated with antibody TW2.3 at 4 °C for 30 min, washed in PBS, and then stained with AlexaFluor 647 anti-mouse antibody at 4 °C for 30 min. All flow cytometry experiments were conducted using an LSR II flow cytometer (BD Biosciences) and analyzed by Flowjo (Tree Star, Inc., Ashland, OR).
**Results**

**Protein Fragmentation Is a Ubiquitous Feature of Fluorescent Proteins**—Given the widespread use of fluorescent proteins in biology, it is important to understand their biogenesis and degradation. Whenever we have used GFP, or GFP genetically appended to the C termini of other proteins, we have detected an anti-GFP antibody-reactive species of ~20 kDa (GFP$_{20}$) in immunoblots of GFP-expressing cells (Fig. 1A, lane 1) (20–22). Notably, a similarly sized fragment is also observed when GFP is synthesized in cell-free translation systems (26), suggesting that it is an intrinsic feature of GFP biogenesis.

We first tested whether GFP$_{20}$ is generated from related GFP variants expressed by HeLa cells from transfected genes (Fig. 1A). These include the following: 1) moxsynGFP, a monomerized human codon-optimized, superfolder GFP lacking cysteines (Fig. 1A, lane 2); 2) mGFP A206K, a monomerized GFP (Fig. 1A, lane 3); 3) Emerald, an eGFP derivative that folds more efficiently at 37 °C (Fig. 1A, lane 4); and 4) Venus, a yellow fluorescent protein (Fig. 1A, lane 5).

For each of the GFPs tested, the anti-GFP antibodies detected both the full-length protein and an abundant species migrating similarly to GFP$_{20}$. The specificity of detection is shown by the absence of these species in cells transfected with an empty vector (Fig. 1A, lane 6).

Red fluorescent proteins (RFPs) are structurally highly similar to GFP but evolutionarily distinct (27). We tested lysates from L-k$^b$ cells (L929 cells expressing the mouse class I molecule H-2k$^b$ from a transgene) infected with rVV$s$ expressing RFP variants appended to the C terminus of influenza A virus NP (Fig. 1B). The chimeric gene product NPStagRFP consists (N to C terminus) of influenza A virus NP, the K$_b$-binding peptide SIINFEKL, and tagRFP, rVV$s$ encoding β-gal (negative control), or also expressing NPS-tagRFP, NPS-tagBFP, NPS-mCherry, and NPS-tdTomato, were tested (Fig. 1B).

Immunoblotting using antibodies to either NP or RFP revealed that a significant fraction of each of the chimeric proteins was detected as an N-terminal fragment (anti-NP antibody) or a C-terminal fragment of ~20 kDa (anti-RFP antibody) (Fig. 1B). Notably, the intensity of the N-terminal fragment was only moderately less than the full-length species (NPS-tagRFP and NPS-tagBFP) or even more abundant than the full-length species (NPS-mCherry and NPS-tdTomato). Thus, for the RFP variants, the fragmentation process is highly robust.

**GFP Fragments as a By-product of Chromophore Maturation**—Our results show that fragmentation is a ubiquitous feature for both GFP and RFP. As the amino acid sequences of RFP$s$ and GFP$s$ we used exhibit little similarity, it is unlikely that the fragments arise from site-specific proteolysis. Together with our previous finding that levels of the fragment are controlled by an N-terminal degron (22), we can safely rule out fragmentation origins from downstream initiation. Because cell-free translation systems also generate the GFP fragment (26), and its degradation is sensitive to proteasome inhibition in cells (20), we can also conclude that it is not generated as a post-lysis artifact.

A simple explanation for fluorescent protein fragmentation is that it is a by-product of chromophore rearrangement (27, 28). The eGFP chromophore is generated by cyclization of tripeptide 65-TYG67. Cleavage between Thr$^{65}$ and Tyr$^{66}$ would leave a 65-amino acid N terminus and a 173-residue C terminus with a calculated molecular mass of 19.9 kDa, consistent with the migration of GFP$_{20}$ in SDS-PAGE.

Mutations of the tripeptide alter GFP rearrangement and lead to different post-translational outcomes (28). To examine the contribution of chromophore maturation to fragmentation, we studied an eGFP mutant (GSGGF$^{−}$) previously described to prevent rearrangement and hence chromophore formation (Fig. 2A) (28). We also studied a mutant (GSGF$^{−}$) reported to induce eGFP cleavage between Gly$^{65}$ and Ser$^{66}$ as a positive control and comparator for fragment formation (Fig. 2A) (28) (note that F$^{+}$ and F$^{−}$ refers to the fragmentation state of GFP, as will be confirmed experimentally below).

We expressed WT and mutant eGFP fused to a C-terminal SIINFEKL (GFPS) or to an N-terminal NP-SIINFEKL reporter protein (NPSGFP) to precisely measure class I peptide genera-
Importantly, treating cells with the proteasome inhibitor MG132 had opposite effects on the generation of GFP<sup>20</sup> WT (increased) and GSG<sup>F−</sup> (decreased) (Fig. 2B, lanes 1 and 2 versus lanes 3 and 4). Because proteasome inhibitors typically decrease protein synthesis (the decrease in NPSGFP is evident), these findings indicate that GSG<sup>F+</sup>-GFP<sup>20</sup> is more stable than WT GFP<sup>20</sup>, which we have previously shown to exhibit a half-life of ~15 min (20, 22).

Quantitating the immunoblot signals in Fig. 2B reveals that the GSG<sup>F+</sup> fragments represent 35% of the total amount of signal (fragments + full length), a value that changes very little with MG132 treatment (33%). The WT fragment increases from 24% of total signal to 38% in the presence of proteasome inhibitor. These findings suggest that for both WT and GSG<sup>F+</sup> fusion proteins, roughly 1/3 of the full-length proteins are fragmented, which is consistent with our previous pulse labeling studies (see Fig. 6B in Ref. 20).

We made essentially similar findings when using SIINFEKL fused to the C terminus of eGFP (Fig. 2C) as follows: WT and GSG<sup>F−</sup> GFP fragment but GSSG<sup>F−</sup> does not, and only the WT fragment increased when we treated cells with MG132. Thus, appending GFP to NP does not affect its fragmentation.

Based on the above findings of GFP expression in cells, we conclude that about one-third of the WT GFP protein fragments. This is due to the chromophore maturation process.

**Similar GFP Fragments Display Large Differences in Biochemical Behavior**—We further characterized WT and GSG<sup>F+</sup> fragments by measuring their stability via immunoblotting after treating rVV-infected L-K<sup>+</sup> cells with the protein synthesis inhibitors cycloheximide and emetine to immediately stop translation (Fig. 3, A and B). For the WT C-terminal fragment, the data nearly perfectly fitted first-order decay kinetics, with a calculated 18.5 min half-life (t<sub>1/2</sub>). By contrast, the GSG<sup>F+</sup> fragment increased for the first 60 min until reaching steady state. Because any potential degradation is offset by ongoing fragment generation, we cannot calculate precise half-lives for degradation or creation, but it is clear that degradation is slower for GSG<sup>F+</sup> than WT fragment. Importantly, the N-terminal fragment, detected either using anti-GFP or anti-NP antibodies, behaved similarly to the C-terminal fragment, with the WT fragment demonstrating rapid degradation and the GSG<sup>F+</sup> fragment demonstrating continued generation (Fig. 3C).

Immunoblotting Triton X-100-soluble and -insoluble fractions with anti-GFP C-terminally specific antibodies revealed a similar pattern in each fraction for WT and GSG<sup>F+</sup>, although GSSG<sup>F−</sup> again failed to exhibit detectable fragmentation (Fig. 3D).Treating cells with MG132 between 0.5 and 2.5 h post-infection recapitulated the pattern of WT (increased), GSG<sup>F+</sup> (decreased), and GSSG<sup>F−</sup> (none observed) fragment expression and revealed that the entire increase of the WT fragment was accounted for in the Triton X-100-insoluble fraction (Fig. 4A), consistent with our previous findings (20). We observed a similar pattern for the N-terminal fragment, with nearly all of the fragments recovered from the Triton X-100-insoluble fraction following MG132 treatment (Fig. 4B).

The pattern of C-terminal fragment recovery was nearly identical in L-K<sup>+</sup> cells infected with the GFPS constructs (Figs. 3E and 4C). The only significant difference between NPSGFP
and GFPS constructs is that although a cohort of NPSGFP was Triton X-100-insoluble without proteasome treatment (expected, due to the nuclear localization of NP and partial extraction of nuclear proteins by Triton X-100 (29)), GFPS was only present in insoluble fractions when cells were treated with proteasome inhibitors. This occurred essentially equally for WT, GSGF/H11001, and GSGGF/H11002 forms.

These findings demonstrate that although the GSGF/H11001 and GSGGF/H11002 mutations have no detectable effect on full-length NPSGFP or GFPS stability or detergent solubility, there are substantial effects on the kinetics of fragment generation and degradation (Table 1). To further explore the mechanism for this difference, we pulse-labeled rVV-infected cells with [35S]Met for 5 min, recovered N-terminal fragments by immunoprecipitation using the anti-native NP antibody HB-65 (29), and then quantitated the association of the C-terminal fragment with the N-terminal fragment using a PhosphorImager to quantitate the SDS-PAGE species (Fig. 5).

Over three experiments, the stoichiometry of N- versus C-terminal fragment recovery was consistently different between WT and GSGF/E+ (Fig. 5A). Remarkably, accounting for the number of Met residues in the N- versus C-terminal fragments (25 versus 5, respectively), their ratio in GSGF/E+ is close to 1, indicating that essentially all C-terminal fragments remain attached to the N terminus. By contrast, for WT NPSGFP, fewer C-terminal fragments were recovered, demonstrating that fragmentation generates free fragments. Because such fragments are unlikely to maintain native structure, this accounts for their rapid degradation and detergent insolubility. By contrast, because the GSGF/E+ fragments remain associated in presumably native or near native form, they exhibit the metabolic stability of full-length NPSGFP.

WT GFP Fragments Are an Important Source of DRiP Peptides—As a cohort of translation products that are clearly defective, WT NPSGFP and GFPS fragments fully meet the definition of DRiPs. Because they are rapidly degraded, they represent a potential source of peptides, particularly given their abundance and their sensitivity to proteasome inhibitors (Kb-SIINFEKL generation from NPSGFP and GFPS is completely inhibited by MG132 (Fig. 6)).

To precisely measure Kb-SIINFEKL cell surface expression by flow cytometry, we used Alexa647-conjugated 25-D1.16, a monoclonal antibody specific for this complex (24). VV gene expression is highly dependent on infectious dose. Because the GSGF/E+ and GSGGF/E+ constructs express nonfluorescent GFP

![Figure 3](Image)

**FIGURE 3.** WT fragments are rapidly degraded although GSGF/E+ fragments are stable. We infected L-Kb cells with rVVs at an m.o.i. of 10 for 30 min, added protein synthesis inhibitors (20 μg/ml cycloheximide and 20 μg/ml emetine) to cell cultures at 2.5 hpi, and collected cells at the indicated time points after the addition of protein synthesis inhibitors (PSI). A, by immunoblotting whole cell lysates, we measured levels of NPSGFP (upper panel) and C-terminal fragments (middle panel) using anti-GFP C-terminal antibody and determined levels of actin as a loading control (lower panel) using anti-actin antibody. B, quantitation of WT GFP C-terminal fragment degradation after the addition of protein synthesis inhibitors. We normalized the signals representing WT GFP C-terminal fragment at 30, 60, 120, and 180 min to the signal at time 0. Mean values and standard deviations from three independent experiments are given. We used GraphPad Prism software to fit the curve to a nonlinear regression model with a half-life of 18.5 min. C, by immunoblotting, we measured levels of NPSGFP and NPSGFP N-terminal fragment using anti-GFP N-terminal antibody (upper panel) and anti-NP antibody (lower panel). D, we used Triton X-100 to fractionate whole cell lysates into soluble and insoluble fractions and measured levels of NPSGFP and NPSGFP C-terminal fragment using anti-GFP C-terminal antibody. E as in A, but using rVVs expressing GFPS(WT), GFPS(GSGF/H11001), and GFPS(GSGGF/H11002).
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(Fig. 7A), we could not normalize infection values to GFP expression. Therefore, we recreated the rVV panel using a backbone VV expressing tagBFP from a different early VV promoter as a means to normalize VV gene expression following infection of L-Kb cells (Fig. 7, B and C). We inserted NPSGFP and GFPS coding sequences into the thymidine kinase locus with tagBFP controlled by a synthetic optimized early/late poxviral promoter (30). Because fluorescent BFP signals were similar among cells infected with different rVVs at the same m.o.i. (Fig. 7B) as expected, we used these values to normalize antigen expression levels at the single cell level.

As seen in Fig. 7D, at 4 h post-infection, blocking chromophore maturation and fragment generation (GSGF<sup>+</sup>) reduced Kb-SIINFEKL by 50% after normalizing based on BFP expression. We could directly attribute this effect to degradation of the C-terminal fragment because GSGF<sup>+</sup>, which generates stable fragments, also demonstrated a 50% decrease in Kb-SIINFEKL expression.

To confirm the results we obtained from rVVs containing tagBFP, we used intracellular antibody staining of the VV early protein E3L to normalize gene expression. The different efficiency of peptide generation from WT versus GSGF<sup>+</sup> and GSSGF<sup>-</sup> constructs occurred over the entire time course of expression (Fig. 7E). Cementing these findings, we observed a highly similar effect in GFPS constructs (Fig. 7F).

In Fig. 7G, we directly compared Kb-SIINFEKL generation from NPSGFP (N-terminal fragment) and GFPS (C-terminal fragment). Peptide generation from the C-terminal fragment is more efficient in this case, which could result from the different flanking sequences in each set of constructs.

Taken together, these data show that a natural DRiP generated from GFP autocleavage accounts for up to 50% of the peptide generation from this reporter protein that is commonly used as a fusion partner for antigen processing studies. Notably, the peptide can either be on the N-terminal fragment (for NPS-GFP) or the C-terminal fragment (for GFPS).

Discussion

A PubMed search for GFP or similar fluorescent proteins returns more than 45,000 publications, with a still rising yearly rate. Based on the importance of these proteins to modern biomedical research, it is critical to understand how GFP might perturb its genetic fusion partners in the cells or organisms that express it.

Here, we show that for a wide variety of GFP and RFPs, approximately a third of the proteins synthesized autofragment in cells, likely as a consequence of chromophore maturation, as we directly demonstrate for eGFP. We cannot determine whether fragmentation is an inevitable chemical event in generating GFP and RFP chromophores or whether it results from expressing these proteins outside of their naturally evolved contexts (marine organisms living at relatively low ambient temperatures). Although it seems unlikely, we cannot rule out generating GFP and RFP chromophores or whether it results from expressing these proteins outside of their naturally evolved contexts (marine organisms living at relatively low ambient temperatures). Although it seems unlikely, we cannot rule out that the fragments have a biological function in the host organism. There would not necessarily be tremendous evolutionary selection pressure for efficient chromophore maturation, because the natural organisms could simply just synthesize more of this very unusual protein and deal with the fragments generated by the natural degradation machinery. Paraphrasing Samuel Johnson: “A fluorescent protein is like a dog walking on

**TABLE 1**

Properties for GFP and its mutants

| Variant | WT | T65G/Y66S | GSGF<sup>+</sup> | GSGG<sup>+</sup> |
|---------|----|-----------|-----------------|-----------------|
| Chromophore tripeptide | TYG | T65G/Y66S | GSGF<sup>+</sup> | GSGG<sup>+</sup> |
| Mutations | Yes | Yes | No | No |
| Fluorescent | Yes | No | Yes | No |
| Fragment properties | 1. Rapid degradation | 1. Slow degradation | 2. Decrease with proteasome inhibition | 3. Fragments are free |
| | 2. Increase with proteasome inhibition, accumulating in Triton X-100-insoluble fraction | 3. C-terminal fragment remain attached to the N-terminal fragment | 4. Fragments contribute to DRiP antigen presentation | |

**FIGURE 4.** Proteasome inhibition rescues WT but not GSGF<sup>+</sup> fragments into the Triton X-100-insoluble fraction. We infected L-Kb cells with rVVs at an m.o.i. of 10 for 30 min, cultured cells with (+) or without (−) 20 μM MG132, harvested cells at 5 hpi, and fractionated cells with Triton X-100 (TX-100). A, by immunoblotting we determined levels of NPSGFP (upper panel) and C-terminal fragments (middle panel) using anti-GFP C-terminal antibody and measured levels of actin as a loading control (lower panel) using anti-actin antibody. B, as in A but detecting N-terminal fragments with anti-GFP N-terminal antibody. Note that the lower levels of the GSGF<sup>+</sup> fragment recovered after MG132 treatment likely result from MG132 inhibition of translation. C, as in A but using rVVs expressing GFPS(WT), GFPS(GSGF<sup>+</sup>), and GFPS(GSGG<sup>+</sup>).
his hind legs. It is not done well; but you are surprised to find it done at all."

Fragmentation has been reported for GFP mutants (28) and WT RFP family members (31–34), but only in vitro and typically after subjecting the proteins to harsh conditions (chemical denaturants and heat). Here, we show that all commonly used denaturants and heat). Here, we show that all commonly used

GFP and RFP variants we examined exhibit extensive fragmentation is likely a consequence of ribosomal synthesis per se. Here (see Fig. 5) and in previous studies (20, 22), we show that considerable WT GFP fragmentation occurs during a 5-min radiolabeling period, demonstrating that it is a rapid event.

Most interestingly, changing just two chromophore amino acids (65TY to 65GS) completely changes the nature and fate of GFP fragmentation. We show that GSGF fragments remain associated (Fig. 5) (as originally described by Barondeau et al. (28)) and exhibit the metabolic stability characteristic of the full-length native protein, by-passing the rapid and destructive fragmentation characteristic of WT GFP. Together with the lack of GFP fragmentation from the GSGGG non-rearranging mutants, this clearly demonstrates that WT GFP fragmentation is a by-product of chromophore generation.

We are uncertain as to how fragmentation occurs in WT fluorescent proteins, and we note that the precise cleavage site in nascent WT GFP remains to be determined more directly. Presumably, chromophore rearrangement (and fragmentation) requires that folding of the characteristic barrel structure is completed, which occurs within seconds following release from ribosomes (35), consistent with the rapid generation of GFP fragmentation we observe. In vitro, the final step of chromophore formation, the formation of the imidazolinone ring by addition of molecular oxygen, exhibits a half-life on the order of 10 min for eGFP (36). GFP maturation in cells may occur more slowly than in vitro (nascent eGFP becomes fluorescent with a t½ of ~20 min in cells following protein synthesis inhibition (see Fig. 2C in Princiotta et al. (18)). Because WT fragment generation is largely complete during the 5-min pulse labeling, this suggests that fragmentation occurs prior to the final step of chromophore maturation.

However they are generated, both the WT GFP N- and C-terminal fragments represent a substantial source of peptides for antigen presentation. This is clearly shown by the 30–50% reduction in K<sub>β</sub>-SIINFEKL generation from NPSGFP (N-terminal fragment) and GFPS (C-terminal fragment) associated with changing the WT chromophore to GSGF<sup>−</sup> or GSGGG<sup>−</sup> mutants. Importantly, presentation of antigen from these fragments is an inevitable consequence of GFP maturation.

Farfán-Arribas et al. (37) used intein catalysis in GFP fusion proteins to demonstrate that antigenic peptides can be generated by protein auto-splicing. Although the authors concluded that the intein-generated peptides must derive from retirees, spliced antigenic peptide-containing fragments are clearly abundant in their pulse-chase experiments (see Fig. 2 in Ref. 37), and are a near certain source of peptides. Thus, when using GFP fusion proteins to study the contributions of retirees to antigen presentation, experimental measures must be employed to segregate rapid presentation from GFP fragments (and other GFP-derived DRiPs) (11, 22).

Knowing that GFP fragments approximately account for 33% of total GFP synthesis and 50% of K<sub>β</sub>-SIINFEKL generation, we can revisit our previous studies on antigen processing efficiency (11, 18). Lacking knowledge of the abundance of relevant rapidly degraded DRiPs, we were unable to calculate the efficiency of peptide generation from DRiPs. In Princiotta et al. (18), we found that 300 K<sub>β</sub>-SIINFEKL complexes are generated for every 6 × 10<sup>5</sup> NPSGFP molecules synthesized. Our present findings indicate that 150 complexes are derived from 3 × 10<sup>5</sup> NPSGFP...
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FIGURE 7. Rapidly degraded WT GFP fragments are a major source of antigenically relevant DRiPs. A–C, we infected L-Kb cells with rVV expressing both BFP and NPSGFP at an m.o.i. of 10 for 30 min at 37°C and incubated cells for 4 h. After staining live cells with AlexaFluor 647-conjugated 25D1.16 monoclonal antibody against Kb-SIINFEKL, we measured fluorescence of GFP (A), Kb-SIINFEKL (B), or BFP (C) by flow cytometry. Mean values and standard deviation for all measurements are derived from three independent experiments. MFI, mean fluorescence intensity. D, we replotted data from 8 and C to normalize complex formation to account for the difference in gene expression between different rVV. We used GraphPad Prism software to calculate p value (Student’s t test). E, we infected L-Kb cells with rVV expressing NPSGFP at an m.o.i. of 10 for 30 min at 37°C and then cultured cells for the indicated times. We determined levels of surface Kb-SIINFEKL and intracellular VV E3L by flow cytometry. We normalized Kb-SIINFEKL complex formation to E3L levels and then calculated the normalized complex formation relative to Kb-SIINFEKL expression from NPSGFP(WT) at 6 hpi. F, as in E, but using rVV expressing GFPS(WT), GFPS(GSGFG), and GFPS(GSGGF). G, as in E and F, we measured levels of surface Kb-SIINFEKL and intracellular VV E3L by flow cytometry using rVV expressing NPSGFP(WT) and GFPS(WT) in parallel.

fragments. This is equal to the calculated efficiency of one complex generated for every 2000 fragments degraded. Similarly, revisiting the data in Dolan et al. (11), we can now calculate that the efficiency of generating Kb-SIINFEKL from fragments derived from SCRAP (a GFP-degron fusion protein that is stabilized by shield-1, a cell permeant drug that binds to the degron) is one complex generated for 50 fragments degraded, when SCRAP is expressed from a transfected gene, and 1 in 500 when SCRAP is expressed by VV or VSV.

As noted previously, a 1:2000 efficiency is consistent with peptide supply from a 30% RDP fraction (38), whereas a 1:50 efficiency from either DRiPs or retirees is far too high to account for peptides to be the rate-limiting step in class I export (16) or transporter associated with antigen processing (39). Clearly, further studies are needed to clarify this situation, but we do note that this will require quantitating the various substrates. Establishing the fraction of GFP that fragments and its contribution to the antigenic peptide pool are the first steps in biochemically defining the relevant DRiP population and represents a significant step in the right direction.

Finally, it is important to discuss the implications that a commonly used reporter protein generates a large fraction of defective fragmented protein that must be handled by cellular degradation pathways. Inasmuch as GFP fragments are typical DRiPs, cells should have little difficulty in dealing with them, because they are a drop in the bucket of proteasome substrates, even if RDPs were as small as a few percent of total translation. But unlike normal RDPs, GFP did not evolve in yeast or mammalian cells and did not have to satisfy the first rule of protein evolution in the context of these organisms, which is to play well with others.

Indeed, Drummond and co-workers (40) reported that expression of just 50,000 copies of GFP per yeast cell (or <0.1% of total protein) results in a 3% fitness cost (as measured by competitive growth rates) when expressing a misfolded GFP. Relevant to our study, WT GFP exhibited half of the fitness cost. Could this be due to cytostatic effects of the GFP fragments? In any event, it would be of interest and potentially of great use to find or engineer fluorescent proteins that could generate a chromophore without the attendant costs of producing non-fluorescent and potentially damaging fragments.

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References
1. Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173, 33–38
2. Poole, B., and Wibo, M. (1973) Protein degradation in cultured cells. The effect of fresh medium, fluoride, and iodoacetate on the digestion of cellular protein of rat fibroblasts. J. Biol. Chem. 248, 6221–6226
3. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78, 761–771
4. Yewdell, J. W., Lascina, J. R., Rechsteiner, M. C., and Nicchitta, C. V. (2011) Out with the old, in with the new? Comparing methods for measuring protein degradation. Cell Biol. Int. 35, 457–462
5. Yewdell, J. W., Anton, L. C., and Bennink, J. R. (1996) Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules? J. Immunol. 157, 1823–1826
6. Anton, L. C., and Yewdell, J. W. (2014) Translating DRiPs: MHC class I immunosurveillance of pathogens and tumors. J. Leukocyte Biol. 95, 551–562
7. Dolan, B. P., Li, L., Takeda, K., Bennink, J. R., and Yewdell, J. W. (2010) Defective ribosomal products are the major source of antigenic peptides endogenously generated from influenza A virus neuraminidase. J. Immunol. 184, 1419–1424
8. Apecher, S., Millot, G., Daskalogianni, C., Scherl, A., Manoury, B., and Fäthauer, R. (2013) Translation of pre-spliced RNAs in the nuclear compartment generates peptides for the MHC class I pathway. Proc. Natl. Acad. Sci. U.S.A. 110, 17951–17956
9. David, A., Dolan, B. P., Hickman, H. D., Knowlton, J. J., Clavirano, G., Pierre, P., Bennink, J. R., and Yewdell, J. W. (2012) Nuclear translation visualized by ribosome-bound nascent chain puromycylation. J. Cell Biol. 197, 45–57
10. Hosaka, Y., Sasafo, F., Yamanaka, K., Bennink, J. R., and Yewdell, J. W. (1988) Recognition of noninfectious influenza virus by class I-restricted
murine cytotoxic T lymphocytes. J. Immunol. 140, 606–610

11. Dolan, B. P., Sharma, A. A., Gibbs, J. S., Cunningham, T. J., Bennink, J. R., and Yewdell, J. W. (2012) MHC class I antigen processing distinguishes endogenous antigens based on their translation from cellular vs. viral mRNAs. Proc. Natl. Acad. Sci. U.S.A. 109, 7025–7030

12. Croft, N. P., Smith, S. A., Wong, Y. C., Tan, C. T., Dudek, N. L., Fleisch, I. E., Lin, L. C., Tscharké, D. C., and Purcell, A. W. (2013) Kinetics of antigen expression and epitope presentation during virus infection. PLoS Pathog. 9, e1003129

13. Duttler, S., Pechmann, S., and Frydman, J. (2013) Principles of cotranslational ubiquitination and quality control at the ribosome. Mol. Cell 50, 379–393

14. Brandman, O., Stewart-Ornstein, J., Wong, D., Larson, A., Williams, C. C., Li, G. W., Zhou, S., King, D., Shen, P. S., Weibelzahn, J., Dunn, J. G., Roussin, S., Inada, T., Frost, A., and Weissman, J. S. (2012) A ribosome-bound quality control complex triggers degradation of nascent peptides and signals translation stress. Cell 151, 1042–1054

15. Wheatley, D. N., Giddings, M. R., and Inglis, M. S. (1980) Kinetics of degradation of “short” - and “long-lived” -proteins in cultured mammalian cells. Cell Biol. Int. Rep. 4, 1081–1090

16. Schubert, U., Antón, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W., and Bennink, J. R. (2000) Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature 404, 770–774

17. Vabulas, R. M., and Hartl, F. U. (2005) Protein synthesis upon acute nutrient restriction relies on proteasome function. Science 310, 1960–1963

18. Princiotta, M. F., Finzi, D., Qian, S. B., Gibbs, J., Schuchmann, S., Buttgereit, F., Bennink, J. R., and Yewdell, J. W. (2003) Quantitating protein synthesis, degradation, and endogenous antigen processing. Immunity 18, 343–354

19. Qian, S. B., Reits, E., Neefjes, J., Deslich, J. M., Bennink, J. R., and Yewdell, J. W. (2006) Tight linkage between translation and MHC class I peptide ligand generation implies specialized antigen processing for defective ribosomal products. J. Immunol. 177, 227–233

20. Qian, S. B., Princiotta, M. F., Bennink, J. R., and Yewdell, J. W. (2006) Characterization of rapidly degraded polypeptides in mammalian cells reveals a novel layer of nascent protein quality control. J. Biol. Chem. 281, 392–400

21. Qian, S. B., Ott, D. E., Schubert, U., Bennink, J. R., and Yewdell, J. W. (2002) Fusion proteins with COOH-terminal ubiquitin are stable and maintain dual functionality in vivo. J. Biol. Chem. 277, 38818–38826

22. Dolan, B. P., Li, L., Veltri, C. A., Ireland, C. M., Bennink, J. R., and Yewdell, J. W. (2011) Distinct pathways generate peptides from defective ribosomal products for CD8+ T cell immunosurveillance. J. Immunol. 186, 2065–2072

23. Chakrabarti, S., Brechling, K., and Moss, B. (1985) Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. 5, 3403–3409

24. Porgador, A., Yewdell, J. W., Deng, Y., Bennink, J. R., and Germain, R. N. (1997) Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. Immunity 6, 715–726

25. Qian, S. B., Bennink, J. R., and Yewdell, J. W. (2005) Quantitating defective ribosome products. Methods Mol. Biol. 301, 271–281

26. Sawasawa, T., Hasegawa, Y., Tsuchimochi, M., Kamura, N., Ogawara, T., Kuroita, T., and Endo, Y. (2002) A bilayer cell-free protein synthesis system for high-throughput screening of gene products. FEBS Lett. 514, 102–105

27. Remington, S. J. (2006) Fluorescent proteins: maturation, photochemistry and photophysics. Curr. Opin. Struct. Biol. 16, 714–721

28. Barondeau, D. P., Kassmann, C. I., Tainer, J. A., and Getzoff, E. D. (2006) Understanding GFP posttranslational chemistry: structures of designed variants that achieve backbone fragmentation, hydration, and decaX05bylation. J. Am. Chem. Soc. 128, 4685–4693

29. Antón, L. C., Schubert, U., Bacik, I., Princiotta, M. F., Wearsch, P. A., Gibbs, J., Day, P. M., Realini, C., Rechsteiner, M. C., Bennink, J. R., and Yewdell, J. W. (1999) Intracellular localization of proteasomal degradation of a viral antigen. J. Cell Biol. 146, 113–124

30. Blasco, R., and Moss, B. (1991) Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-dalton outer envelope protein. J. Virol. 65, 5910–5920

31. Gross, L. A., Baird, G. S., Hoffman, R. C., Baldridge, K. K., and Tsien, R. Y. (2000) The structure of the chromophore within DsRed, a red fluorescent protein from coral. Proc. Natl. Acad. Sci. U.S.A. 97, 11990–11995

32. Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A. (2002) An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. Proc. Natl. Acad. Sci. U.S.A. 99, 12651–12656

33. Martynov, V. I., Savitsky, A. P., Martynova, N. Y., Savitsky, P. A., Lukyanov, K. A., and Lukyanov, S. A. (2001) Alternative cyclization in GFP-like proteins family. The formation and structure of the chromophore of a purple chromoprotein from Anemonia sulcata. J. Biol. Chem. 276, 21012–21016

34. Remington, S. J., Wachter, R. M., Yarbrough, D. K., Branchaud, B., Anderson, D. C., Kallio, K., and Lukyanov, K. A. (2005) zFP538, a yellow-fluorescent protein from Zoanthus, contains a novel three-ring-chromophore. Biochemistry 44, 202–212

35. Kelkar, D. A., Khushoo, A., Yang, Z., and Skach, W. R. (2012) Kinetic analysis of ribosome-bound fluorescent proteins reveals an early, stable, cotranslational folding intermediate. J. Biol. Chem. 287, 2568–2578

36. Iizuka, R., Yamagishi-Shirasaki, M., and Funatsu, T. (2011) Kinetic study of de novo chromophore maturation of fluorescent proteins. Anal. Biochem. 414, 173–178

37. Farfán-Arribas, D. J., Stern, L. J., and Rock, K. L. (2012) Using in vitro catalysis to probe the origin of major histocompatibility complex class I-presented peptides. Proc. Natl. Acad. Sci. U.S.A. 109, 16998–17003

38. Yewdell, J. W., Reits, E., and Neefjes, J. (2003) Making sense of mass destruction: quantitating MHC class I antigen presentation. Nat. Rev. Immunol. 3, 952–961

39. Reits, E. A., Vos, J. C., Grommé, M., and Neefjes, J. (2000) The major substrates for TAP in vivo are derived from newly synthesized proteins. Nature 404, 774–778

40. Geiler-Samerotte, K. A., Dion, M. F., Budnik, B. A., Wang, S. M., Hartl, D. L., and Drummond, D. A. (2011) Misfolded proteins impose a dosage-dependent fitness cost and trigger a cytosolic unfolded protein response in yeast. Proc. Natl. Acad. Sci. U.S.A. 108, 680–685