Cell. Mol. Life Sci. (2011) 68:1481–1489
DOI 10.1007/s00018-011-0656-z

Cellular and Molecular Life Sciences

MULTI-AUTHOR REVIEW

Translating DRiPs: progress in understanding viral and cellular sources of MHC class I peptide ligands

Brian P. Dolan · Jack R. Bennink · Jonathan W. Yewdell

Received: 17 February 2011 / Revised: 17 February 2011 / Accepted: 18 February 2011 / Published online: 17 March 2011

Abstract It has been 15 years since we proposed the defective ribosomal product (DRiP) hypothesis to explain the rapid presentation of viral peptides by MHC class I molecules on the surface of infected cells. Here, we review the evidence for the contribution of DRiPs to antigen processing, pointing to the uncertainties regarding the physical nature of DRiPs, and emphasizing recent findings suggesting that peptide generation is a specialized process involving compartmentalized translation.

Keywords Antigen processing · MHC class I · Proteasome · Translation · Virus

> *That we find out the cause of this effect. Or rather say, the cause of this defect. For this effect, defective comes by cause.*
> Polonius, regarding Hamlet's insanity
> *Hamlet, Prince of Denmark,*
> William Shakespeare

The race goes to the swift

Doherty and Zinkernagel's demonstration of the dual viral-antigen and MHC-restricted nature of cytotoxic T cell recognition of virus-infected cells [1] triggered a mad scramble to understand the molecular nature of the recognition event. Unanue [2] and Grey [3] provided the initial conceptual breakthrough, in demonstrating that MHC class II molecules bind to short peptide sequences and present them to CD4 T cells. This work was quickly extended by Townsend [4] to MHC class I molecules and viral peptides. Because Townsend [4] arrived at this answer by demonstrating CTL recognition of cells expressing truncated metabolically unstable forms of a viral protein (a phenomenon reported initially by Tevethia [5]), it was immediately clear that peptides were derived from protease products of biosynthesized proteins.

The problem of presenting peptides from metabolically stable viral proteins with sufficient alacrity was given little thought, but this poses a major problem for immunosurveillance. A single cell has $\sim 3 \times 10^9$ copies of proteins, yet only expresses $\sim 1 \times 10^5$ class I molecules [6]. Were it necessary to wait for the degradation of a viral protein with a half-life measured in days, antigen presentation would initiate only well after viral replication was completed. Standard CTL assays through the 1980 with lytic viruses like influenza A virus (IAV) or vaccinia virus (VV) were performed over a period of 10–16 h postinfection, giving little reason to consider the kinetics of peptide generation. By the late 1980, however, the introduction of inhibitors like cycloheximide (CHX) [7] and brefeldin A [8] to antigen presentation studies led naturally to consideration of the kinetics of antigen presentation. In the guise of quantitating the requirement for TAP in endogenous viral-antigen presentation, we found that a sufficient vesicular stomatitis virus (VSV) nucleocapsid protein (N) is synthesized within the initial 45 min of infection to enable CTL killing of target cells [9].

Nucleocapsid protein, like most viral proteins, is extremely stable in infected cells, so how could enough N be made in 45 min to enable T cell recognition? Further, how could we explain why CHX treatment greatly retarded the export of nascent class I molecules from the ER, enabling

B. P. Dolan · J. R. Bennink · J. W. Yewdell (✉)
Laboratory of Viral Diseases, NIAID, Bethesda, MD 20892, USA
e-mail: jyewdell@nih.gov

antigen presentation of cytosolically-delivered exogenous viral proteins more than 24 h after addition of CHX to cells [10]? These findings prompted the DRiP hypothesis, which posited that peptides are generated from defective ribosomal products, i.e. defective forms of newly synthesized proteins that are degraded rapidly due to inability to properly fold, assemble, or traffic [11].

DRiP: it's not just semantics

After 15 years, what is the status of the DRiP hypothesis? Antigen presentation kinetics inspired the DRiP hypothesis, and kinetics provides the most compelling evidence for relevance of DRiPs to antigen presentation. This evidence, which continues to deepen [12–14] has been reviewed recently [15–17]. In short, studies in both viral and host antigen systems indicate that the generation peptides relevant for antigen processing is closely tied kinetically to increases and decreases in protein synthesis, strongly implying that many/most peptides derive from pool degraded with a half-life two orders of magnitude less than the average half-life of proteins, namely, 10 min versus 24 h (1,440 min).

It is worth digressing on the nature of the evidence. A key strategy in gauging the contribution of DRiPs to peptide generation is to examine the effect of abrogating protein synthesis with translation inhibiting drugs, typically CHX, which blocks polypeptide elongation. Based on the immediacy of the shut of peptide generation, the half-life of the peptide precursor pool can be calculated. A potential limitation, however, to interpreting the action of CHX on peptide generation is that it effects protein degradation, or other features of class I assembly or transport unrelated to its effects on precursor pool size.

A number of recent studies support the conclusion that CHX treatment does not inhibit the antigen processing and presentation machinery. We reported that presentation of the influenza virus peptide $PA_{224–233}$ synthesized as a VV minigene product or Ub-liberated peptide continues for hours in the presence of CHX due the unusual ability of $PA_{224–233}$ to generate a sizable intracellular pool [18]. This extended our prior demonstration [19] that CHX has no significant effect on degradation of RDPs or slowly degraded proteins measured by pulse chase experiments, and that peptide generation is prolonged after CHX treatment if a pool of precursors (presumably DRiPs) is expanded by treating cells with a proteasome inhibitor. Similarly, Neefjes and colleagues showed that peptide generation continues unabated in the presence of CHX for at least 7 h if cells are exposed to γ-irradiation [19]. Taken together, these findings provide solid support for the validity of short term CHX-shutdown (on the order of hours) as a means of measuring the metabolic stability of peptidogenic substrates.

While the evidence supporting an important role for DRiPs in antigen processing mounts, there has been somewhat less progress in understanding the physical nature of DRiPs. As this is a key area for future studies, it is useful to discuss the meaning of "DRiP", which ultimately must have a physical definition, or more accurately, definitions, since DRiPs almost certainly comprise different categories of substrates. Casting a wide net, we originally defined DRiPs as any non-functional form of a gene product, including forms that would not qualify as proteins per se, which is why the P in DRiP is product and not protein. This includes unfolded or misfolded proteins of the proper length and sequence, proteins with sequence errors or errors in posttranslational modification, prematurely terminated proteins, and proteins translated from the wrong start codon. We later extended DRiPs to include wrong reading frames, and sequences that do not encode traditional proteins, as encoded by introns, upstream sequences etc., all of which are now known to be sources of antigenic peptides [17].

Based, however, on the identification of thousands of defined viral peptides by functional assays and thousands of cellular peptides by mass spectrometry, it is reasonably clear that nearly all abundant peptides derive from standard open reading frames. Eisenlohr [20] and colleagues recently revisited the DRiP hypothesis, insightfully raising the issue that many misfolded proteins are degraded with kinetics that are approximately 10-fold slower than DRiPs relevant to antigen processing (i.e. on the order of hours). They propose that peptides are generated from otherwise normal full length gene products that are stochastically ignored by the folding machinery and targeted for immediate destruction by 20S proteasomes (more on this below). As it is essentially impossible experimentally to know whether such substrates are not defective in some essential manner, there seems to be little purpose in not terming them DRiPs. It might be worth pointing to the original DRiP model, which proposed that a subset of DRiPs *"may be directly derived from ribosomes by association with "anti-chaperones" that target the polypeptide to proteases"* [11], which seems to jibe closely with the thinking of Eisenlohr.

Physically defining DRiPs

In 2000 we unwittingly rediscovered the original finding of Denys Wheatley that a large fraction (nearly 40% in Wheatley's dexterous hands) of nascent total proteins are degraded with a half-life of 10 min after 1 min labeling with [^{3}H]-Leu [21, 22]. The true fraction of such rapidly

degraded polypeptides (RDPs) is subject to some uncertainty, as we pointed out in our original review of DRiPs [23], and questioned experimentally by Vabulas and Hartl [24] (but see Yewdell and Nicchitta [17] for rebuttal). The size of the RDP fraction, which is of great interest and importance to cell function and evolution, is, however, a red herring in terms of the contribution of DRiPs to antigen processing. This is from Yewdell and Nicchitta [17]:

> *It is crucial to stress that the RDP fraction is only tangentially related to the fraction of MHC class I peptide ligands that derive from DRiPs. In other words, RDPs could constitute 1% (or less) of newly synthesized proteins, yet still provide all of the peptides presented by class I molecules.*

Readers might feel that we are being a tad slippery with our words here. In our defense, nature itself is slippery, since there is not a simple digital distinction between DRiPs and native proteins. An entire class of proteins, termed intrinsically disordered proteins (IDPs-we swear, not our acronym!) are naturally disordered, and fold into stable structures only conditionally, typically when bound to other proteins [25, 26]. It is thought that more than 30% of eukaryotic proteins contain at least one disordered domain of more than 50 residues [26].

That not all RDPs contribute equally to antigen processing is suggested by our findings that RDPs can be divided into two classes based on their solubility following their rescue from proteasome degradation [27]. The major fraction ($\sim$75%) of RDPs is soluble in mild detergent, modulated by HSC70 levels, and degraded by the classical ubiquitin (Ub)-26S proteasome pathway. The remainder is insensitive to HSC70 levels, become insoluble within an hour of blocking proteasome activity, and are degraded independently of ubiquitylation by proteasomes lacking 19S regulators. The latter appear to represent the bulk of DRiPs from standard proteins, while the former appear to be the principal source of peptides with exogenous degradation signals that interfere with folding or directly target the proteins to the Ub-proteasome pathway. What are these insoluble products? We found one clue in this study. A COOH-fragment of GFP, probably generated by autocatalytic cleavage associated with fluorophore maturation [28], exhibited all the characteristics of the bulk insoluble fraction [27].

Such fragments might be a common source of DRiPs. We provided evidence that common downstream initiation events generates peptidogenic DRiPs when antigens are expressed by standard Semliki forest virus vectors [16]. Downstream initiation in this case is due to SFV modification of translations factors, and is dependent on eIF2a phosphorylation. Gu et al. [29] used shRNAs directed against a target antigenic protein to cleave its mRNA. When SIINFEKL was inserted upstream of the interfering target, K^b-SIINFEKL complex expression was enhanced relative to cells expressing control shRNA, despite decrease expression of full length protein. Cardinaud et al. [14] investigated the long known ability of the Gly Ala repeat (GAr) of the gamma herpes virus EBNA-1 protein to interfere with immunosurveillance of the EBNA-1 gene [30]. It was originally believed that the GAr completely abrogated antigen processing of EBNA-1 by blocking proteasome mediated peptide generation in a cis acting manner [31]. Subsequently, it was shown that EBNA-1 is immunogenic in humans and antigenic in cell lines [32–35], and critically, that EBNA-1 diminishes antigen presentation by interfering with translation [36]. Cardinaud et al. found that the GAr interferes with translation by inducing premature termination in or just upstream of the GAr, an effect that is likely due to the secondary structure of mRNA encoding the repeat, since altering codon usage of this segment abrogates its activity [37]. Most importantly, Cardinaud et al. found that regardless of the presence of the GAr, truncated polypeptides were generated in substantial amounts, and tied their degradation to peptide generation (surprisingly in cells, as opposed to in vitro translation, the amount of truncated protein was greatly increased by removal of the GAr).

A key challenge for future studies is to determine the overall contribution of downstream initiation and premature termination to DRiP generation. If one is the predominant mechanism for generating DRiPs, there should be respectively, COOH-terminal or NH_2-terminal bias in class I peptide ligands defined structurally and class I antigens defined functionally. Given the ever-increasing number of identified class I ligands, there clear opportunities for bioinformatics, particularly combined with quantitative mass spectrometry [38], to provide important clues as the biochemical nature of DRiPs.

Mass action: it's not for everyone

The law of mass action holds that the concentration of reactants is proportional to the reaction rate and at equilibrium to the concentration of product. Given a finite supply of class I molecules, as peptides approach saturating concentrations, they should compete for binding, assuming they are in a common compartment. To test this, we took advantage of the ability of VV to increase viral gene expression in proportion to the multiplicity of infection (10-fold increase in multiplicity leading to a 3-fold increase in gene expression) [39]. We found that it was possible to saturate class I presentation at a relatively low MOI using viruses expressing class I binding peptides as minigene products or Ub-liberated products. This enabled us to

perform competition experiments by co-infecting cells expressing different defined peptides. As predicted from the law of mass action, the high affinity K^b binding peptide VSV $N_{52–59}$ ("RGY") competed for presentation of K^b-SIINFEKL complexes as determined flow cytometrically using the 25-D1.16 mAb to directly quantitate cell surface K^b-SIINFEKL complexes. Competition occurred at the level of class I (and not TAP, for example) since RGY did not compete with presentation of D^b complexed with a rVV-expressed D^b-binding peptide. But, we made a curious observation when SIINFEKL was expressed in a protein context requiring proteasomal liberation. Despite the expression of a saturating amount of competing RGY, we were unable to reduce K^b-SIINFEKL complex expression. We interpret this to mean that peptides introduced into the cytosol by direct ribosomal synthesis or liberation from nascent protein by Ub-hydrolases are at a competitive disadvantage for loading class I molecules in the ER relative to peptides liberated from DRiPs by proteasomes.

We can think of two explanations for this finding. First, the NH_2-terminal extensions present on naturally processed peptides provide some sort of "handle" that enables much more efficient loading onto class I molecules. Second, cells are compartmentalized in some manner to prevent cytosolic peptides from competing with naturally processed peptides. Taking this a step further, the fact that DRiPs are the principal source of peptides despite the near equal amount of degradation of retirees [22, 23, 40], strongly suggests heterogeneity in the access of proteasome substrates to this putative compartment.

This idea that classes of proteasome substrates differ widely in their efficiency of accessing the class I pathway is central to the current thinking in our laboratory about antigen processing. As discussed above, slowly degraded DRiPs generated by misfolding are a less efficient source of peptides than rapidly degraded DRiPs. Even among rapidly degraded DRiPs there can distinctions. NP targeted for rapid and complete destruction by the N-end pathway appears to be a far less efficient source of peptide than natural NP DRiPs, since increasing the immediate degradation to 100% only increase peptide generation 3-fold [41]. Since we cannot detect any difference in NP expression in the presence of proteasome inhibitors (which block peptide generation), this suggests that true DRiPs are many fold more efficient than the artificially generated DRiPs.

We recently extended these findings using a system developed by the Wandless lab to rapidly and reversibly control the folding status of reporter proteins using the drug Shield-1 [42]. Creating a Shield-1-controlled SIINFEKL-tagged GFP-fusion protein, we found that K^b-SIINFEKL complexes are generated much more efficiently from Shield-1 insensitive DRiPs than from either Shield-1-rescued nascent or "aged" proteins [43]. In all cases, antigen presentation was completely blocked by proteasome inhibitors.

This provides yet another (Orwellian) example that not all proteasome substrates are treated equally: peptides liberated from "true" DRiPs have more efficient access to the pathway than peptides liberated from retirees or "mildly" misfolded nascent proteins. The key idea is that DRiP processing may be compartmentalized to enhance efficiency.

Compartments within the cell

What type of compartment might house high efficiency antigen processing and presentation machinery? As the fundamental unit of life, cells are highly organized structures that contain organelles and sub-organelle domains evolved to increase the efficiency of metabolic processes. Not all domains form structures that can be visualized by current microscopic techniques. Restricted domains are proposed to explain H_2O_2-mediated signaling in the face of cytosolic peroxiredoxin levels that should immediately destroy nascent H_2O_2 [44]. Protein translation itself seems to be compartmentalized to ensure that aminoacylated-tRNAs are in close proximity to the translation machinery and do not have to passively diffuse to ribosomes after their generation by tRNA aminoacyl synthetases [45, 46].

One physically defined compartment is related, at least tangentially, to DRiP processing. Dendritic cells segregate and store newly synthesized and polyubiquitylated proteins in dendritic cell aggresome-like induced structures (DALIS) during the process of maturation [47, 48], demonstrating that molecular mechanisms exist to ensure compartmentalization of at least a subset of DRiPs. Stress induces similar structures in other cell types, providing clear precedent for cellular compartmentalization of DRiPs [49].

There is also evidence for compartmentalized processing of cross-presented antigens. Guermonprez et al. [50] made the initial observation that class I complexes were selectively generated in the compartment containing phagocytosed antigen. Since peptide generation was both TAP- and proteasome-dependent, they proposed that peptides were generated locally and transported into their source compartment. Nearly identical observations were made by Burgdorf et al. [51] studying antigen endocytosed via the mannose receptor.

Though Occam's razor can be a weapon of mass destruction when applied to biological systems, it is tempting to speculate a connection between the organization of the cross-presentation- and DRiP-presentation compartments. Both face the common problem of avoiding

competition from peptides generated by turnover of normal cellular proteins, and both appear to be able to generate peptides at high efficiency relative to the efficiency of generating peptides from retirees and proteins targeted for more rapid destruction [19, 41, 52].

ERAD or PrERAD?

Since the electron microscope was first employed to scrutinize cells, the presence large numbers of ribosomes on the endoplasmic reticulum (classically referred to as rough ER) has repeatedly demonstrated that a substantial fraction of cellular protein translation is associated with this organelle. The recent work of Nicchitta and colleagues puts a new twist on this finding, as it appears that a substantial fraction of proteins synthesized on ER-associated ribosomes are not imported into the ER by Blobel's signal based mechanism [53], but rather are destined for the cytosol/nucleus [54–56].

Still, a surprisingly large fraction of proteins, more than 25% of individual gene products [57] and total synthesized protein [58] are targeted to the ER. ER proteins are a rich source of class I peptides. This is particularly well established for viral proteins. Indeed, at the dawn of the-peptide-as-epitopes era (i.e. pre-Unanue), the first peptides identified as CTL antigens, derived from viral membrane proteins [59, 60].

Although there are exceptions [61], presentation of most ER-targeted proteins is TAP- and proteasome-dependent, implicating cytosolic processing in peptide generation. We recently performed careful kinetic studies on the presentation of the SIINFEKL peptide embedded in the neuraminidase (NA) stalk of recombinant IAVs [13]. Although SIINFEKL insertion has subtle effects on IAV pathogenesis in mice [62], it has no significant effect on viral growth in cultured cells, and NA biogenesis is not detectably affected. Antigen presentation and NA accumulation at the surface of infected cells occurred with nearly identical kinetics and required ongoing synthesis of NA protein. Remarkably, only 12 min separated abrogation of K^b-SIINFEKL presentation when we treated cells with proteasome inhibitor versus CHX. Assuming that this would account for $\sim$3 half-lives of the relevant substrate (=87.5% of the available antigen), these data imply a $\sim$4 min half-life of the NA-DRiP pool. Notably, we previously found similar kinetics for ovalbumin, that natural source of SIINFEKL, which is also an ER-targeted secreted protein.

For typical ER-targeted proteins like NA and Ova, that are not known to be re-imported into the cytosol after achieving native state, but rather are either secreted/released from cells or degraded in endo/lysosomal compartments, it is hardly surprising, on moment's reflection, that peptides derive from DRiPs. We can divide such DRiPs into two bins: those that never entered the ER, and those that were re-exported from the ER into the cytosol by ER-associated degradation (ERAD). ERAD is actually a misnomer, since it was coined by Klausner and colleagues to described the proteolysis of misassembled TCRs (DRiPs!), which they believed occurred in the ER itself [63]. It was first shown by Brodsky and McCracken in yeast that ERAD actually occurs in the cytosol [64]. Independently of these findings, our group and Engelhard's group showed that peptides from ER-targeted proteins almost were certainly generated by ERAD since they were TAP-dependent and were affected by N-linked glycosylation, a process that exclusively (a dangerous word!) occurs in the ER [65, 66]. Engelhard later showed that in their tyrosinase antigen system, substrates that enhance folding in the ER reduce antigen presentation, demonstrating that at least some of the sources of peptide are not irretrievably defective [67].

A key issue in relating these findings to our concept that DRiPs are frequently not salvageable is quantitation and kinetics. T cell activation-based assays are poorly suited to generate these data, and the application of flow cytometry based quantitation of peptide class I complexes is needed to gauge the real degree of differences between our findings with SIINFEKL based antigens and other systems.

We would be remiss if we left the reader with the impression that ERAD is the major route of presentation of ER-targeted antigens, since its relative importance in antigen processing is uncertain. Groettrup and colleagues provided solid evidence that for prostate stem cells antigen, the major source of antigen is a pool of DRiPs that were not properly targeted to the ER and were degraded by proteasomes (a process we term preERAD or PrERAD) [68]. To the extent that available ERAD inhibitors (drugs and siRNA) effectively inhibit overall ERAD (they probably do not), evidence would suggest that non-targeting is the major route of presentation, since blocking ERAD has little effect on peptide generation from endogenous sources of peptide antigens, though ERAD has been implicated in antigen cross-presentation [69, 70].

Given the vagaries, the only solid conclusion regarding the nature of DRiPs for ER-targeted proteins is that the major pathways have been lightly sketched out and much remains to be learned.

Translation at the center of it all

Given the critical role of translation in the class I pathway, the clear potential for compartmentalization, and over-representation of minor sources of antigen processed with

high efficiency, it is worth considering all sources of nascent peptides, with particular emphasis on the oddballs. In a recent report we used RNA polymerase II inhibitors to "trap" influenza late mRNAs in the nucleus [71] and observed on-going antigen presentation of IAV NA peptides (albeit at lower levels than untreated cells) despite the almost complete lack of NA in treated cells [72]. We proposed that NA DRiPs synthesized in the nuclei of drug treated cells are a source of peptides. But can proteins be translated in the nucleus?

Nuclear translation was initially reported in 1957 [73], re-discovered a decade ago [74–76], but is widely considered by translation mavens to be an artifact [77]. As more and more of the components of translation are detected in the nucleus, such as charged-tRNAs [78], amino acyl tRNA synthetases [79, 80], and translation initiation factors [76, 81–84] the case for nuclear translation mounts. What might be translated in the nucleus? Perennial suspects are pioneer translation products from nonsense mediated decay (NMD) of mRNAs [85]. The NMD pathway is a quality control mechanism that degrades mutant or mis-spliced mRNAs by detecting premature termination codons in a process that entails translation of nascent messages (the pioneer round). We previously suggested NMD as a source of peptides for extremely low abundance antigens, whose presentation is difficult to square with the law of mass action [17]. Interest in the potential contribution of NMD to antigen processing is heightened by the exciting recent findings linking NMD to tumor rejection [86].

Met-misacylation? probably not

In collaboration with Tao Pan, we recently reported that up to 14% of Met is attached to the "wrong", i.e. non-cognate tRNA by aminoacyl synthetases (probably Met tRNA synthetase when cells are subjected to stressful conditions, including viral infections [87]). We were testing the hypothesis that tRNA-misacylation is a source of DRiPs, with the idea that misincorporation of amino acids leads to misfolding. Indeed, we also provided multiple lines of evidence that non-cognate Met is incorporated into proteins (our strongest evidence is the similar off rate of Met from cognate and non-cognate tRNA and the complete blockade of both when CHX is added to block protein synthesis).

While it is certainly plausible that Met-misacylation contributes to peptidogenic DRiPs, our available evidence suggests this is not a major effect of Met replacement. First, misacylation is induced by a number of conditions that seem to be unrelated to any need to enhance peptide generation, including exposure to LPS, oxidants, and even overcrowding of cells. Second, as far as we can determine, Met-misacylation is induced by intracellular ROS generation by NADPH oxidases, a common signalling event in the cellular response to infectious, chemical, and physical stress. Third, as Met is known as a "bodyguard" residue that protects proteins against ROS mediated damage [88, 89], and Met misacylates specific tRNAs, we have proposed that Met-misacylation functions (ironically) as an anti-DRiP, i.e. to reduce degradation of nascent proteins, and not to enhance it.

While do not favor the idea that Met-misacylation is a major contributor to DRiPs, we are keeping an open mind on the issue, and encourage others, particularly mass spectrometrists, to do so as well. When encountering class I associated peptides with masses that do not match the predicted proteome, it would be a useful exercise to research using an algorithm that replaces the mass of the 19 other common amino acids with Met.

The ribosome-proteasome link

The close kinetic relationship between translation and peptide generation along with our surprising (non-) competition results [39], suggests a potential linkage between ribosomes and proteasomes (and other cytosolic proteases implicated in antigen processing). Detailed analysis of the yeast proteasome interaction network revealed several proteins involved in translation [90]. eIF3 [91] and EGD [92] have been found to interact with both ribosomes and proteasomes by biochemical analysis. An additional translation factor, eIF1A is thought to interact with the Rpt1 subunit of the proteasome to facilitate co-translational degradation of mis-folded proteins [93].

Analysis of mammalian cells is more limited, but two translation factors, eIF2 and eIF1α, are reported to associate with proteasomes isolated from cells [94]. It is of obvious interest to examine the interaction of ribosomes and proteasomes via microscopy, particularly in living cells with fluorescent versions of the protagonists.

What's next?

Fifteen years and counting, there is solid evidence that DRiPs account for the majority of viral peptides. There is compelling, but less extensive evidence for a major contribution of DRiPs to cellular peptides. With the accretion of Ab/TCR-based probes with TCR-like specificity it will be important to extend flow cytometry based kinetic studies comparing antigen expression and generation of class I peptide complexes using genetically unmodified versions of antigen. It is particularly important to compare the properties of antigen presentation between cell lines and real differentiated cells, ideally analyzed in vivo, but

initially ex vivo using dissociated tissues. For example, it should be possible to measure levels of a nominal class I peptide complex on epithelial cells at various times after initiating infections.

The most significant weakness in the DRiP hypothesis is the poor biochemical definition of DRiPs. Though it is reasonably clear that a subset of DRiPs are generated via premature termination [14] and downstream initiation [16], the relative contribution of these processes to DRiPs is uncertain, not to mention other myriad sources, including NMD and other transcription error sources. Eisenlohr et al.'s [20, 95] point is well taken that misfolded proteins are typically degraded too slowly to account for the rapid kinetics of peptidogenic DRiPs, but half-lives are based on the assumption of uniform exponential decay (being modeled on the law of mass action), and it is possible that a subset of misfolded proteins are degraded with much more rapid kinetics than the majority population. Indeed, we found that although the amino acid canavanine induced slowly degraded antigen, it still enhanced presentation from a cohort that was present with kinetics identical to non-canavanyl DRiPs in CHX shut down experiments [19]. As the number of defined IDPs increases and their average degradation kinetics emerges, this will provide a handle on gauging the contribution of this potentially important subset of proteins to the RDP pool and to class I peptide ligands.

It is highly unlikely that we have identified all of the important gene products that participate in class I peptide ligand generation. With a little luck, a few surprises are still in store for the field, and will likely come from high throughput screens for compounds/siRNAs that modulate antigen presentation. Our recent results suggest a role for deubiquitylases in peptide generation [43], which is consistent with our prior findings that ubiquitylation is not required for generating peptide class I complexes [27, 96].

Given the role of MHC class I molecules in multiple "alternative" processes (which may actually be more important evolutionarily than anti-pathogen immunity) that now include tumor immunosurveillance, NK recognition, mate selection, and neuronal organization, it seems increasingly likely that the repertoire of presented peptides that regulate these processes is not simply left to chance. Despite the doubts of some of our colleagues dismayed by our slow progress, we will continue to investigate the immunoribosome hypothesis that cells segregate a subset of mRNAs for high efficiency conversion to antigenic peptides.

Finally, the forest: why come to grip with DRiPs? A better understanding of the cellular and molecular biology of DRiP antigen presentation will lead to novel insights into myriad aspects of cellular function, and is required for practical applications, including rational vaccine design, enhancing immune eradication of pathogens and tumors, and blocking autoimmunity.

Acknowledgments This work was generously supported by the Division of Intramural Research, NIAID, Bethesda, MD

References

1. Oxenius A, Bachmann MF, Ashton-Rickardt PG et al (1995) Presentation of endogenous viral proteins in association with major histocompatibility complex class II: on the role of intracellular compartmentalization, invariant chain and the TAP transporter system. Eur J Immunol 25(12):3402–3411
2. Babbitt BP, Allen PM, Matsueda GR et al (1985) Binding of immunogenic peptides to Ia histocompatibility molecules. Nature 317:359–361
3. Buus S, Sette A, Colon SM et al (1986) Isolation and characterization of antigen-Ia complexes involved in T cell recognition. Cell 47:1071–1077
4. Townsend A, Bastin J, Gould K et al (1988) Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. J Exp Med 168(4):1211–1224
5. Tevethia S, Tevethia M, Lewis A et al (1983) Biology of simian virus 40 (SV40) transplantation antigen (TrAg). IX. Analysis of TrAg in mouse cells synthesizing truncated SV40 large T antigen. Virology 128:319–330
6. Yewdell JW, Reits E, Neefjes J (2003) Making sense of mass destruction: quantitating MHC class I antigen presentation. Nat Rev Immunol 3(12):952–961
7. Yewdell JW, Bennink JR, Hosaka Y (1988) Cells process exogenous proteins for recognition by cytotoxic T lymphocytes. Science 239:637–640
8. Yewdell JW, Bennink JR (1989) Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. Science 244:1072–1075
9. Esquivel F, Yewdell J, Bennink J (1992) RMA/S cells present endogenously synthesized cytosolic proteins to class I-restricted cytotoxic T lymphocytes. J Exp Med 175(1):163–168
10. Yewdell JW, Eisenlohr LC, Bennink JR (1990) Where do antigens associate with MHC class I molecules? In: Janeway CA Jr, Sprent J, Sercarz E (eds) Immunogenicity UCLA symposia on molecular and cellular biology, new series. Wiley-Liss, New York, pp 1–6
11. Yewdell JW, Anton LC, Bennink JR (1996) Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules? J Immunol 157(5):1823–1826
12. Mackay LK, Long HM, Brooks JM et al (2009) T cell detection of a B-cell tropic virus infection: newly-synthesised versus mature viral proteins as antigen sources for CD4 and CD8 epitope display. PLoS Pathog 5(12):e1000699
13. Dolan BP, Li L, Takeda K et al (2010) Defective ribosomal products are the major source of antigenic peptides endogenously generated from influenza A virus neuraminidase. J Immunol 184(3):1419–1424
14. Cardinaud S, Starck SR, Chandra P, Shastri N (2010) The synthesis of truncated polypeptides for immune surveillance and viral evasion. PLoS One 5(1):e8692
15. Shastri N, Cardinaud S, Schwab SR et al (2005) All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway. Immunol Rev 207:31–41
16. Berglund P, Finzi D, Bennink JR, Yewdell JW (2007) Viral alteration of cellular translational machinery increases defective ribosomal products. J Virol 81(13):7220–7229
17. Yewdell JW, Nicchitta CV (2006) The DRiP hypothesis decennial: support, controversy, refinement and extension. Trends Immunol 27(8):368–373

18. Lev A, Takeda K, Zanker D et al (2008) The exception that reinforces the rule: crosspriming by cytosolic peptides that escape degradation. Immunity 28(6):787–798
19. Qian SB, Reits E, Neefjes J et al (2006) Tight linkage between translation and MHC class I peptide ligand generation implies specialized antigen processing for defective ribosomal products. J Immunol 177(1):227–233
20. Eisenlohr LC, Huang L, Golovina TN (2007) Rethinking peptide supply to MHC class I molecules. Nat Rev Immunol 7(5):403–410
21. Wheatley DN, Giddings MR, Inglis MS (1980) Kinetics of degradation of "short-" and "long-lived" proteins in cultured mammalian cells. Cell Biol Int Rep 4(12):1081–1090
22. Schubert U, Anton LC, Gibbs J et al (2000) Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature 404(6779):770–774
23. Chen W, Calvo PA, Malide D et al (2001) A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7(12):1306–1312
24. Vabulas RM, Hartl FU (2005) Protein synthesis upon acute nutrient restriction relies on proteasome function. Science 310(5756):1960–1963
25. Wright PE, Dyson HJ (1999) Intrinsically unstructured proteins: re-assessing the protein structure-function paradigm. J Mol Biol 293(2):321–331
26. Dunker AK, Lawson JD, Brown CJ et al (2001) Intrinsically disordered protein. J Mol Graph Model 19(1):26–59
27. Qian SB, Princiotta MF, Bennink JR, Yewdell JW (2006) Characterization of rapidly degraded polypeptides in mammalian cells reveals a novel layer of nascent protein quality control. J Biol Chem 281(1):392–400
28. Barondeau DP, Kassmann CJ, Tainer JA, Getzoff ED (2006) Understanding GFP posttranslational chemistry: structures of designed variants that achieve backbone fragmentation, hydrolysis, and decarboxylation. J Am Chem Soc 128(14):4685–4693
29. Gu WY, Cochrane M, Leggatt GR et al (2009) Both treated and untreated tumors are eliminated by short hairpin RNA-based induction of target-specific immune responses. Proc Natl Acad Sci USA 106(20):8314–8319
30. Levitskaya J, Coram M, Levitsky V et al (1995) Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. Nature 375:685–688
31. Levitskaya J, Sharipo A, Leonchiks A et al (1997) Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen + 1. Proc Natl Acad Sci USA 94(23):12616–12621
32. Tellam J, Connolly G, Green KJ et al (2004) Endogenous presentation of CD8 + T cell epitopes from Epstein-Barr virus-encoded nuclear antigen 1 1. J Exp Med 199(10):1421–1431
33. Voo KS, Fu T, Wang HY et al (2004) Evidence for the presentation of major histocompatibility complex class I-restricted Epstein-Barr virus nuclear antigen 1 peptides to CD8 + T lymphocytes. J Exp Med 199(4):459–470
34. Lee SP, Brooks JM, Al-Jarrah H et al (2004) CD8 T cell recognition of endogenously expressed Epstein-Barr virus nuclear antigen 1. J Exp Med 199(10):1409–1420
35. Jones RJ, Smith LJ, Dawson CW et al (2003) Epstein-Barr virus nuclear antigen 1 (EBNA1) induced cytotoxicity in epithelial cells is associated with EBNA1 degradation and processing 2. Virology 313(2):663–676
36. Yin Y, Manoury B, Fahraeus R (2003) Self-inhibition of synthesis and antigen presentation by Epstein-Barr virus-encoded EBNA1. Science 301(5638):1371–1374
37. Tellam J, Smith C, Rist M et al (2008) Regulation of protein translation through mRNA structure influences MHC class I loading and T cell recognition. Proc Natl Acad Sci USA 105(27):9319–9324
38. Fortier MH, Caron E, Hardy MP et al (2008) The MHC class I peptide repertoire is molded by the transcriptome. J Exp Med 205(3):595–610
39. Lev A, Princiotta MF, Zanker D et al (2010) Compartmentalized MHC class I antigen processing enhances immunosurveillance by circumventing the law of mass action. Proc Natl Acad Sci USA 107(15):6964–6969
40. Reits EA, Vos JC, Gromme M, Neefjes J (2000) The major substrates for TAP in vivo are derived from newly synthesized proteins. Nature 404(6779):774–778
41. Princiotta MF, Finzi D, Qian SB et al (2003) Quantitating protein synthesis, degradation, and endogenous antigen processing. Immunity 18(3):343–354
42. Banaszynski LA, Chen LC, Maynard-Smith LA et al (2006) A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. Cell 126(5):995–1004
43. Dolan BP, Li L, Veltri CA et al (2011) Distinct pathways generate peptides from defective ribosomal products for CD8 + T cell immunosurveillance. J Immunol 186(4):2065–2072
44. Woo HA, Yim SH, Shin DH et al (2010) Inactivation of peroxiredoxin I by phosphorylation allows localized H(2)O(2) accumulation for cell signaling. Cell 140(4):517–528
45. Negrutskii BS, Stapulionis R, Deutscher MP (1994) Supramolecular organization of the mammalian translation system. Proc Natl Acad Sci USA 91(3):964–968
46. Stapulionis R, Deutscher MP (1995) A channeled tRNA cycle during mammalian protein synthesis. Proc Natl Acad Sci USA 92(16):7158–7161
47. Lelouard H, Gatti E, Cappello F et al (2002) Transient aggregation of ubiquitinated proteins during dendritic cell maturation. Nature 417(6885):177–182
48. Lelouard H, Ferrand V, Marguet D et al (2004) Dendritic cell aggresome-like induced structures are dedicated areas for ubiquitination and storage of newly synthesized defective proteins. J Cell Biol 164(5):667–675
49. Szeto J, Kaniuk NA, Canadien V et al (2006) ALIS are stress-induced protein storage compartments for substrates of the proteasome and autophagy. Autophagy 2(3):189–199
50. Guermonprez P, Saveanu L, Kleijmeer M et al (2003) ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature 425(6956):397–402
51. Burgdorf S, Lukacs-Kornek V, Kurts C (2006) The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. J Immunol 176(11):6770–6776
52. Villanueva MS, Fischer P, Feen K, Pamer EG (1994) Efficiency of MHC class I antigen processing: a quantitative analysis. Immunity 1(6):479–489
53. Blobel G (2000) Protein targeting (Nobel lecture). Chembiochem 1(2):86–102
54. Stephens SB, Nicchitta CV (2008) Divergent regulation of protein synthesis in the cytosol and endoplasmic reticulum compartments of mammalian cells. Mol Biol Cell 19(2):623–632
55. Pyhtila B, Zheng T, Lager PJ et al (2008) Signal sequence- and translation-independent mRNA localization to the endoplasmic reticulum. RNA 14(3):445–453
56. Lerner RS, Nicchitta CV (2006) mRNA translation is compartmentalized to the endoplasmic reticulum following physiological inhibition of cap-dependent translation. RNA 12(5):775–789
57. Diehn M, Bhattacharya R, Botstein D, Brown PO (2006) Genome-scale identification of membrane-associated human mRNAs. PLoS Genet 2(1):e11
58. Shaffer AL, Shapiro-Shelef M, Iwakoshi NN et al (2004) XBP1, downstream of Blimp-1, expands the secretory apparatus and

other organelles, and increases protein synthesis in plasma cell differentiation. Immunity 21(1):81–93
59. Guertin DP, Fan DP (1980) Stimulation of cytolytic T cell by isolated viral peptides and HN protein coupled to agarose beads. Nature 283:308–313
60. Wabuke-Bunoti MAN, Taku A, Fan DP et al (1984) Cytolytic T lymphocyte and antibody responses to synthetic peptides of influenza virus hemagglutinin. J Immunol 133:2194–2201
61. Hammond SA, Bollinger RC, Tobery TW, Siliciano RF (1993) Transporter-independent processing of HIV-1 envelope protein for recognition by CD8 + T cells. Nature 364:158–161
62. Jenkins MR, Webby R, Doherty PC, Turner SJ (2006) Addition of a prominent epitope affects influenza A virus-specific CD8 + T cell immunodominance hierarchies when antigen is limiting. J Immunol 177(5):2917–2925
63. Lippincott-Schwartz J, Bonifacino JS, Yuan LC, Klausner RD (1988) Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. Cell 54:209–220
64. Werner ED, Brodsky JL, McCracken AA (1996) Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. Proc Natl Acad Sci USA 93(24):13797–13801
65. Bacik I, Snyder HL, Anton LC et al (1997) Introduction of a glycosylation site into a secreted protein provides evidence for an alternative antigen processing pathway: transport of precursors of major histocompatibility complex class I-restricted peptides from the endoplasmic reticulum to the cytosol. J Exp Med 186(4):479–487
66. Skipper JCA, Hendrickson RC, Gulden PH et al (1996) An HLA-A2-restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins. J Exp Med 183:527–534
67. Ostankovitch M, Robila V, Engelhard VH (2005) Regulated folding of tyrosinase in the endoplasmic reticulum demonstrates that misfolded full-length proteins are efficient substrates for class I processing and presentation. J Immunol 174(5):2544–2551
68. Schlosser E, Otero C, Wuensch C et al (2007) A novel cytosolic class I antigen-processing pathway for endoplasmic-reticulum-targeted proteins. EMBO Rep 8(10):945–951
69. Giodini A, Rahner C, Cresswell P (2009) Receptor-mediated phagocytosis elicits cross-presentation in nonprofessional antigen-presenting cells. Proc Natl Acad Sci USA 106(9):3324–3329
70. Ackerman AL, Giodini A, Cresswell P (2006) A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. Immunity 25(4):607–617
71. Amorim MJ, Read EK, Dalton RM et al (2007) Nuclear export of influenza A virus mRNAs requires ongoing RNA polymerase II activity. Traffic 8(1):1–11
72. Dolan BP, Knowlton JJ, David A et al (2010) RNA polymerase II inhibitors dissociate antigenic peptide generation from normal viral protein synthesis: a role for nuclear translation in defective ribosomal product synthesis? J Immunol 185(11):6728–6733
73. Allfrey VG, Mirsky AE, Osawa S (1957) Protein synthesis in isolated cell nuclei. J Gen Physiol 40(3):451–490
74. Goidl JA, Canaani D, Boublik M et al (1975) Polyanion-induced release of polyribosomes from HeLa cell nuclei. J Biol Chem 250(23):9198–9205
75. Gozes I, Walker MD, Kaye AM, Littauer UZ (1977) Synthesis of tubulin and actin by neuronal and glial nuclear preparations from devloping rat brain. J Biol Chem 252(5):1819–1825
76. Iborra FJ, Jackson DA, Cook PR (2001) Coupled transcription and translation within nuclei of mammalian cells. Science 293(5532):1139–1142
77. Dahlberg JE, Lund E, Goodwin EB (2003) Nuclear translation: what is the evidence? RNA 9(1):1–8
78. Lund E, Dahlberg JE (1998) Proofreading and aminoacylation of tRNAs before export from the nucleus. Science 282(5396):2082–2085
79. Gunasekera N, Lee SW, Kim S et al (2004) Nuclear localization of aminoacyl-tRNA synthetases using single-cell capillary electrophoresis laser-induced fluorescence analysis. Anal Chem 76(16):4741–4746
80. Nathanson L, Deutscher MP (2000) Active aminoacyl-tRNA synthetases are present in nuclei as a high molecular weight multienzyme complex. J Biol Chem 275(41):31559–31562
81. Dostie J, Lejbkowicz F, Sonenberg N (2000) Nuclear eukaryotic initiation factor 4E (eIF4E) colocalizes with splicing factors in speckles. J Cell Biol 148(2):239–247
82. Ferraiuolo MA, Lee CS, Ler LW et al (2004) A nuclear translation-like factor eIF4AIII is recruited to the mRNA during splicing and functions in nonsense-mediated decay. Proc Natl Acad Sci USA 101(12):4118–4123
83. Lejbkowicz F, Goyer C, Darveau A et al (1992) A fraction of the mRNA 5' cap-binding protein, eukaryotic initiation factor 4E, localizes to the nucleus. Proc Natl Acad Sci USA 89(20):9612–9616
84. McKendrick L, Thompson E, Ferreira J et al (2001) Interaction of eukaryotic translation initiation factor 4G with the nuclear cap-binding complex provides a link between nuclear and cytoplasmic functions of the m(7) guanosine cap. Mol Cell Biol 21(11):3632–3641
85. Ishigaki Y, Li X, Serin G, Maquat LE (2001) Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. Cell 106(5):607–617
86. Pastor F, Kolonias D, Giangrande PH, Gilboa E (2010) Induction of tumour immunity by targeted inhibition of nonsense-mediated mRNA decay. Nature 465(7295):227–230
87. Netzer N, Goodenbour JM, David A et al (2009) Innate immune and chemically triggered oxidative stress modifies translational fidelity. Nature 462(7272):522–526
88. Luo S, Levine RL (2009) Methionine in proteins defends against oxidative stress. FASEB J 23(2):464–472
89. Levine RL, Mosoni L, Berlett BS, Stadtman ER (1996) Methionine residues as endogenous antioxidants in proteins. Proc Natl Acad Sci USA 93(26):15036–15040
90. Guerrero C, Milenkovic T, Przulj N et al (2008) Characterization of the proteasome interaction network using a QTAX-based tag-team strategy and protein interaction network analysis. Proc Natl Acad Sci USA 105(36):13333–13338
91. Sha Z, Brill LM, Cabrera R et al (2009) The eIF3 interactome reveals the translasome, a supercomplex linking protein synthesis and degradation machineries. Mol Cell 36(1):141–152
92. Panasenko O, Landrieux E, Feuermann M et al (2006) The yeast Ccr4-Not complex controls ubiquitination of the nascent-associated polypeptide (NAC-EGD) complex. J Biol Chem 281(42): 31389–31398
93. Chuang SM, Chen L, Lambertson D et al (2005) Proteasome-mediated degradation of cotranslationally damaged proteins involves translation elongation factor 1A. Mol Cell Biol 25(1): 403–413
94. Besche H, Haas W, Gygi S, Goldberg A (2009) Isolation of mammalian 26S proteasomes and p97/VCP complexes using the ubiquitin-like domain from HHR23B reveals novel proteasome-associated proteins. Biochemistry
95. Golovina TN, Morrison SE, Eisenlohr LC (2005) The impact of misfolding versus targeted degradation on the efficiency of the MHC class I-restricted antigen processing. J Immunol 174(5): 2763–2769
96. Cox JH, Galardy P, Bennink JR, Yewdell JW (1995) Presentation of endogenous and exogenous antigens is not affected by inactivation of E1 ubiquitin-activating enzyme in temperature-sensitive cell lines. J Immunol 154(2):511–519