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DRiPs Solidify: Progress in Understanding Endogenous MHC Class I Antigen Processing

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Abstract

Defective Ribosomal Products (DRiPs) are a subset of rapidly degraded polypeptides that provide peptide ligands for MHC class I molecules. Here, I review recent progress in understanding DRiP biogenesis. These findings place DRiPs at the center of the MHC class I antigen processing pathway, linking immunosurveillance of viruses and tumors to mechanisms of specialized translation and cellular compartmentalization. DRiPs enable the immune system to rapidly and sensitively detect alterations in cellular gene expression.

Classical MHC class I molecules play a central role in CD8+ T cell recognition of intracellular pathogens, tumors, and allogeneic and autoimmune targeted cells, and participate in neuronal function [1] and mate selection [2]. Class I molecules consist of three non-covalently bound individual polypeptides: MHC encoded heavy chains (HLA-A, B, and C in humans, H2-K, D, L in mice), β_2 -microglobulin (β_2m), and an oligopeptide, typically of 8 to 10 residues.

Peptides are generated from polypeptides synthesized (endogenous antigens) or acquired (exogenous antigens) by cells. All nucleated cells in jawed vertebrates are capable of endogenous antigen presentation, which occurs constitutively in nearly all cell types and is enhanced by interferons and other cytokines. The nature of endogenous antigens is surprisingly poorly defined, and is a topic that encompasses central aspects of cell biology (fidelity and efficiency of gene expression, protein degradation, cellular sub-compartmentalization), rational vaccinology (optimal design of CD8+ T cell vaccines for pathogens and tumors), and autoimmunity (understanding/blocking self-peptide generation).

In 1996 my colleagues and I hypothesized that many endogenous peptides derive from defective ribosomal products (DRiPs), defined as “prematurely terminated polypeptides and misfolded polypeptides produced from translation of *bona fide* mRNAs in the proper reading frame” [3]. DRiPs were proposed to explain first, that truncating [4, 5] or mistargeting [6] viral genes maintains or enhances antigenicity, and second, that peptides are generated rapidly (within an hour) from highly stable viral proteins in the context of a normal viral infection [7].

DRiPs were considered as one of many potential sources of peptides, including other forms of defective proteins, as well as normal protein turnover (so-called “retirees” [8]). The original DRiP hypothesis was predictably incomplete, and DRiPs have evolved to include defective polypeptides arising from alternative/defective mRNAs [9, 10], ribosomal frame

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shifting [11, 12], downstream initiation on *bona fide* mRNAs [13], and all other errors that occur in converting genetic information into proteins (including tRNA-amino acid misacylation [14]). An important update to the DRiP hypothesis distinguishes DRiPs as the subset of rapidly degraded polypeptides (RDPs; nascent polypeptides with half-lives of ~ 10 min) that efficiently access the class I pathway [15].

Here, I review progress in understanding the nature of DRiPs and their contribution to presentation of viral and host cell peptides. Readers are directed to a recent collection [16] of outstanding reviews for discussion of other aspects of generating the class I immunopeptidome (*i.e.* the repertoire of peptide presented by class I molecules).

Kinetics, Kinetics, Kinetics

A key approach for gauging the contribution of DRiPs to antigen presentation is to measure the kinetics of peptide presentation relative to source protein synthesis and degradation. Using viral vectors, it is typically easy to achieve rapid synchronized expression of a source antigen. By correlating the kinetics of antigen expression to its cognate peptide MHC class I (pMHC I) complex and their behavior after addition of various inhibitors, the kinetics of the degradation of the peptide source can be inferred [17] (Figure 1). This is done most precisely using reagents that directly detect pMHC I complex by flow cytometry (which also provides a direct ratio per cell between folded source protein expression and pMHC I complex), but T cell assays also provide a reasonable measure of the contribution of DRiPs *vs.* retirees. Indeed, T cell recognition of viral proteins (most of which have half-lives of days) by T cells within hours of infection, observed across viral systems by numerous labs over decades, cogently argues for DRiPs as a major peptide source. The contribution of DRiPs is supported by the stoichiometry and kinetics of viral *vs.* host protein expression: cells typically used in classical CTL assays consist of $\sim 2 \times 10^9$ proteins and only $\sim 10^5$ class I molecules [8, 18, 19]. Given the typical levels of viral gene expression, $< 10^7$ copies of any given protein are synthesized during the course of a CTL assay. Viral proteins are typically degraded with a similar half-life as the total cellular protein pool, *i.e.* ~1–2 days [20, 21]. It is simply not possible for such typical viral proteins to compete with cellular proteins for the limited pool of class I molecules, if peptides derived from cell and viral retirees have equal access to class I molecules.

Still, firmly testing the hypothesis required real numbers.. The first careful kinetic study of antigen presentation used the 25D1-1.16 mAb specific for K^b-SIINFEKL complexes to quantitate antigen presentation from SIINFEKL-containing proteins expressed by recombinant vaccinia virus (VV) infected cells [19]. This revealed that complex generation closely tracked viral protein synthesis, particularly for ovalbumin (the evolutionary source of the SIINFEKL peptide), whose antigen presentation kinetics were identical to that of a rapidly degraded (~ 10 min half-life) SIINFEKL-containing fusion protein. Presentation was somewhat slower for a prototypical stable chimeric protein (NP-S-GFP), but still rapid compared to its very slow degradation rate (no measurable degradation in 10 h [15]).

These findings were extended [22] to influenza A virus (IAV) neuraminidase (NA), with SIINFEKL inserted into the fibrous stalk region of the peptide (NA-SIINFEKL). This relatively minor genetic manipulation had no discernible effect on NA folding, assembly, intracellular trafficking, or specific enzyme activity, reducing the possibility of an artificially high NA-SIINFEKL DRiP fraction. This artifact is a worrisome consideration for experiments with highly artificial chimeric proteins that have experienced no evolutionary pressure to maximize folding efficiency. This is a particular problem for GFP (and related proteins like YFP, see later), which evolved in a different organism in a different environment (a jellyfish living in ~ 12°C seawater), and a large fraction of which is

autocatalytically cleaved in generating its chromophore [15, 23], generating a rapidly degraded highly insoluble fragment, that makes GFP a poster-protein for DRiPs [15].

Infection of cells with IAV-NA-SIINFEKL resulted in a near perfect correlation between the onset and increase in the relative cell surface expression of NA and K^b-SIINFEKL complexes. As K^b-SIINFEKL generation was dependent on both TAP expression and proteasome activity, it is clearly generated by the classical cytosolic pathway. Moreover, shut-off of protein synthesis with cycloheximide rapidly abrogated K^b-SIINFEKL and NA surface expression (in fact K^b-SIINFEKL shutdown was more rapid, because K^b-SIINFEKL complexes traverse the secretory pathway more rapidly than NA). From the difference in the shutdown kinetics after inhibiting protein synthesis and proteasome degradation (Figure 1), the half-life of SIINFEKLogenic DRiPs was estimated to be ~ 5 min.

A potential pitfall of using cycloheximide as a measure of the contribution of DRiPs to the immunopeptidome is its possible downstream effects on protein degradation, class I levels in the ER, and other processes that contribute to antigen processing. These effects increase with prolonged cycloheximide incubation. The relevant period of concern is the time in which antigen presentation is measured. It was shown in relevant antigen presenting cells in a relevant time frame for antigen presentation that cycloheximide has no detectable effects on RDPs or a slowly degraded vaccinia virus (VV)-encoded antigen [24]. Further, a pool of RDPs saved from degradation by transient proteasome inhibition, generated total cellular peptides (measured by TAP mobility, which slows when transporting peptides [25]), or K^b-SIINFEKL when the proteasome inhibition was lifted in the presence of cycloheximide. Similarly, it was demonstrated that the cycloheximide shut down of TAP transported peptides is superseded following cellular γ -irradiation, showing that peptide generation also occurs in the presence of cycloheximide in uninfected cells [26]. While cycloheximide and other protein synthesis inhibitors are compatible with prolonged antigen presentation in many systems (for example, they were used to demonstrate that presentation of exogenous IAV structural proteins delivered to the cytosol is not due to artifactual viral gene expression [27]), it has been reported to block presentation of exogenous Ova delivered to the cytosol [28].

Remaining doubts regarding the capacity of VV-infected cells to generate class I complexes in the presence of cycloheximide were put to rest by two studies. In the first [29], cells expressing an IAV peptide (PA₂₂₄₋₂₃₃) as a Met-initiated minigene product, or a peptide liberated from a Ub-fusion protein were shown to generate a large cytosolic PA₂₂₄₋₂₃₃ pool. Critically, a bacteriophage phage library Ab specific for the D^b-PA₂₂₄₋₂₃₃ complex showed that for at least 2h post- cycloheximide addition, the rate of complex delivery to the cell surface is steady. The second study [30] reported that K^b-SIINFEKL generation from ERAD (endoplasmic reticulum associated degradation) substrates continues for at least 3 h in the presence of cycloheximide. Thus, in the VV-system the rapid cycloheximide-mediated abrogation of presentation of K^b-SIINFEKL from numerous protein contexts can be attributed to a lack of peptidogenic substrates and not other factors often invoked to argue against the contribution of DRiPs to antigen presentation.

An alternative kinetic approach for gauging the contribution of DRiPs to peptide generation is to control mRNA expression. Initial results correlating antigen presentation with the amount of cognate mRNA rather than protein [28, 31] were extended in a comprehensive study using human Epstein-Barr Virus (EBV) transformed B cells that express EBV mRNA under the control of doxycycline [32]. Examining recognition of seven class I- and four class II-restricted peptides revealed that CD8⁺ T cell recognition uniformly depends on mRNA levels, while CD4⁺ T cell recognition uniformly depends on protein levels, providing an elegant internal control for the overall antigen presentation capacity of cells over the 192 h

experimental time course. Remarkably, the loss of CD8+ T cell recognition following mRNA transcriptional shutdown closely paralleled the decay of pMHC I complex generated by limited peptide loading, strengthening the relationship between protein synthesis and antigen presentation. Furthermore, γ -irradiation of cells enabled the generation from the pre-existing protein pool of the one CD8+ T cell determinant examined, extending the previous irradiation study [26]. Expressing a doxycycline-inducible EBNA-I SIINFEKL fusion protein, a close kinetic relationship was found between antigen synthesis and amount of SIINFEKL and proteasome-generated SIINFEKL precursor peptide as measured directly in HPLC fractions [33]. Similarly, following transfection with mRNA encoding Ova, K^b-SIINFEKL complexes were demonstrated to reach maximal cell surface expression in parallel with Ova translation and not Ova steady state levels [34].

Taken together, the presentation kinetics of multiple peptides, presented from multiple contexts, by multiple class I allomorphs, in multiple cell types, studied by multiple laboratories, robustly point to DRiPs as the predominant, if not the sole detectable source of antigenic peptides generated from virus- and transfectant-encoded gene products.

Quantitating DRiPs

Defining the DRiPome

Given accurate 'omic'-quantitation of cellular processes that create and destroy polypeptides (transcriptome, RNAiome, translome, proteome, degradome, peptidome, and immunopeptidome), it would be possible to simply calculate the contribution of various substrate pools to antigen processing. These data will likely be forthcoming in the next decade or two, given the rapid technological advances that are enabling direct measurements of the relevant processes and end products. Although present knowledge is woefully incomplete the initial 'omics studies give tantalizing clues to the underlying complexity of peptide generation.

Stable isotope labeling with amino acids in cell culture (SILAC) spectrometry. is a key approach for understanding peptide generation, because it uniquely provides direct information regarding the generation/synthesis/degradation kinetics of multiple peptide and source proteins (potentially tens of thousands with improvements in instrumentation and computation). As previously reviewed [17], the initial SILAC findings [35] are consistent with a major contribution of rapidly degraded DRiPs to the immunopeptidome, and a significant contribution of slowly degraded proteins, including one clear retiree, with the rest being either retirees or slowly degraded DRiPs. Extending these initial SILAC pulse chase-labeling findings is essential to understanding endogenous antigen presentation.

Another important -omics-approach combines microarrays with mass spectrometry to relate the immunopeptidome to the transcriptome [36]. Comparing 270 peptides identified in a renal carcinoma and autologous normal kidney tissue revealed an overall weak relationship between peptide and mRNA abundance. Twenty peptides were expressed uniquely in tumor or normal tissues, typically with little change in mRNA levels. For 15 peptides, there was a complete absence of detected mRNA.

This approach was also used to compare mouse thymocytes vs. EL4 lymphoma cells. Perhaps due to the use of real time PCR quantitation of mRNA, a better relationship between the transcriptome and immunopeptidome was found, but there were still many discrepancies. Of 189 thymocyte peptides characterized, 42% derive from the 9% of highly abundant mRNAs, while 20% derived from the 62% of low abundance mRNAs. EL4 cells showed similar expression of 75% of these peptides: with half of the disparate peptides showing over expression and half under-expression. Remarkably, half of the EL4-disparate

peptides derive from genes implicated in tumorigenesis, and ~ 75% of these do not demonstrate alterations in mRNA expression. A further study revealed large disparities in the abundance of peptides expressed by thymocytes vs. bone marrow derived DCs, consistent with considerable immunopeptidome tissue specificity [37].

Taken together, these studies establish the important practical point that transcriptome analysis alone missed ~ 75% of potential immunotherapy target peptides up-regulated in tumor tissues. Importantly, the lack of clear correlation between the transcriptome and immunopeptidome suggests a selective mechanism for choosing RNAs for translation into DRiPs based on quality and not quantity. This implies that cells employ special mechanisms to enhance immunosurveillance of tumor related gene products by controlling access to the MHC I pathway, consistent with compartmentalization, a concept expanded below.

DRiPonomics Revisited

Understanding the contribution of DRiPs to antigen presentation and the cellular protein economy requires multiple values: the number of RDPs synthesized per unit time (which equals their degradation rate at steady state), the fraction of RDPs that are DRiPs, the efficiency of converting DRiPs to pMHC I complexes, and the number of pMHC I complexes generated per unit time. Based on measured values in a fibroblast cell [19] of 4×10^6 proteins synthesized min^{-1} , a RDP rate 25%, one pMHC I complex generated per ~2,000 proteins degraded, 150 class I complexes generated min^{-1} it was possible to broadly reconcile peptide generation with overall protein degradation [18]_[ET1]. Little thought, however, was given to the critical relationship between DRiPs and RDPs, or to the calculated value of peptides generated to peptides degraded and actual steady state peptide levels. To this day, little is known about the amounts, location, and nature of proteasome products in cells, other than those that constitute the immunopeptidome. And, what about the measured values; are they correct or generally applicable?

A phage library derived Ab specific for hCMV pp65₄₉₅₋₅₀₃ complexed to HLA-A2 was used to determine that 40% of intracellular A2 molecules were occupied by this one peptide, derived from a protein that is not particularly abundant [38]. Given the deviously clever nature of viruses, this remarkable occupancy could be related to a viral evolutionary strategy, but this finding is not particularly unusual. The SYPETHEI peptide database takes its name from a JAK1 peptide that occupies 10% of K^d molecules in P815 cells [39]. There are other examples of viral (measles F protein) [40] and cellular (tyrosinase) [41, 42] peptides that occupy 10% or more of their restricting class I molecule. Even VV-expressed ovalbumin derived-K^b-SIINFEKL complexes, at 3,500 copies/cell, represent 9% of total cell surface K^b 5 h post-infection, implying an even higher fractional occupancy of K^b molecules exported from the ER during infection [43]. Copy numbers of viral peptides in the thousands is common [44–47], so SIINFEKL, while clearly above average in its representation in the immunopeptidome, is not extraordinary. Notably, in each of these cases, pMHC I complex complexes are expressed in vast excess relative to the fractional value of source protein synthesis relative to total synthesis. Whether this is truly related to special handling of DRiPs in a gene product specific manner (as implied for SIMP, which has a number of over-represented peptides [42]), or simply means that the peptides are exceptional in their liberation or accessing class I molecules is unclear, and remains a critical issue for further investigation.

Never the less, per cell, at an efficiency of 1/2000, 2×10^6 DRiPs would need to be degraded to generate 1,000 complexes. For model antigens like IAV nucleoprotein (NP) [13, 48] and NA [22, 49] or SCRAP [50], DRiPs have not been clearly detected biochemically in the presence of proteasome inhibitors. It is typically easy to detect proteins in the million-copy number per cell. This strongly suggests a much higher efficiency for generating

antigenic peptides from true DRiPs vs. the model DRiP (N-end rule targeted NP) used for efficiency calculations [19].

And if this is generally true for DRiPs, it implies that the very high fraction of RDPs detected in pulse-chase amino acid radiolabeling experiments [19, 51–53] is either incorrect or irrelevant for antigen processing, since antigenic peptides are likely to be limiting in cells [25, 26], and not saturating as calculating from the original numbers [18]. Indeed, a contradictory study [54] concluded that RDPs represent only a few percent (or less) of nascent proteins, although the RDP fraction varied depending on the pulse chase radiolabeling protocol employed (see reference [55]) for a more thorough discussion). The pulse-chase approach was updated in a study showing that following IFN- γ treatment, a large (though unenumerated) fraction of nascent proteins (many of which are damaged by IFN- γ induced-oxidative stress) are rapidly degraded by immunoproteasomes and presumably contribute to the increased peptide pool that accompanies the increase in class I expression [56] (a specialized role for immunoproteasomes in degrading oxidatively damaged proteins is supported by an independent study [57]).

At this point, it is simply not possible to definitively explain the discrepancies in RDP rates reported in different studies. As with many techniques, pulse-chase radiolabeling is based on reasonable assumptions that are nearly impossible to conclusively validate [58, 59]. Better estimates of the RDP fraction of nascent proteins awaits introduction of new methods for global measurement of protein turnover (though all that methodologically glitters [60] is not necessarily gold [59, 61, 62]).

One of the troubling issues raised by a 30% RDP fraction is that since protein synthesis is so energy intensive, 10% of cellular energy would be consumed synthesizing defective or unneeded proteins [19]. Are cells this wasteful? A recent study suggests that yeast, at least, may not be. By expressing an inducible misfolded form of YFP in yeast the fitness costs of protein expression were measured simply by measuring yeast growth [63]. Despite representing 0.1% of total cellular protein, misfolded YFP caused a 3% decrease in proliferation, likely related to induction of chronic stress response measured by a selective increase in the cytosolic chaperone machinery. There are two general explanations for this result: first, misfolding is an extremely unusual event in unstressed yeast, or second, YFP is highly unusual in its properties as a misfolded protein. In support of the latter, misfolded YFP was inefficiently degraded, accumulating in insoluble aggregates. Perhaps tellingly, even expression of *wt* YFP (as discussed above, likely to significantly auto-fragment) incurred a 1% fitness cost, suggesting that YFP is not an ideal model protein, since its expression is not innocuous.

These remarkable findings imply that there is constant strong selection in cells for expression of well-behaved proteins, *i.e.* proteins that don't interfere with cellular function by inadvertently binding other proteins or cellular material, and that proteins evolved in different organisms play by incompletely overlapping rules. While the data favor the idea that misfolding of nascent proteins is an uncommon event, it is equally possible that there is robust selection for genes whose aberrant products can be efficiently handled by the quality control machinery. Consistent with the latter possibility, an extremely high error rate was reported in bacteria as measured by expression of GFP with introduced stop codons and frame shifts [64].

The bottom line is that the RDP fraction is uncertain. At the same time, it is important to recognize that the fraction of proteins that represent immunologically relevant DRiPs can be minute (1% of translation or less) if the efficiency of generating pMHC I complexes from degraded substrates is high. With 2% efficiency, it would take just 50,000 substrates

degraded to generate 1000 complexes. In a typical viral infection, cells can synthesize millions of copies of a viral gene product in a few hours. Diversion of a few percent of translation products to DRiP generation would be difficult to detect biochemically, yet sufficient for immunosurveillance. The problem is even more acute for tumor/self antigenic peptides, typically present at low copy numbers per cell. Under these circumstances, mechanisms of DRiP genesis must be defined by genetic approaches.

Defining DRiPs

Destination: Rome

The close kinetic relationship between translation and K^b-SIINFEKL generation and the kinetics of cycloheximide shutdown implies a ~ 5 min half-life for source DRiPs [19, 22, 24]. Importantly, the shut down in peptide supply for TAP in uninfected and IAV infected MelJuso cells [25] is consistent with a similar half-life for typical cellular and viral peptides. It has been insightfully argued that misfolded proteins typically demonstrate much slower degradation kinetics and do not increase peptide supply greatly, and therefore that most DRiPs are likely to be fundamentally different from typically misfolded proteins [65]. Instead it was proposed that DRiPs are predominantly produced by ribosomes lacking associated chaperones and are directly shunted for proteasome degradation, extending the concept of the “immunoribosome”, a ribosome subset generating proteins more efficiently targeted to antigen processing [55, 66, 67].

If DRiPs are dedicated to antigen processing, and Occam’s Razor is sharp, DRiPs should be subject to minimal post-translational modification in terms of folding, interaction with enzymes *etc.* Dolan *et al.* [50] expressed a cytosolic SIINFEKL-chimeric protein (SCRAP) whose stability is controlled by a rapidly acting reversible drug (Shield-1) and whose antigenicity is proteasome dependent. Incubation of cells with Shield-1 generates a pool of stable protein rapidly degraded at will by removal of Shield-1. Although this pool generates K^b-SIINFEKL complexes in the presence of cycloheximide when Shield-1 is removed, Shield-1 has only a minor effect on the kinetics of K^b-SIINFEKL generation (30% reduction), despite complete inhibition of SCRAP degradation.

These findings lead to two insights. First, 70% of pMHC I complex complexes in this system derive from DRiPs that resist folding. These DRiPs are undetected by standard biochemical methods, so likely of extremely low abundance, implying high efficiency for antigen processing. This reinforces many examples where targeting proteins for complete and rapid destruction has only a modest effect on peptide generation [19, 30, 34, 47, 68, 69]. Second, peptides are also generated from a standard form of the protein, extending findings that misfolded NP degraded with a 70 min half-life is actually a more efficient source of peptides on a molecule degraded basis than N-end rule-NP targeted for immediate destruction [19].

Since retirees also access the class I pathway, it follows that evidence that peptides derive from non-defective proteins (*i.e.* they were glycosylated or phosphorylated [70], or sensitive to agents that promote folding [41, 71]) does not disprove or even discount the contribution of DRiPs to antigen processing. Rather it re-confirms [72] that there are multiple sources of peptides, and that for any given protein, one pathway or another might dominate. Case in point: it was [73] found that the effect of cycloheximide on BMDC class I expression (inferred to relate to peptide supply) varied from complete dependence at 4 h post LPS activation to partial dependence 12 h later, corresponding to variation in the rate of total protein synthesis. Again, the critical evidence for interpreting the relative contribution of new *vs.* old gene products must come from kinetic analysis relating protein synthesis and shut down to peptide production, no less because Occam’s razor can be rather dull [74].

DRiP Biochemistry: Finally!

Due in large part to the uncertain (and likely high) efficiency of natural DRiP antigen processing, biochemical characterization of potential source of antigenic peptides must be closely linked to generating the cognate pMHC I complex. An unsettling example: N-end rule targeting of NP for rapid destruction by proteasomes enhances generation of peptides restricted by D^b, K^d and K^k, but only presentation of the D^b peptide is blocked by concentrations of proteasome inhibitors that completely block degradation of the detected form of NP [75]. Here, the relevant source of K^k- and K^d-restricted peptides is clearly not the substantial biochemically detected proteasome inhibitor-sensitive pool, despite the paradoxical fact that their presentation is enhanced by proteasome targeting.

Proteasome dependent-K^b-SIINFEKL generation from stable VV-encoded NP-S-GFP correlated with the behavior of a highly hydrophobic (TX-100 insoluble) fraction of RDPs, demonstrating independence from E1-dependent ubiquitylation and 19S proteasome regulator expression [15]. By contrast, K^b-SIINFEKL generation from slowly degraded NP-S-GFP was reduced to the same level as stable NP-S-GFP, consistent with a contribution of “classical” DRiPs processed via 20S proteasomes. Extending these results in the VV K^b-SIINFEKL system, it was shown that cytosolic antigens are resistant to dominant-negative Ub expression, while ER-targeted versions of the same antigens are sensitive, and also selectively sensitive to knockdown of HRD-1, the major ERADE3 Ub-ligase [76].

These findings point to contributions from Ub dependent- and independent-peptide generation. Complicating matters, a potential role for ubiquitin hydrolases in SCRAP DRiP presentation and overall cellular peptide generation was uncovered, [50]. This segues with an intriguing report [77] characterizing BAG-6, a MHC-encoded protein that targets ubiquitylated RDPs to 26S proteasomes and whose knock-down reduces class I cell surface expression, consistent with a role for BAG-6 in DRiP processing. These findings provide a clear launching point for unraveling the link between DRiPs, ubiquitin and degradation by various incarnations of the proteasome/immunoproteasome with different regulatory subunits [56, 78].

A key feature of natural DRiPs may be their hydrophobicity [15]. When progressively longer hydrophobic sequences were appended to cytosolic proteins, progressively rapid degradation and enhanced generation of K^b-SIINFEKL complexes occurred [30]. These antigens were, however, less efficient sources than an equivalent N-end rule targeted antigen, which itself is likely to be a far less efficient peptide source than natural DRiPs.

The nature of DRiPs in the substantial subclass of proteins that are targeted to the ER or other membranous organelles (~25% of human genes [62]), providing immunodominant peptides for many viruses) has also been addressed [30]. DRiPs from ER-targeted proteins can arise from either ERAD export of unfolded protein back to the cytosol [79, 80], or a failure to be imported [81]. Notably, the efficiency of ER-import and folding can be sufficient to prevent detectable peptide generation [82], and targeting of cytosolic proteins to the ER can diminish antigen presentation [75, 80]. ERAD substrates degrade slowly (hours) following their import into the cytosol, particularly when lacking their transmembrane domain, providing a steady supply of antigenic peptides (implying that the rapid presentation of NA DRiPs [22] is due to a failure of import into the ER during synthesis [30]). Most importantly, the clear kinetic link between peptide generation with the amount and degradation rate of a biochemical species provides the best biochemical definition of a naturally processed DRiP to date [30].

Translation: The Heart of the Matter

The close kinetic link between translation and peptide generation stressed in this review, strongly implicates a critical role specialized/non-traditional translation in antigen processing. The harbinger of the weirdness of antigen presentation in its independence from traditionally measured gene expression came decades ago while identifying tumor specific peptides from cells transfected with genes in “non-translational” contexts [83]. Soon after, frame-shifting a transgene was reported to abrogate IAV NP protein expression with shockingly little effect on antigen presentation [12]. There are now many examples of cellular/viral peptide generation from natural or experimentally introduced alternative reading frames (ARFs), introns, “non-translated regions”, downstream initiation, and stop codon read-through [84].

Antigenic peptides can be generated from alternative translation on short reading frames (*e.g.* Leu initiation on CUG codons [85, 86]). These peptides, while potentially of great importance medically as tumor and transplantation peptides, probably constitute a minority of the immunopeptidome (though their relative contribution could increase markedly under stress conditions, which favors their translation as other genes are translationally blocked [87]). Based on mass-spectrometric analysis and antigenic peptide mapping, the vast majority of defined peptides in online databases (syfpeithi.de; iedb.org) derive from the normal reading frame of traditional proteins. While there is no doubt that ARF peptides have been overlooked by investigator bias, until proven otherwise these are likely to be an exception [88] (counterpoint: there are now a considerable number of biologically relevant ARF CD8+ T cell epitopes defined for HIV and SIV [89–91]).

In considering translational mechanisms that might favor peptide generation, it is important to distinguish, at least until we know better, natural from artificial situations. We should be wary of translating proteins in unnatural contexts, particularly from transfected genes, because cells might have a mechanism for distinguishing mRNA transcribed from foreign DNA and employ special mechanisms to enhance immunosurveillance. It is obviously also safer to study a natural protein sequence rather than a chimeric protein, whose translation is likely less efficient/odd due to the absence of an evolutionary optimizing filter for not interfering with cellular function. Similarly, swapping genes between viruses runs the risk of running afoul of each virus’ unique translation plan, as clearly shown regarding the mistranslation and misfolding of IAV NP in the context of alphavirus gene expression [13] or the distinct processing pathways (TAP-dependent *vs.* -independent) used by respiratory syncytial virus (RSV) F protein when expressed by RSV *vs.* VV [92].

Several groups have carefully characterized the translational control of the Epstein Barr virus protein EBNA-1, which evades immunosurveillance by a Gly-Ala repeat that blocks translation in a *cis*-acting manner [93], and not by blocking its proteasome degradation, as originally proposed [94] (in fact, the repeat has little effect on proteasome degradation of EBNA-1 [95]). Several studies show that that EBNA-1 antigen presentation is governed by the rate of translation [96, 97], but it is worth noting, that regardless of the mechanism of peptide generation, making less of the precursor will result in diminished antigen presentation.

Taking a critical step forward, it was reported that rather than modifying *in vitro* translation initiation of an EBNA-1 SIINFEKL chimera, the Gly-Ala repeat greatly increased the frequency of terminating translation, during or just after its own translation [33]. As predicted from the *in vitro* translation studies, the Gly-Ala repeat diminished cellular generation of downstream antigenic precursors (detected by proteolytically liberating the peptide and measuring its activity by T cell activation), which in an ironic twist, were

principally recovered as truncated polypeptides ranging from ~ 50 to 150 amino acids. These clearly qualify as DRiPs based on their kinetics of appearance and disappearance with transcription and protein synthesis. It is likely that these fragments arise from premature termination, but based on the absence of NH₂-terminal sequences on some of the fragments, limited proteolysis and/or downstream initiation is probably involved as well.

Compartmentalization: The Key to Immuno-peptidome Diversity?

Since oligopeptides are small and typically highly diffusible [98] (but see [29] for an exception), it has been generally assumed that proteasome-generated peptides have equal access to TAP and hence, loading onto class I molecules. How then to explain the enhanced presentation of DRiPs *vs.* retirees (especially if the fraction of DRiPs is very low)? Or natural DRiPs *vs.* proteins engineered for rapid degradation? Or peptides from low abundance mRNAs, ARFs or unusual initiation sites, non-coding regions, in the face of competition from normal protein turnover? Or peptides generated by proteasome mediated splicing, which ought to be generated in minute amounts [99]?

A likely explanation is that all peptides are not created equal, because some proteasomes are more adept at generating precursor peptides, or have more direct access to TAP. This could be the case if DRiP processing is compartmentalized in some manner to limit competition for peptide access to TAP. Precedent for compartmentalized presentation comes from studies on exogenous antigen presentation, where peptides appear to be locally generated in the cytosol and loaded via TAP-onto class I molecules present in the source phago/endosomal compartment [100–102]. Exogenous antigen presentation is hard to understand without compartmentalization, because the amount of antigen reaching the cytosol is miniscule compared to the amount of protein present in the cytosol. Even IAV PB1, present at a few copies per virion, and nearly certainly in native conformation as a component of the polymerase complex packaged into the virion, is rapidly presented to T cells when delivered to the cytosol during viral penetration [27].

To study compartmentalized endogenous antigen presentation, cells were co-infected with rVVs expressing either cytosolically liberated SIINFPEKL (synthesized as MSIINFPEKL or GFP-Ub-SIINFPEKL and liberated by Ub-hydrolases [103]) or SIINFPEKL in the context of full-length proteins [104]. K^b-SIINFPEKL expression from cytosolic SIINFPEKL was reduced by law of mass action effects by excess cytosolic expression of another high affinity K^b binding-peptide. Remarkably, however, the same peptide did not compete for K^b-SIINFPEKL generation from Ova itself (a secreted protein), or stable, slowly- or rapidly degraded cytosolic chimeric proteins. As full-length proteins provide far less SIINFPEKL than cytosolically liberated SIINFPEKL, competition should have been enhanced, not inhibited. Similarly, the cytosolic competing peptide did not reduce generation of complexes of seven K^b- VV-encoded peptides eluting in distinct HPLC fractions.

These findings are consistent with the existence of two general compartments: one for presentation of DRiPs, the other for presentation of peptides liberated directly into the cytosol by synthesis (MSIINFPEKL) or hydrolysis from Ub-fusions proteins. The latter are synthesized from self and even from bacterial ribosomes, because K^b-SIINFPEKL complexes are generated with similar efficiency from GFP-Ub-SIINFPEKL synthesized from VV or intracellular *Listeria* [105]. Where might such compartments be located?

Although TAP is widely considered to be exclusively localized to the ER, compelling evidence now also places TAP in the Golgi-ER intermediate compartment and cis-Golgi Complex [106], where it functions to load peptides onto class I molecules that have exited the ER. An attractive model is that free cytosolic peptides are loaded in the Golgi Complex, while DRiP-derived peptides are loaded in the ER itself, where the bulk of cellular

ribosomes reside (including immunoribosomes?) that synthesize nuclear and cytosolic proteins in addition to ER-targeted proteins [107, 108]. These non-traditional ER-ribosomes [109], like CUG start codon decoding ribosomes [87], resist stress-induced protein synthesis inhibition, consistent with an immunoribosome like-function.

Notably, TAP is present in both the outer nuclear membrane and the inner nuclear membrane [110]. Blocking IAV NA-SIINFELK mRNA export from the nucleus diminishes NA biosynthesis disproportionately to blocking K^b-SIINFELK surface expression, and it was proposed that peptides were generated by nuclear protein synthesis[49]. Although nuclear translation is contentious [111, 112], a new technique that localizes actively synthesizing ribosomes via standard immunofluorescence, has shown that that nuclear translation is robust in cultured cells (although absent in VV-infected cells, due to shut down of host protein synthesis), with high activity in the nucleolus. Intriguingly, the ER extends tunnels into and through the nucleus that often course next to nucleoli [113], a potential means for nuclear/nucleolus-generated peptides to conveniently access TAP.

It remains to be determined what is actually translated in the nucleus, but it has long been suspected that pioneer translation associated with mRNA nonsense mediated decay (NMD) occurs in the nucleus, could be a potential source of DRiPs [55]. Blocking mRNA synthesis rapidly reduces class I cell surface expression, consistent with a contribution of short-lived mRNAs destroyed by the NMD pathway [49]. Furthermore, interfering with a number of elements of the NMD pathway reduces overall peptide supply [114]. Extending these intriguing results, an elegant study demonstrated that NMD plays a critical role in generating peptides from model constructs, and that this occurs in an eIF4G dependent/eIF4E independent manner. *i.e.* distinct from typical translation [34]. RNA regulation was previously implicated in DRiP generation when it was reported that RNAi targeted downstream of a reporter peptide results in translation of truncated proteins with enhanced antigen presentation *in vitro*, with increased tumor destruction *in vivo* [115]. More work is needed, but these findings point to an important involvement of NMD and RNAi pathways in immunoribosome based-DRiP generation, with a potential role for nuclear translation.

If DRiP processing is compartmentalized, it is a safe bet that viruses have devised schemes for subverting it to avoid T cell recognition. The leading candidates would be herpesviruses, which during their long co-evolution in vertebrates solved the problem of establishing persistent infections in the face of vigorous T cell responses. The KSHV LANA1 protein minimizes antigen presentation based on the cis-acting properties of a Q E D repeat region [116]. Could this sequence act by uncoupling high efficiency compartmentalized DRiP presentation?

What's Next?

While the centrality of DRiPs to antigen processing is solidifying, there is much to learn (Box 2). There is compelling evidence for an important contribution of DRiPs to host and viral immunopeptidomes. It is important to extend the kinetic evidence to increasingly relevant situations. This will entail continued progress in generating TCR like- reagents for accurate measurement of specific pMHC I complexes to enable measurement of complexes in their natural contexts of expression, ultimately, in fully differentiated cells *in vivo*. It will also require SILAC labeling experiments to directly measure the kinetics of generating hundreds to thousands of defined peptides, which should eventually be possible to perform *in vivo*, perhaps by isolating peptides from class I molecules present in serum, recently described as an exciting new human biomarker [117].

If immunoribosomes exist as ribosome subsets, genome wide siRNA screens should reveal gene products whose knock down selectively reduces DRiP presentation due to effects on

the immunoribosome itself, or adapter proteins that facilitate its compartmentalization. Such screens, which have successfully been used to study the class II pathway [118], should reveal other unknown participants in the various aspects of class I presentation, and also possible links between the endogenous, exogenous and autophagy pathways [119, 120].

A particularly promising area is the involvement of microRNAs in antigen processing, both as a direct source of peptides [121] as well as in creating DRiPogenic mRNAs from standard mRNAs. The seminal findings implicating miRNA and NMD in peptide generation [114, 122, 123] point towards integrating peptide generation to mRNA quality control mechanisms (including “nonstop” and “no go translation”, *i.e.* translation of mRNAs lacking a stop codon, and stalled translation [124]), possibly with the involvement of nuclear translation.

Progress in these areas will go hand-in-hand with progress in biochemically defining DRiPs. Having a genetic handle on antigen processing pathways will generate testable predictions regarding the intracellular location of DRiPs and their physical properties.

A remaining mystery is the extent to which cells discriminate antigens generated from truly self genomic material *vs.* genes introduced via transfection or intracellular pathogens. A finding that begs explanation is the enhanced generation of cytosolic peptides following IAV infection [25]. This suggests the working of an innate immune sensor that enhances DRiP formation by modulating cytosolic protease activity to favor oligopeptide survival, increasing peptide accessibility to TAP, or increasing immunoribosome translation. Could cells also have a means for sensing proteins synthesized by their own ribosomes *vs.* proteins introduced from exogenous sources?

The *yang* of DRiPs is their potential involvement in thymic self-tolerance, where essentially nothing is known. If ever there was an organ needing a specialized DRiP apparatus, it would be the thymus particularly with the existence thymus-specific gene products, AIRE and thymoproteasomes pointing to thymus-specific tricks for creating a unique tolerizing immunopeptidome. It is also possible that neurons have specific mechanisms for generating peptides presented by class I molecules at synapses that contribute to neuronal communication [125, 126]. Here, class I molecules might function as “translatometers”, enabling neurons to monitor translation activity of their synaptic partners [126].

The MHC class I processing pathway, in addition to its own obvious intrinsic interest and medical importance, continues to provide a unique platform for making basic cell biology discoveries in numerous areas, including membrane protein trafficking, biogenesis of ER proteins, ERAD and cytosolic protein degradation, and peptide trafficking and processing. It now promises to continue this tradition in providing fundamental insights into specialized translation and cytosolic compartmentalization (Box 2), and makes an excellent argument for basic research as the key to unlocking doors to the unknown.

Box 1 Solidifying DRiPs: Known unknowns and future directions

- Systems DRiPology: using quantitative mass spectrometry studies to relate the immunopeptidome to the translome, and degradome.
- Defining the range of efficiencies of pMHC complex formation from different antigen sources.
- Defining the mechanisms of alternative translation that funnel into the MHC I antigen processing pathway. These include NMD, RNAi, non-stop and no-go translation and likely still be discovered processes that translate “non translated” regions of viral and host genomes. A key approach will be genome wide

knockdown screens to identify novel gene products involved in immunosurveillance.

- Defining the biochemical nature of DRiPs generated during translation of standard reading frames. Weighing the contribution of downstream initiation, premature initiation, misfolding of full length gene products due to stochastic and deliberate failure of chaperoning nascent gene products.
- Defining compartmentalization of antigen processing that enables peptides from minor translation products to successfully compete with peptides from major translation products for binding to class I molecules.
- Defining the intersection of endogenous antigen processing and exogenous antigen processing mechanisms in DCs and other professional APCs.
- Extending knowledge gleaned from cultured cells to cells in living animals. Discovering features of antigen processing specialized for thymic selection, neuronal function, and other tissue-specific functions.

Box 2 Insights into Basic Cell Function Contributed by MHC Class I Endogenous Processing Studies

- Degradation of oligopeptides within seconds unless bound to a MHC class I or II molecule.
- Absence of carboxypeptidase activity in cytosol and secretory compartments of mammalian cells.
- Proteins are degraded in the nucleus by proteasomes, with high activity at specific nuclear organelles.
- Special role of immunoproteasomes in degrading oxidized proteins.
- Proteasome specificity.
- Proteasome splicing of oligopeptides.
- ERAD.
- High fraction of proteins destroyed within minutes of synthesis by proteasomes.
- High frequency of translational errors, including premature termination, read through and downstream initiation.
- Translation of “non-translated” regions of genes.
- Translation can initiate with Leu decoded on Leu codons.
- Oxidative stress induced modification of the genetic code.
- Retrograde transport between GC and ER.
- Specificity and function of ER chaperones.

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Figure 1. Idealized Kinetic Analysis of Antigen Presentation

Depicted are graphs of cells expressing a source antigen at a uniform rate starting at time zero.

(a) presentation kinetics. If antigenic peptides derive exclusively from rapidly degraded DRiPs, the substrate pool will reach steady state rapidly and class I complexes will be generated at linear kinetics from near time zero, with a lag dependent on the time for degradation, loading, and transport from the site of loading in the secretory pathway. If peptides derive exclusively from retirees, there will be a much longer lag prior to detection on the cell surface, and the rate of complex delivery will accelerate over a much longer time, as the pool of substrate reaches its steady state level.

(b) and (c) presentation shut off kinetics. From adding various inhibitors, half-lives can be calculated for antigen presentation. BFA provides a measure of the transit time from post-BFA sensitive compartments to the cell surface. Proteasome inhibitors provide a measure of the time for peptide loading and transport to the cell surface. Protein synthesis inhibitors provide a measure of the half-life of the relevant substrate pool. If peptides derive from DRiPs, blocking protein synthesis will rapidly shut off delivery of pMHC I complexes to the cell surface (b). If peptides derive from long lived retirees, blocking protein synthesis will have no effect on complex delivery to the plasma membrane, at least as long as cells maintain a sufficient supply of class I molecules in the ER, likely to be the limiting factor in continue antigen presentation.