Translating DRiPs: MHC class I immunosurveillance of pathogens and tumors

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ABSTRACT

MHC class I molecules display oligopeptides on the cell surface to enable T cell immunosurveillance of intracellular pathogens and tumors. Speed is of the essence in detecting viruses, which can complete a full replication cycle in just hours, whereas tumor detection is typically a finding-the-needle-in-the-haystack exercise. We review current evidence supporting a nonrandom, compartmentalized rejection of peptide substrates that focuses on rapidly degraded translation products as a main source of peptide precursors to optimize immunosurveillance of pathogens and tumors.

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Introduction

Classical MHC class I molecules (in man, HLA-A, -B, and -C; in mouse, H-2-K, -D, and -L allomorphs) play an important role in gnathostome biology, including immunosurveillance [1], mate selection [2], and neuronal development and function [3]. Class I molecules provide a window to the peptidyl informational profile (translatome, proteome) of individual cells by binding and presenting oligopeptides at the cell surface. This enables lymphocyte-mediated immunosurveillance, whose importance in viral and tumor immunity is underscored, respectively, by the ever growing numbers of viral proteins that interfere with the antigen-processing pathway [4] and frequent down-regulation of genes involved in MHC class I antigen processing in tumor cells [5].

Class I molecules consist of an extremely polymorphic heavy chain, a conserved light chain (β2-microglobulin), and an oligopeptide (typically 8–13 aa). MHC class I is constitutively expressed by nearly all gnathostome-nucleated cells and is upregulated quickly upon exposure to IFNs and other proinflammatory cytokines. Classical studies in the 1980s established that MHC class I (and class II) molecules present oligopeptides [6–8], but much remains to be learned about how peptides are generated from proteins synthesized at the APC. Here, we review recent progress in understanding such “endogenous” antigen presentation, with emphasis on the insights the field has generated on central processes in cell biology. Figure 1 outlines the class I pathway and provides a preview of what is to follow. Readers are directed to previous reviews [9–11] for a comprehensive view of earlier findings regarding the generation of endogenous peptides.

DRiPs GERMINATE

In 1996, we (with Jack Bennink) proposed the DRiP hypothesis [12] to explain kinetic observations [13] that conflicted with the ruling concept that class I peptide ligands (hereafter, referred to as peptides) derive from the regular turnover of source proteins. Virus-infected cells were recognized by antigen-specific CD8+ cytotoxic T-lymphocytes soon after infection, long before source protein could be detected. This was true regardless of the stability of the source protein; in fact, nearly all of the viral proteins examined are metabolically stable, which made degradation of their “retirees” an unlikely source of the precursors (proteins are typically degraded with first-order kinetics with a characteristic half-life). Whether the stochastic “retirement” is a result of random fluctuations on protein structure that are recognized by cellular degradative machinery or random damage to the protein is a central, although little-studied, question in proteostasis [14].
What was/is a DRiP? Originally [12], DRiPs were defined as ribosomal products that do not attain mature (stable) conformation but rather are degraded during or shortly after translation. At the time, we focused on translation of standard mRNAs in the standard reading frame, positing that DRiPs derived from deliberate and entropic premature termination or nascent-chain misfolding. Over the years as the evidence mounted [9], we broadened DRiPs to include defective poly-peptides arising from alternative/defective mRNAs [15, 16], ribosomal frame shifting [17, 18], downstream initiation on bona fide mRNAs [19], and indeed, all other errors that occur in converting genetic information into proteins (including tRNA-amino acid misacylation [20] or transcription errors [21]). A recent expansion of the DRiP hypothesis posits “immunoribosomes” as a subset of ribosomes, specialized for generating immunologically relevant DRiPs [22, 23].

**DRiPs FLOWER: KINETIC IMMUNOPEPTIDOMICS**

In principle, the simplest method to gauge the contribution of DRiPs to the class I immunopeptidome (the set of peptides presented by MHC class I molecules) [24] would be to incubate cells with radiolabeled amino acids for a few minutes and measure the kinetic appearance of radiolabeled peptides recovered from MHC class I molecules. This experiment, simple in principle, has yet to be reported (although, ironically, the first natural peptide to be characterized was a radiolabeled peptide derived from VSV nucleocapsid protein (N), a highly stable viral protein [25]).

Better yet, however, is to perform this kinetic approach with SILAC, since due to the miracle of MS, the production kinetics of thousands of peptides can be studied simultaneously. Pioneering this approach, Milner et al. [26] found a poor correlation between source protein abundance and presentation, with some peptides appearing shortly after their synthesis, regardless of the turnover of their source proteins. Similar discrepancies were observed after treating cells with proteasome inhibitors [27]. This concept was strengthened recently in a massive SILAC analysis of the B-lymphocyte immunopeptidome, where peptide abundance was poorly correlated with the amount or turnover of the source gene product [28].

In a landmark study, Croft et al. [29] used VV to present most definitive evidence to date correlating viral protein synthesis with peptide generation. Building on previous work, identifying H-2b-presented VV epitopes [30, 31], they used a novel MS technology, selection-reaction monitoring [32], to correlate the kinetics of source VV protein expression with...
peptide generation. Each of eight peptides studied demonstrated a striking correlation between protein synthesis and peptide generation, providing the strongest evidence to date for the DRiP hypothesis. For early VV proteins, peptides were detected at the first time-point examined, 30 min postinfection. For the most part, peptide levels stabilized or fell as source antigen synthetic rates declined (VV choreographs expression of its genes; early gene products typically make way to intermediate and late gene products), presumably reflecting the balance of peptide synthesis versus the stability of the peptide-MHC complex. At the same time, the B8\textsubscript{20-27} peptide demonstrated complex kinetics consistent with an important contribution from retirees. Peptides from retirees are not surprising themselves, as cytosolic retirees were the first definitive source of peptides [33]. B8, however, is a secreted protein, raising the issue of whether the retirees are actually slowly degraded DRiPs versus native forms that re-enter the cytosol or are processed in an endosomal compartment [34]. We will return to the topic of retiree presentation below, discussing new findings that shake the very foundation of our quantitative understanding of antigen processing.

The linkage between translation and peptide generation enables rapid recognition of virus-infected cells and immunosurveillance of acute changes in cellular gene expression. Perrault and colleagues [35] insightfully suggested that the self-immunopeptidome is a signature of the tissue type and metabolic status of a cell and provided supporting evidence by treating cells with rapamycin, which induces global changes in cellular translation [36]. With the use of MS, they found several instances of increases in peptides without a corresponding increase in source protein levels. In the case of rictor, an integral component of one of the mTOR complexes, mTORC2, they could correlate an increase in peptides with rapid degradation of ubiquitylated forms of the nascent protein. Creatively combining proteomics with transcriptomics, they discovered that transcripts targeted by miRNAs are over-represented as a source of peptides [37]. It is uncertain whether actively translating ribosomes can be targeted by miRNAs [38], although it has been shown that for small interfering RNAs, ongoing translation is required for degrading their target mRNAs [39]. In any event, it seems likely that these results are mechanistically related to the finding of Gu et al. [40] that shRNAs enhance peptide generation from targeted transcripts if the presented epitope is encoded upstream of the shRNA-targeted sequence.

In toto, the evidence points to nonrandom selection of source proteins as a central feature of peptidogenic DRiPs. Indeed, in designing immunosurveillance, an optimal system would sample peptides based on mRNA identity, not abundance. In the latter case, the peptidome would be dominated by peptides derived from the most abundant transcripts, which typically encode housekeeping proteins or proteins with exocrine functions. In other words, antigen processing should be modeled after the U.S. Senate, not the House of Representatives. Such nonrepresentational sampling, whereas of debatable value for democracy, is perfectly suited to detecting alterations in the metabolic state of tissues [41, 42], including virus-infected cells, where a large fraction of altered peptides can be derived from host genes [43, 44].

**DRiPs, SLiPs: ALL IN THE RDP FAMILY**

When we posed the DRiP hypothesis, we had given little thought to the overall economics of protein synthesis. This only came after [45, 46] we discovered that upward of 30% of proteins in pulse-chase labeling experiments are degraded within minutes of their synthesis [47]. With improved methodology, we revised the fraction of RDPs to closer to 25% [46, 48, 49] and gave more thought to the basic concepts of nascent protein biogenesis [14]. Using plain English, we termed the RDPs detected in pulse-chase experiments just that (RDPs), and distinguished between DRiPs and SLiPs, which through essentially no fault of their own, are degraded rapidly. SLiPs include excess subunits of multiprotein complexes that failing to assemble and hence, achieve a stable conformation, are treated as defective proteins. Similarly included are intrinsically disordered proteins, which only assume a stable conformation upon finding a suitable binding partner. These are not minor classes of proteins. Although strictly computational, it is estimated that 28% of mouse proteins are mostly disordered [50]. Based on empirical evidence, no less than 20% of encoded proteins are believed to exist in multisubunit assemblies [51], with increases in excess subunits in aneuploid cells as a result of gene-dosage imbalances [52].

The high RDP fraction in cultured cells was challenged by Vabulas and Hartl [53], who using a different strategy (measuring the amount of protein synthesized over short times in the presence and absence of proteasome inhibitors), concluded that RDPs represented, at most, a few percent of total synthesis. Ironically, the initial study to confirm Schubert et al. [47] was published by Denys Wheatley et al. [54], 20 years earlier. Wheatley and colleagues [54] reported that after labeling for 1 min with radiolabeled Leu, ~30% of proteins are degraded within 30 min of synthesis (repploting 1-min RDP data and fitting to first-order degradation kinetics reveal a calculated half-life of 10 min). More recently, Cenci et al. [55] found in traditional pulse-chase experiments that the RDP fraction varied between 1% and 30% among human myeloma cell lines and was altered by cellular activation. As we have emphasized [56, 57], pulse-chase labeling, like every technique, is subject to potential artifacts, necessitating the application of other methods to validate the true RDP fraction.

Given the enormous energetic costs of protein synthesis (more than one-third of cellular energy consumption in actively dividing cells [46]), a high RDP fraction seems incompatible with evolutionary pressure to maximize efficiency, given inevitable limitations in food sources (given unlimited food, unlimited procreation restores scarcity). On the other hand, it may simply be difficult to synthesize tens of thousands of different proteins, and a high RDP fraction may be the inescapable price for complexity. Consistent with this idea, Ha et al. [58] report a similarly high RDP fraction in yeast, extending the phenomenon to a unicellular organism with no need to generate antigenic peptides. This finding echoes the origi-
nal report of Turner and Varshavsky [59] that up to 50% of β-galactosidase is degraded in yeast.

Cogitating based on first principles is fun and sometimes even useful for generating hypotheses. In the end, however, conclusions must be evidence-based. Clearly, establishing the true RDP fraction requires new methodology. Two groups recently developed techniques that measure the fraction of ribosome-bound nascent chains that are ubiquitylated for degradation. Wang et al. [60] reported that in human cells, up to 15% of nascent chains are ubiquitylated, through K48 and to a lesser extent, K11 linkages (those associated to proteasomal degradation), whereas in yeast, this number is lower (~6%). The ubiquitylated fraction was enriched in cytosolic versus ER-associated polysomes. Intriguingly, it has been suggested that the immunopeptidome is biased against membrane proteins [61]. Increasing translation errors coordinately increased the fraction of nascent polypeptide ubiquitylation. In unstressed cells, nascent-chain ubiquitylation occurred mostly with actively translating versus stalled ribosomes. Working strictly with yeast, Duttler et al. [62] reported that 1–1.5% of nascent polypeptides are ubiquitylated, increasing to 5% under proteasome blockade. Ubiquitylation may not be involved in degradation of all nascent chains, as Ha et al. [58] found that 30% of yeast nascent chains are degraded by proteasomes and further demonstrated the ubiquitin-independent, cotranslational proteasomal degradation of a selected yeast gene product. We note that several studies support an important role for ubiquitin-independent generation of antigenic peptides [48, 63], particularly from non-ER-targeted proteins [64].

These findings spectacularly confirm the principle of cotranslational degradation proposed by Turner and Varshavsky [59] and fit into a larger picture, linking translation with degradation on a local and global scale [65–68], with a significant contribution from translational stalling/pausing that is typically increased under stress [69]. A key question in linking cotranslational degradation with the pulse-chase findings is the kinetics of degradation of cotranslationally ubiquitylated nascent chains. If degradation is very rapid, it would barely register in pulse labels longer than 1 min, although it certainly could account for the 10% difference in RDPs (out of total translation) that Wheatley et al. [54] observed between cells labeled for 1 min versus 10 min. On the other hand, it is possible that degradation takes longer, resulting in a larger pool of nascent ubiquitylated proteins.

Indeed, quantitating the various pools of ubiquitylated proteins awaiting destruction is key to accurately accounting for the fates of nascent and retired proteins. At the dawn of the ubiquitin era, Hershko et al. [70] reported the initial evidence that nascent proteins provide a significant fraction of ubiquitylated proteins in mammalian cells. We extended these findings by showing that blocking protein synthesis for as briefly as 1 min significantly reduces levels of ubiquitylated proteins in total cell extracts [47]. Analyzing the ubiquitylome by MS, Kim et al. [71] confirmed the critical contribution from RDPs and paved the way to deeper understanding of the contribution of RDPs versus retirees by identifying ubiquitylated peptides from thousands of gene products. Indeed, although the quantitation is rough, their evidence suggests that RDPs represent 70% of ubiquitylated protein species degraded by proteasomes, which is in agreement (although possibly spuriously) with our calculation that 72% of proteasome substrates are RDPs [46].

Taken together, these findings strongly support the conclusion that a large fraction of nascent proteins is degraded in cultured cells. We note, however, that mammalian cells evolved in mammals, not on plastic flasks in artificial media with 21% oxygen (oxygen levels in tissues range from 13% in arterial blood, 3–10% in tissues, and likely, 1% in cells; see Table 2 in ref. [72]). It is critical in the future to move from cultured cells to cells in living animals. This poses technical challenges but is not impossible, and it is useful to remind young scientists that most of the earliest pulse-radioactive metabolic-labeling studies were performed intravitaly.

**RNA SURVEILLANCE**

Peptides convey information specified by mRNA. Why doesn’t the immune system survey mRNAs themselves? In other words, why didn’t class I molecules (or equivalent) evolve to present oligonucleotides to T cells? Direct RNA surveillance would provide the enormous benefit of monitoring nontranslated RNAs (including negative-stranded virus RNAs), which are abundant in cells. Whereas proteins can bind RNA in a highly specific manner, perhaps fragmenting and trafficking RNA or discriminating fine distinctions between RNAs by alternative TCRs pose insurmountable problems. Or maybe we just haven’t discovered adaptive immunity to RNA yet.

Although removed from direct RNA recognition, recent findings point strongly to a crucial role for RNA quality control in antigen processing. As discussed above, nascent-chain cotranslational ubiquitylation occurs on stalled and actively translating ribosomes. Almost by definition, ribosomes stall as a result of defects in RNA. Cells deploy multiple mechanisms to detect and degrade nonfunctional mRNA:

1. **NMD**, detecting and degrading mRNAs with a premature stop codon;
2. **NSD**, detecting mRNA lacking a stop codon on ribosomes that stall while translating the polyA tail;
3. **NGD**, targeting mRNA on stalled ribosomes [73].

NMD is believed to take place immediately after mRNA is exported from the nucleus to the cytosol [74], although nuclear NMD remains possible [75, 76]. In theory, ribosomes make just a single “pioneer” round of translation before the NMD machinery detects the premature stop codon. A member of the NMD complex, Upf1 is implicated in the degradation of the nascent polypeptide [77]. Pioneer translation occurs when the mRNA remains bound to the cap-binding complex (mostly nuclear) before transfer to eIF4E, which plays a central role in canonical cytoplasmic translation [77].

The contribution of NMD to antigen processing was pioneered by Apcher et al. [78], who found that similar amounts of peptides were generated when peptides were translated from functional, spliced mRNA that could produce reporter protein versus mRNA where the peptides were upstream of a premature stop codon that induced NMD and prevented translation of the standard gene product. Through clever experimental strategies to dissect the contributions of various transla-
tion factors, they could show that eIF4G, but not eIF4E, is required for peptide generation from functional and NMD-targeted mRNAs, consistent with the idea that pioneer translation is important in peptide generation.

Although these and other findings [78] strongly support the concept that alternative translation, unrelated to standard protein production, is a major source of antigenic peptides, the precise role and function of pioneer translation remain a mystery. If pioneer translation generates a single copy of the polypeptide, the efficiency of antigen presentation would seem to be far too low (even under the best of circumstances) to generate a sufficient number of peptides to account for the T cell activation reported or for true in vivo relevance. It is likely, however, that models of NMD are overly simplified and that multiple rounds of translation occur and with many complications [79–81].

It is of obvious importance to extend these intriguing findings to overall peptide generation. Although their findings are indirect, Dolan et al. [82] found that blocking mRNA synthesis with RNA polymerase II inhibitors reduces overall class I expression by ~35%, consistent with substantial peptide generation from nascent mRNAs. On the other hand, Pastor et al. [83] found that shRNA-mediated interference with factors required for NMD (Smg1 or Upf2), enhanced in vivo elimination from nascent mRNAs. As nascent chains by OT-I cells (T cells specific for H-2Kd;H9253) by siRNA targeting eIF4G, but not eIF4E, enhanced in vivo elimination of tumor cells by OT-I cells (T cells specific for Kb-SIINFEKL complexes) if SIINFEKL were expressed upstream of a premature termination codon. Presumably, this is a result of increased levels of mRNA that are canonically translated into a RDP and pointing to the likelihood that multiple translation pathways contribute peptides for immunosurveillance.

NSD identifies ribosomes translating the polyA tail by virtue of arrest, resulting from translating long strings of Lys [84]. In yeast, the interaction of nascent-chain-positive charges with the negatively charged ribosome exit tunnel is the major cause of translational pausing, with codon use/RNA abundance having a minor effect, at most, on translational speed [84], reinforcing findings in mouse cells using ribosome profiling [85]. As nascent chains on stalled ribosomes are targeted for destruction [67, 86], presumably contributing to the pool of ubiquitylated nascent-chain polypeptide degradation [60, 62], this is a potentially rich source of antigenically relevant DRiPs.

NGD recognizes stalled ribosomes, usually as a consequence of mRNA features or damaged nucleotides; it shares with NSD some of the factors involved in the recognition and degradation of the mRNAs. Interestingly, the protein SIMP/STT3B, which is the source of at least three different, very abundant peptides in mice and humans, has a stretch in the C-terminus with high content in basic residues [87], which may slow translation and target it for presentation. Presumably, NGD functions in the degradation of proteins, truncated as a result of knotted mRNA secondary structure, a known source of antigenic peptides [88].

**BORN TO DIE: TARGETING NASCENT-CHAIN DESTRUCTION**

There are a growing number of factors involved in recognition and degradation of nascent or newly synthesized polypeptides, including chaperones, cochaperones, and ubiquitylation/deubiquitylation systems. All potentially participate in antigen processing. Several gene products target stalled nascent chains for destruction, including Listerin/Ltn1 and Ubr1. E3 ubiquitin ligases and downstream, Cdc48/p97 [68, 89]. Cdc48/p97 is an abundant motor ATPase involved in multiple quality-control pathways, both in the cytosol and in ER-associated degradation [90, 91]. Despite suggestive evidence from a prior study [92], active p97 does not appear to be essential for DRiP-dependent class I ligand generation [93].

The first steps of chaperone-assisted folding are mediated by ribosome-associated Hsc70 and accompanying cochaperone flotilla, including J-domain-containing proteins (DNAJC2 in mammals) favoring substrate folding and carboxyl-terminus of Hsp70-interacting protein, an E3 ligase, promoting degradation [94]. Bag6 is a particularly intriguing candidate for linking DRiPs to antigen processing. Bag6 functions in nascent protein-quality control [95, 96], with selectivity for exposed hydrophobic domains in substrates.

Bag6 associates with proteasomes and immunoproteasomes and also participates in the formation of ALIS—stress-induced depots for ubiquitylated proteins awaiting degradation [97–99]. Intriguingly, Bag6 is encoded within the MHC, is strongly induced by IFN-γ [100], and appears to participate in peptide generation [95, 101]. Although the effect of knocking down Bag6 on DRiP processing appears to be minor to moderate so far, it is clearly worthy of more detailed study. We should add, however, a note of caution when interpreting data from these studies, given the general role of Bag6 in protein-quality control and in light of its recently identified role in the assembly of the 19S and 20S subunits of the proteasome [102].

Another intriguing candidate for DRiP processing is eEF1A, which is known to interact with [103], and mediate proteosomal degradation of [104], newly synthesized proteins in a process stimulated by ribosome-associated JNK [103, 105]. When substrate degradation is prevented, eEF1A mediates their accumulation in ALIS [106].

ALIS have a yin-yang role in antigen presentation. They function as a storage depot for DRiPs that can be re-targeted to the class I pathway when cells recover from stress, and ALIS are disassembled [98, 99]. Alternatively, clearing ALIS by autophagy destroys DRiPs without generating class I peptide complexes. DRiPs sequestered in autophagosomes can be an efficient source for cross-presentation by DCs [107].

**COMPARTMENTALIZED TRANSLATION, DEGRADATION, AND ANTIGEN PROCESSING**

Compartmentalized translation, first described in 1983 [108], has clear, functional benefits [109]. Spatial organization of protein synthesis and degradation is well-established in neurons, adjusting the synaptic proteome to enable synaptic plasticity [110, 111]. Global proteomic analysis supports a general role for compartmentalized degradation by demonstrating varying protein half-life in nuclear, cytoskeletal, membrane, and cytosolic fractions [112]. An intriguing example of mRNA
selectivity is the translation of nuclear and cytosolic proteins by ER-associated ribosomes [113] and their selective regulation under proteotoxic stress [114], which otherwise, globally inhibits translation. Notably, stress resistance is a feature of noncanonical translation of CUG-leucine-initiated antigenic peptides [115, 116]. ER-associated, noncanonically translating ribosomes are obvious candidates for immunoribosomes, particularly given the association of standard and immunoproteinometabolites with the ER [117, 118], where transporter associated with antigen-processing transports peptides to nascent class I molecules.

One of the most remarkable features of antigen processing is that proteasome substrates do not have equal access to the class I pathway. This was implied by the dependence of overall peptide generation on protein synthesis [47, 119], despite the unabated degradation of the proteome during acute GHX-induced protein synthesis blockade [57, 120] (see Fig. 1 in ref. [120]). Differential substrate access was observed when expressing rapidly versus relatively slowly degraded, full-length proteasome substrates (half-lives of 10 min vs. 70 min), the latter of which is presented at twice the efficiency per protein degraded [46], or with canavanine-misfolded proteins, where RDPs were preferentially presented over slowly degraded forms [120]. Furthermore, competition studies [121] revealed that DRP-derived SIINFEKL is presented in a manner that completely avoids competition with high-affinity, Kb-binding, 8-mer peptides, generated in excess amounts in the cytosol from direct synthesis of minigenes or by proteolytic liberation from ubiquitin-fusion proteins.

Unexpected, functional supporting evidence for compartmentalized antigen processing comes from studying the localization of specific class I/peptide complexes. Staining cells with reagents specific for Kb bound to SIINFEKL or SIYRYYGL, expressed by recombinant vaccinia viruses revealed that Kb molecules are expressed on the cell surface in clusters highly enriched in each peptide [122]. This is unlikely to be based on sorting postloading, as incubating cells with synthetic peptides results in colocalized staining. Peptide-specific clusters are stable for hours on the cell surface, and clusters are associated with increased T cell sensitivity, presumably by increasing interaction with clustered TCRs at the immune synapse. Cluster formation also occurs in VV-infected DCs and is associated with increased T cell sensitivity [123]. Class II molecules also present processed peptides in clusters [124], pointing to a possible common mechanism for enhancing immunosurveillance. Although Kb-peptide loading almost certainly occurs in the ER, complexes are first detected in the cis-Golgi complex, with different peptides already separated spatially.

These findings are consistent with the idea that peptides are generated and loaded from individual mRNAs to create homogeneous clusters that then traffic together through the secretory pathway. It is important to test the extent to which these findings extend to other peptides, class I molecules, viruses, and host gene products. A critical test of the model is to express two peptides in a single protein: if peptide generation is compartmentalized on the basis of the source mRNA, then class I complexes with the different peptides should colocalize.

**THE RIBOSOME: COMPLEX MACHINE FOR A COMPLEX TASK**

Imagine a single machine that can produce all man’s mechanical inventions from bicycles to aircraft carriers. Such is the ribosome, which creates virtually all of nature’s polypeptides. It is easy to predict that this universality entails a degree of specialization, and evidence is steadily mounting that the basic RNA machine (protein-free ribosomes are perfectly capable of protein synthesis [125]) is modified in myriad ways to increase the efficiency, according to the gene product synthesized.

The immunoribosome hypothesis extends the ribosome filter hypothesis, which posits that ribosomes are specialized to translate specific mRNA subsets [126, 127]. Specialized ribosomes are best defined in yeast, where many ribosomal proteins are encoded by multiple genes with nonredundant functions [128, 129]. In mammals, paralogues are exceptional, but when present, they are relevant, as evidenced by the selective effect of their deletion on development [130]. Ribosomal pseudogenes, common in mammals, are conserved and are actively transcribed [151], which argues for a nonpseudo status (the very term pseudo, like “junk”, as discussed below, is a barrier to discovery and underscores the many dangers of nameing). Clearly, we have just scratched the surface of how post-translational modifications of ribosomal proteins, such as ubiquitylation [132], as well as post-transcriptional modifications of ribosomal RNA and tRNAs [133], will modify the function and selectivity of ribosomes.

Ribosomes might also be specialized to modulate decoding of mRNAs. ARS are associated with ribosomes [134–136] and mRNAs [137]. ARS specificity can be modulated by cell stress to attach their cognate amino acids to noncanonical tRNA [20]. This has been termed “adaptive translation” [138], as it likely has a physiological function. David et al. [137] proposed that ARS are modified in a mRNA-specific manner to selectively modulate the amino acid composition of specific gene products. To enhance this process, ribosomes might recruit and retain a selective set of tRNAs, creating a self-contained translation unit [139, 140] that enables compartmentalized translation.

Given the central importance of translation in cell biology—their ancient status, as among the first RNA machines, and their sheer abundance (millions of copies/cell)—the ribosome must function as the central information hub in cells. The trickle of papers regarding the role of ribosomes in monitoring and regulating stress, energy metabolism, or cellular proliferation [141] precedes an incipient flood of information regarding the function of ribosomes in myriad cellular processes, not the least of which is generation of class I and class II [123]-restricted antigens.

**NUCLEAR TRANSLATION: WHO ORDERED THAT?**

Pioneering studies localizing incorporation of very brief pulses of radiolabeled amino acids by electron and light microscopy identified the nucleoplasm and particularly, the nucleolus as fractionally minor but clear sites of protein synthesis [142–
A classic study from Wu and Warner [146] showed that most rapidly labeled nuclear proteins are synthesized in the cytoplasm. Although the authors left open the possibility of nuclear synthesis representing up to 10% of total protein synthesis, dogma somehow quickly dictated that nuclei were devoid of protein synthesis.

Thirty years later, Iborra et al. [76, 147] resurrected the concept of nuclear translation, although their conclusions were disputed and largely dismissed [148, 149]. Almost 10 years later, Dolan et al. [82] found that blocking nuclear export of influenza A virus neuraminidase mRNA disproportionately spared peptide versus native protein expression and proposed that nuclear translation participates in immunosurveillance. David et al. [150] developed the RPM to identify translating ribosomes and found a robust signal in the nucleoplasm and nucleus. Although skepticism remains [151, 152], two recent studies provide strong support for nuclear translation.

With the extension of previous findings, localizing ribosomes with transcripts in drosophila genome [153], Brogna and colleagues [154] used split GFP constructs to detect elegantly assembled ribosomes exhibiting all of the hallmarks of translation (appropriate sensitivity and resistance to various protein synthesis inhibitors), which correlated with RPM staining. In a remarkable study, Apcher et al. [155] reported that OT-I T cells equally recognize cells expressing SIINFEKL from intron versus exon sequences in transfected genes. The shift of the reading frame of the intronic peptide nearly abrogated recognition, consistent with the idea that translation of the intron is based on initiation at the AUG of the standard ORF. Remarkably, the blocking of mRNA export from the nucleus in any of several clever ways increased K60-SIINFEKL generation from both exonic and intronic SIINFEKL, pointing to predominant peptide generation from nuclear translation of prespliced mRNA. To reinforce this conclusion, the blocking of mRNA splicing enhanced presentation from both constructs, which further provide direct evidence for synthesis of an intron-encoded reporter B cell epitope-tagged construct in the nucleolus by immunofluorescence. The intronic-encoded epitope colocalized with ribosomes using antibodies specific for a ribosomal subunit and with actively translating ribosomes identified by RPM.

Taken together, these findings strongly support the concept of nuclear translation and suggest further that nuclear ribosomes translate diverse classes of RNAs, depending on their location in the nucleolus and chromatin or nonchromatin-associated nucleoplasm. Most excitingly, it appears that nuclear ribosomes may play a major role in immunosurveillance of nuclear RNA and could represent one type of the long sought-after immunoribosome.

JUNK? REALLY?

The concept of wasted biological information (most notably, junk DNA) is naive and pragmatically disastrous, as it seriously discourages experimentation and consideration of potential functions. Nowhere is this clearer with class Ib molecules, originally deemed to be junk [156], but now, clearly more important to vertebrate biology than class Ia molecules.

As technology for detecting nucleic acids and polypeptides advances, it is clear that ribosomes are translating a wide variety of noncanonical RNAs. Indeed, even mitochondrial ribosomes synthesize a large amount of a peptide encoded by the very limited mitochondrial genome that is biologically active as an extracellular hormone [157]. The wide diversity of translation products poses a methodological problem for characterizing the immunopeptidome. Analysis is routinely based on protein databases to identify the sources of the masses identified, ignoring the products of noncanonical translation. The problem is exacerbated by adaptive coding, whereby the specificity of ARS is altered to increase the incorporation of methionine when viruses and other stressors increase ROS [20].

Noncanonical gene products include those encoded by uORFs, defined as upstream translation-initiation sites, typically out-of-frame with the canonical initiation AUG codon. There are ~20,000 transcripts with uORFs expressed in mammalian cells, and a major function is to interfere with the translation of the standard ORF [158]. Proteome analysis based on ribosome profiling has identified translation products from uORFs, although less frequently than expected, which may be a result of an expected, short half-life [159].

Mass spectrometric analysis of ~50 residue peptides reveals peptides encoded by short ORFs, including uORFs, as others within the coding DNA sequence and from the 3' untranslated region, as well as from “noncoding” RNAs [160]. The abundance-alternative ORFs were corroborated [161], supporting the idea that alternative ORFs provide unexpected complexity for immunosurveillance to exploit. Some peptides identified derive from initiation at a near-cognate non-AUG codon [159, 160]. The use of noncognate start codons has been documented extensively by the elegant studies of members of the Shastri laboratory [10, 116, 162], who in the process, have laid the groundwork for understanding the importance of such alternative translation.

DRiPs + RETIREES = 1

Whereas the evidence that DRiPs are a major source of antigenic peptides in cultured cells is nearly irrefutable, peptides also derive from the degradation of mature, native proteins. Despite claims to the contrary [163], this is not controversial. Peptide generation from native proteins was first clearly shown using influenza A virions to deliver internal virion proteins to the cytosol under conditions where viral proteins cannot be synthesized [33]. T cell recognition required viral fusion with cellular membranes, pointing to peptide derivation from native proteins, as virions rigorously select native proteins for incorporation (note that this is not necessarily true in a subsequent study that introduced purified OVA via osmotic loading, as non-native OVA was, no doubt, present in the preparation and furthermore, could have been created in endosomes) [164].

The generality of retiree contribution to peptide generation is best supported by the SILAC MS studies of the Admon lab [26, 27, 165]. Indeed, in its power to identify thousands of
peptides and relate the kinetics of protein versus peptide synthesis, this remains the best approach for weighing the contribution of peptides under various circumstances. The complications of peptide generation from DRiPs versus retirees are perfectly illustrated by studying the effects of γ-irradiation on peptide generation. Extensive remodeling of the stressed cells results in considerable peptide generation from retirees, and then a burst of DRiP-derived peptides from a large wave of mTOR induced translation [166].

Several recent studies have examined the relative contribution of retirees and DRiPs to peptide generation. Farfán-Arribas et al. [163] cleverly exploited intein catalysis to study peptides that can be created when the peptide-containing intein domain is properly folded. Based on the clear generation of peptides, they concluded that DRiPs play no role in peptide generation. Not to belabor the point, but as discussed in the previous paragraph, peptide generation from retirees is not in question (more about this below).

However, can the authors definitively conclude that DRiPs play no role in peptide generation from intein constructs? The clear fact that the intein must fold to generate the peptide does not guarantee that the protein is native. It is well-known that misfolding of individual protein domains of proteins leads to the degradation of the entire protein. Indeed, this is the basis of drug-controlled degron systems pioneered by the Wandless lab [167]. Likely as a result of autoproteolysis-associated fluorophore formation [168], a significant fraction of nascent GFP is converted to a detergent-insoluble DRiP within minutes of its synthesis [48]. Examination of the immunoblots of the intein constructs reveals a large number of lower relative molecular mass species, many of which likely derive from misfolded, cleaved GFP.

The efficiency of peptide generation from retirees, believed to be very low [169], was re-examined recently using the Wandless system [167], in which fusion protein stability (in this case, SIINFEKL-GFP) is controlled by the stabilization of genetically destabilized FK506 binding protein by the cell-permeant drug Shield-1. By expressing the target protein (SCRAP) in the presence of the Shield-1, a native protein pool can be generated that is rapidly degraded by proteasomes when the drug is removed [92]. The enormous decrease in peptide generation from SCRAP upon CHX addition in the presence of Shield-1 reveals the predominant contribution of DRiPs to the system when SCRAP is stabilized. Peptide generation from DRiP versus retired SCRAP exhibited differential sensitivity to several drugs, demonstrating the potential diversity of pathways used for antigen processing of the same gene product.

The real surprise came in calculating the efficiency of peptide generation from retired SCRAP. As a result of the unknown biochemical nature of DRiPs, the efficiency of peptide generation cannot be determined with any accuracy but has been estimated to be approximately one complex/every 2000 proteins degraded (0.05%) [46]. With SCRAP, however, the source of antigen is not in question, as the full-length, mature protein that is sensitive to Shield-1 removal is quantitated easily by immunoblotting or flow cytometry. This revealed that SCRAP, synthesized from a transfected gene, is converted into Kb-SIINFEKL complexes with an efficiency of ~2% or 40-fold higher than the estimated DRiP efficiency [170].

Another surprise is that when the identical SCRAP gene was expressed by VV, the efficiency of Kb-SIINFEKL generation from retirees was now tenfold lower (0.2%), much more in line with estimates of VV-encoded DRiP efficiency. This could not be explained by a VV-induced reduction in retired SCRAP efficiency, which only played a minor role. Thus, the class I antigen-processing machinery can distinguish folded proteins based on the precise details of their synthesis to modulate antigen-presentation efficiency.

Although the 2% efficiency of retired SCRAP processing is in good agreement with the seminal studies of Pamer and colleagues [171, 172] of bacterial proteins naturally delivered to the cytosol during Listeria infection, they are incompatible with the calculated macroeconomics of peptide generation from the total cellular retiree pool [45, 46, 169]. Although SCRAP retiree processing efficiency might be artificially enhanced by the absence of competition from DRiPs (cells are treated with CHX to limit presentation to retirees), these findings suggest that retirees, or at least a subclass of retirees, might be processed at high efficiency in nonstressed cells.

THE RIBOSOME: TRADUTTORE, TRADITORE

The classic Italian adage playfully refers to the impossibility of perfect translation between languages: the translator is bound to mangle the original message, at least a little. The original DRiP hypothesis was based on this premise: application of Murphy’s law (everything that can go wrong, will go wrong) to protein synthesis predicts that there will inevitably be errors in each step of the process of translating nucleic acid-based information into fully functional proteins. The updated DRiP hypothesis extends this idea to deliberate errors, e.g., immunoribosomes translating RNAs to make peptides deliberately. The more directed synthesis of DRiPs seems to be essential for tumor immunosurveillance, which is based on detection of peptides, whose source gene products often defy detection, findings pioneered by Boon and colleagues [173] and elegantly extended by the recent studies of Apcher, Fährnaus, and colleagues [78, 155]. Immunoribosomes likely also contribute to detection of viruses, but Murphy’s law is likely more relevant for viruses that hijack most of the protein synthetic capacity of cells. Under these circumstances, finding defective nascent viral proteins is shooting fish in a barrel as a result of the sheer abundance of viral protein synthesis.

Finally, it is important to consider the implications of recent advances in DRiPology for thymic selection, a process by which T cells are programmed or eliminated to avoid autoimmune recognition of the myriad peptide generated by nonimmune privileged tissues. The transcription factor Aire plays a critical role in expressing the RNA sources of tissue-specific peptides relevant for negative selection [174]. Essentially, nothing is known about how Aire manages this trick or for that matter, how thymic-specific proteasome β5 subunit contributes to this process, as it surely does [175]. It is a safe bet that many forms of alternative translation are in play, as this would avoid thy-
mic epithelial cells from synthesizing large amounts of tissue-specific gene products that are useless and costly at best and highly toxic at worst. At the very least, if noncanonical translation is an abundant source of peptides, as the robust evidence suggests, then the thymus must play along.

Given the attention afforded translational immunology research, now is the time to connect protein translation to antigen processing and other aspects of immune cell function to reveal novel aspects of translation and vice versa.

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Translating DRiPs: MHC class I immunosurveillance of pathogens and tumors

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