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The DRiP hypothesis decennial: support, controversy, refinement and extension

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In 1996, to explain the rapid presentation of viral proteins to CD8$^+$ T cells, it was proposed that peptides presented by MHC class I molecules derive from defective ribosomal products (DRiPs), presumed to be polypeptides arising from in-frame translation that fail to achieve native structure owing to inevitable imperfections in transcription, translation, post-translational modifications or protein folding. Here, we consider findings that address the DRiP hypothesis, and extend the hypothesis by proposing that cells possess specialized machinery, possibly in the form of 'immunoribosomes', to couple protein synthesis to antigen presentation.

The kinetic birth of a hypothesis

> *'Not everything that can be counted counts, and not everything that counts can be counted'* (Albert Einstein).

In jawed vertebrates, CD8$^+$ T cells function to eradicate tumor cells and cells harboring intracellular pathogens. CD8$^+$ T cells recognize MHC class I molecules carrying oligopeptides, typically 8–10 residues long. The recognition of foreign peptides in lymphoid organs activates resting naive CD8$^+$ T cells that rapidly proliferate, synthesize effector molecules and traffic to inflamed organs, where they mediate anti-viral or anti-tumor effector functions.

Individual cells express $\sim 10^4$–10^5 MHC class I molecules, which constitutively present peptides derived from the proteolytic degradation of the polypeptide gene products expressed by cells. Proteins and polypeptides exhibit a wide range of degradation rates: half-lives range from minutes to weeks, with an overall average half-life of 1–2 days [1]. This overall degradation rate would be suitable for maintaining CD8$^+$ T-cell self-tolerance and surveillance of abnormal tumor cell peptides. However, these leisurely degradation rates are poorly suited to detecting virus infections. Even though viral proteins are frequently translated at extremely high levels compared with even the most abundant cellular proteins, MHC class I molecules are faced with a sampling problem. Because viral proteins are generally at least as stable as the average cellular protein, it would take many hours (or days) to accumulate a viral protein pool sufficiently large for viral peptides to successfully compete with the $\sim 3 \times 10^9$ proteins that constitute individual cells. The immune system cannot wait this long. Many viruses have replication times of 6–12 h, so speedy recognition is essential for CD8$^+$ T cells to kill infected cells before progeny viruses are released. Kinetic assays of antigen presentation indicate that MHC class I–viral-peptide complexes can reach sufficient levels to trigger anti-viral CD8$^+$ T-cell activation within one hour of adding rhabdoviruses or influenza viruses to cells [2,3]. This is remarkable, particularly for these negative-stranded RNA viruses, which must enter cells, synthesize a positive strand of mRNA and translate the protein sources of antigenic peptides. Importantly, these rapidly generated MHC class I ligands derive from proteins that are at least as stable as typical cellular proteins, that is, viral proteins with half-lives of the order of days.

To explain the rapidity of viral-antigen presentation from stable viral proteins, Yewdell *et al.* proposed that antigenic peptides originate from defective ribosomal products (DRiPs), defective forms of gene products that are degraded more rapidly than the standard, functional product [4]. In other words, proteins have multiple half-lives: the standard half-life measured for native, functional proteins, and a shorter half-life for each alternative truncated, full length, or extended form that falls short of achieving a stable conformation.

The DRiP hypothesis: pro and con(troversy)

The original experimental evidence supporting the DRiP hypothesis pre-dates it. Studies in the 1980s identified viral membrane proteins to be robust antigens for anti-viral CD8$^+$ T cells [5–7], and it is now apparent that cell-surface proteins, and even secreted proteins (such as ovalbumin and, more naturally, the vaccinia virus B8R protein [8]) are equally efficient and probable sources of antigenic peptides as cytosolic or nuclear proteins. Although there are the inevitable exceptions, the processing of these proteins typically occurs in the cytosol, as demonstrated by their dependence on proteasomal degradation and transport into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) [9,10]. Because the slow turnover of the membrane-bound or secreted proteome typically occurs either extracellularly or in endosomal compartments, antigenic peptides must originate from DRiPs in these conditions. The pathways

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functioning to export protein translocation substrates to the cytosol for proteasomal degradation [11] provide an explanation for the entry of these DRiPs into the MHC class I antigen-processing pathway.

The initial prospective studies to address the DRiP hypothesis were published simultaneously [12,13]. Reits *et al.* [12] devised an ingenious assay to measure antigenic peptide production, in which fluorescence recovery after photobleaching (FRAP) measurements of TAP mobility in the ER membrane are a surrogate measure of peptide transport activity. They established that TAP mobility is proportional to the supply of cytosolic peptides, and that blocking protein synthesis with cycloheximide (CHX) reduces peptide supply to the same extent as proteasome inhibition. In these studies, TAP-transportable peptides were completely depleted 20–35 min after the addition of CHX. Considering that peptides can be degraded with a half-life of <10 s [14], these findings imply that TAP-transportable peptides derive from a pool of substrates degraded with a half-life of ~10 min (3 half-lives to achieve a 90% reduction in TAP-transportable peptides).

Consistent with this finding, Schubert *et al.* [13] found that CHX treatment immediately slowed the export of newly synthesized MHC class I molecules from the ER. In addition, they showed that incubating the cells with proteasome inhibitors increased the recovery of radiolabeled proteins by 25% to 50%, depending on cell type and the conditions used for 2-minute pulse radiolabeling with [^{35}S]Met. They concluded that the excess radiolabel recovered represented rapidly degraded proteins. Subsequent studies established that in cells radiolabeled for 5 min with [^{35}S]Met or [^{3}H]Leu in the absence of proteasome inhibitors, ~20% of proteins are degraded with an overall half-life of <10 min [15–17]. Recognizing that the relative contributions of short-lived proteins and acid-insoluble peptides (e.g. cleaved NH_2-terminal targeting sequences) versus DRiPs to this pool is uncertain, Qian *et al.* [17] considered it best to refer to the pool in literal terms, as rapidly degraded polypeptides (RDPs), distinct from polypeptides degraded with the classical 1–2 day half-life (i.e. slowly degraded polypeptides, SDPs) (Figure 1).

Vabulas and Hartl recently re-examined the fraction of RDPs in nascent proteins, reporting that proteasome inhibitors failed to increase the recovery of newly synthesized radiolabeled proteins that accumulate in cultured cells subjected to continuous labeling conditions for 2–10 min unless cells are pre-starved of the amino acid used for radiolabeling [18]. Under starvation conditions, proteasomes perform a recycling function to supply the amino acid excluded from the media. Vabulas and Hartl concluded that the majority of the putative radiolabeled RDPs reported by Schubert *et al.* [13] were not RDPs but simply the result of increased specific activity of labeled proteins, arising through proteasome-inhibitor-induced increases in aminoacyl-transfer (t)RNA-specific activity of the starved amino acid.

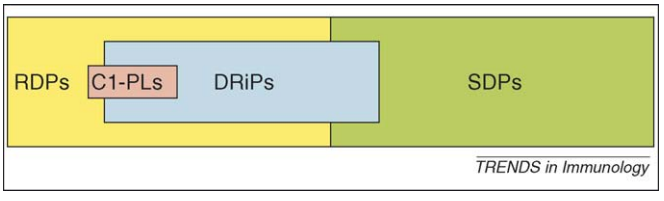

Figure 1. Intracellular polypeptide degradation pools. Polypeptides segregate into two general pools: those degraded with an average half-life of ~10 min (RDPs) and those degraded with an average half-life of ~2000 min (SDPs). Proteins in the SDP pool exhibit a wide range of half-lives, from hours to weeks. Experimentally defined DRiPs belong to the RDP pool, for example, green fluorescent protein (GFP) DRiPs [17], or the SDP pool, for example, canavanyl nucleoprotein-SIINKFEKL-GFP [25], although it seems likely that most naturally generated DRiPs belong to the RDP pool. MHC class I-peptide ligands (C1-PL) seem to be predominantly derived from substrates in the RDP pool. These substrates include DRiPs (e.g. peptides derived from metabolically stable viral and cellular gene products) and cleaved leader sequences (peptides derived from NH_2-terminal ER-targeting sequences).

This explanation could account for the large difference in [^{35}S]Met-labelled RDPs (50% versus 25% of nascent proteins) associated with prior Met starvation noted by Schubert *et al.* [13]. However, Schubert *et al.* simultaneously demonstrated that: (i) pre-incubation in Met-deficient media is not required to detect a high fraction of radiolabeled RDPs (30% RDPs in lymph node cells); (ii) following cell fractionation, RDPs are almost exclusively detected in the insoluble fraction; and (iii) RDPs are enriched in proteins migrating with a high M_r in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

If Vabulas and Hartl's interpretation is generally correct, it would be necessary to postulate that, in the conditions used by Schubert *et al.* [13], cells possess distinct tRNA pools with different specific activities, presumably owing to their unequal access to proteasome-dependent amino-acid recycling. This would require limitations on the free diffusion of tRNAs and amino acids in cells. Although this is generally assumed not to be the case, Stapulionis and Deutscher have published careful studies leading them to propose that 'endogenous tRNA is never free of the protein synthetic machinery at any stage of the translation process and, consequently, that there is a channeled tRNA cycle during protein synthesis in mammalian cells' [19]. Furthermore, Wheatley and Inglis provided evidence that amino acids pools are highly complex, with amino acids existing in freely diffusible and macromolecular-bound forms [20]. (At the end of this article, we return to an interesting twist with regard to the possible intersection between tRNA and DRiPs.)

But, are Vabulas and Hartl correct in concluding that, at most, only 'a few percent of total protein was rapidly degraded immediately upon translation' [18]? Data presented in the same paper document that, using standard pulse-chase techniques, 20% of newly synthesized proteins are degraded within the first 30 min (not 60 min as stated in the text) following 10 min of pulse radiolabeling. These data are remarkably similar to the pulse-chase data of Princiotta *et al.* [15] and Qian *et al.* [17], which, in turn, reproduce the 25-year-old findings of Wheatley and colleagues [21,22]. Importantly, Wheatley *et al.* showed that the RDP fraction increased with decreasing pulse-labeling times; thus, the RDP fraction increased from 11% to 29% to 38% with decreasing labeling times of 30 min, 5 min, and 1 min [22]. Because the average protein takes ~100 s to synthesize (5 residues per second, 500 residues per protein), 1 min of labeling represents something approaching 'flash' labeling and should provide the most