by the only known cytoplasmic palmitoyl protein thioesterase APT1, an enzyme that has broad substrate specificity but no global defect in ionomycin-treated T cells. However, it seems likely that an accessory enzyme is upregulated in response to ionomycin treatment that contributes to the selective inhibition of palmitoylation and resultant hypophosphorylation of LAT. This hypothesis is supported by the fact that LAT hypophosphorylation is titratable with ionomycin and that a small population of LAT in ionomycin-anergized T cells does localize to the DRMs but is not phosphorylated. This unidentified LAT inhibitor could prevent LAT palmitoylation by binding to the cysteine residues or by facilitating the degradation of newly palmitoylated LAT. E3 ligases can discriminate substrates based upon complex posttranslational modifications such as glycosylation or phosphorylation, and LAT has recently been reported to be a target for ubiquitination (Brignatz et al., 2005).

In conclusion, Hundt et al. (2006) have presented new data suggesting in multiple model systems that defective LAT activation is associated with T cell anergy. Although the exact mechanism for this inhibition is still unclear, further work will no doubt clarify the role of LAT in determining the balance between T cell activation or anergy induction.

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Upstream toward the "DRiP"-ing Source of the MHC Class I Pathway

MHC class I binding peptides are generated via cytosolic degradation of a previously undefined substrate. In this issue of *Immunity*, Kunisawa and Shastri (2006) isolate pre-degradation polypeptide intermediates bound to a cytosolic chaperone.

Around 150 years ago, intrepid explorers trekked through the African hinterland to seek the elusive source of the mighty Nile River. After many painful years of hacking their way through previously unexplored jungle, these explorers discovered a source of the river upon which millions of African lives depend. In modern days great wilderness adventurers are few and far between, but within the cell, the search for the source of an intracellular river of peptides, upon which millions of lives also depend, is underway. In this case the "river" is the MHC class I processing pathway, at the mouth of which peptide-MHC class I complexes are presented on the surface of the cell to $CD8^+$ T cells. In this issue of *Immunity*, Kunisawa and Shastri describe their trek upstream and have made substantial progress toward the discovery of a source of peptides in the MHC class I pathway.

Since the original discovery that 9–11 amino acid peptides complexed to MHC class I were the substrate required for $CD8^+$ T cell activation (Townsend et al., 1986), the source of these peptides has generated intense investigation. Peptides complexed to MHC class I can be generated from endogenous or exogenous protein sources in processes referred to as direct presentation or cross-presentation, respectively. Our focus here will be the direct-presentation pathway, in which peptides are generated from an endogenous antigen source. Knowledge of the protein source of peptides in the MHC class I pathway could eventually lead to the development of vaccines that would allow more efficient generation of peptide-MHC class I complexes. The increased numbers of peptide-MHC class I complexes could then exceed the threshold required to trigger low-affinity $CD8^+$ T cells that are not tolerized during exposure to substantial tumor burden or persistent viral infection, providing a potential means of immunotherapy. In addition to this therapeutic potential, two cell-biological observations revealed during ongoing investigations of the MHC class I processing pathway have intrigued researchers. The first observation was that it was not possible to isolate or detect a peptide presented by a specific MHC class I molecule by biochemical means unless the cells examined expressed that MHC class I molecule (Falk et al., 1990). This could lead to the conclusion that MHC class I molecules are actively involved in the generation of the peptides to which they bind. However, Reits et al. demonstrated that cytosolic peptides have a very short half life (Reits et al.,

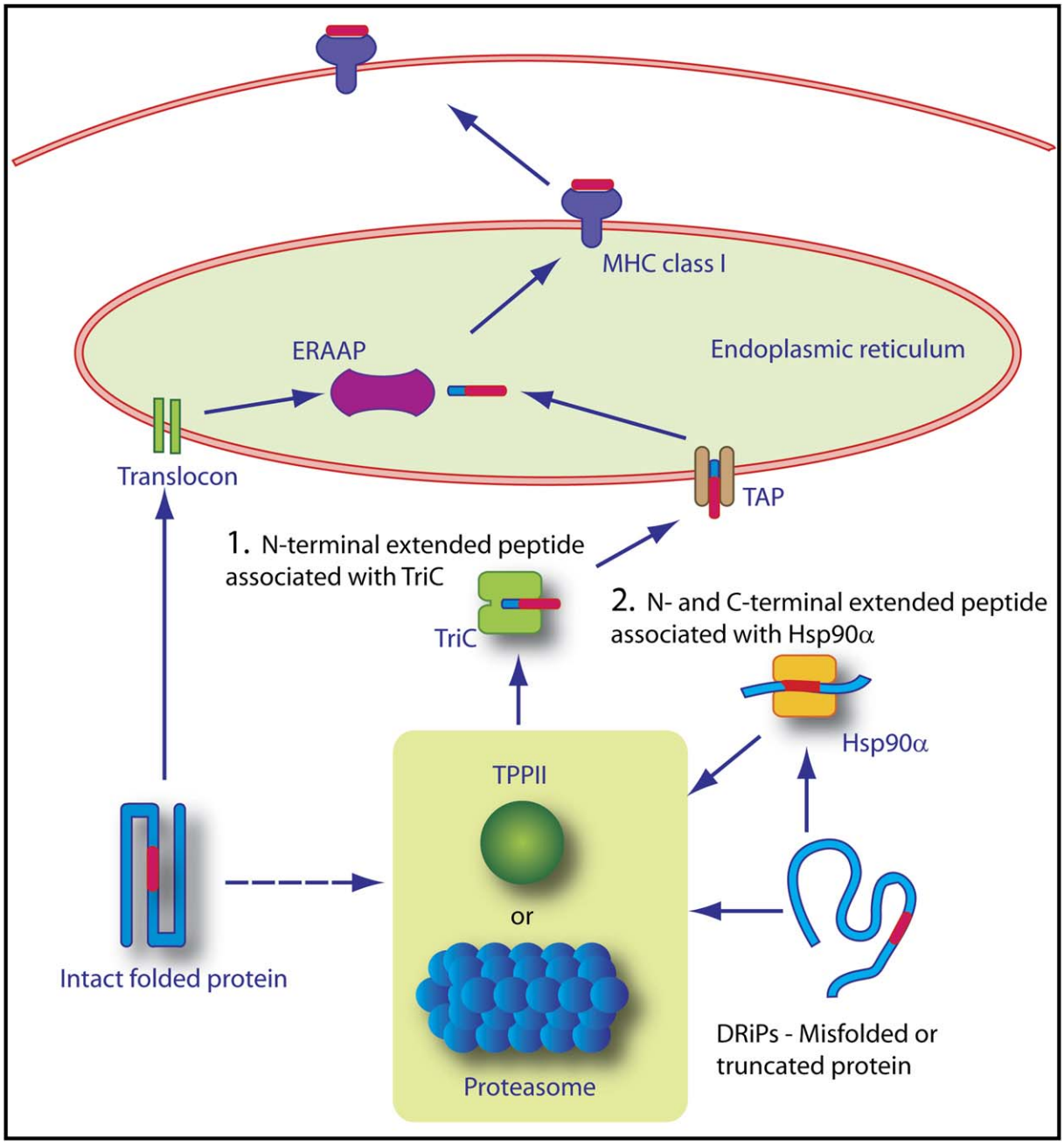

Figure 1. Sources of Endogenous Peptides in MHC Class I Presentation Pathway

MHC class I bound peptides can be derived from two distinct sources in the direct-presentation pathway. Intact protein can be translocated into the endoplasmic reticulum, and peptides can be cleaved from lumenal termini of these proteins and then bind to newly synthesized MHC class I. Folded intact proteins may also be degraded by the proteasome to produce peptides, although this process is inefficient. Alternatively misfolded or truncated proteins may be rapidly degraded by the proteasome or TPPII. Prior to degradation, N- and C-terminally extended peptides associate with Hsp90α. After degradation, N-terminally extended peptides associate with the chaperone TriC and are then transported into the ER via TAP. Once in the ER, final trimming to the antigenic determinant occurs from the N terminus and is mediated by ERAAP. Peptide then binds to MHC class I and transits to the cell surface. Shastri and colleagues have been instrumental in defining both post-degradation (1) and pre-degradation (2) intermediates in this pathway.

2003), and this may only be prolonged after stabilization by either MHC class I molecules or molecular chaperones. A second observation was that efficient presentation of peptides derived from viral proteins could be detected by $CD8^+$ T cells rapidly after virus infection. At the same time point after infection, however, expression of the viral protein from which the peptides were derived was barely detectable by biochemical means, and total amounts of virus-derived protein constituted only a minute fraction of the total pool of cellular proteins (Esquivel et al., 1992). Thus, the amounts of antigen within a cell are not always related to the amounts of peptide-MHC complexes on the cell surface. Taken together, these data lead to two conclusions. First, the

MHC class I pathway is highly specialized to allow efficient presentation of foreign peptides; second, a purely biochemical methodology is unlikely to approach the exquisite sensitivity of naturally selected $CD8^+$ T cells when used to detect physiologically relevant concentrations of peptides or peptide intermediates.

From the discovery that peptides bind to MHC class I, researchers moved upstream toward the source of these peptides by demonstrating that peptides bound in the lumen of the endoplasmic reticulum (ER) and reached this site primarily after transport via the heterodimeric transporter of antigen processing (TAP). Before transport to the ER, minimal peptide determinants that bind to MHC class I are generated by the multicatalytic proteasome and other cytosolic proteases, such as tripeptidyl peptidase. In addition, cytosolic proteases can generate peptides that are extended at the N terminus and more stable than smaller peptides; they can also be transported into the ER by TAP and then further cleaved to the minimal peptide prior to binding to MHC class I. Although cytosolic cleavage could be demonstrated, however, it was still not possible to isolate post-proteolytic peptides in the absence of the MHC class I molecule to which they bind, indicating a discontinuity in the trek upstream. In addition, a further break in the trail stemmed from the inability to identify proteins that were substrates for cytosolic cleavage, and from which peptides could be generated rapidly in the absence of protein accumulation.

A theory, therefore, was proposed that a rapidly degraded subset of proteins that do not accumulate intracellularly was the major source of peptides in the MHC class I pathway. These Defective Ribosomal Products (DRiPs) are proteins targeted for degradation as a result of premature termination or misfolding and could account for the generation of substantial quantities of peptide-MHC complexes in the absence of detectable accumulation of protein antigen (Yewdell et al., 1996). Experimental evidence verified that DRiPs do exist in vitro and in vivo (Schubert et al., 2000). Although increased degradation, premature degradation, or misfolding can increase peptide presentation, there is currently no well-defined mechanistic link between the generation of DRiPs and peptide-MHC class I complex formation. Indeed, the insoluble characteristics of DRiPs have precluded isolation of individual antigenic species, indicating the inherent difficulties associated with a purely biochemical approach.

In 1999, the Shastri laboratory developed the technology to detect proteolytic substrates in the MHC class I pathway. By altering the sequence of a model antigen, chicken egg ovalbumin (OVA), to allow liberation of the minimal antigenic peptide (SIINFEKL ($Ova_{257–264}$)) from longer polypeptides after cleavage by trypsin and carboxypeptidase B, they could use a sensitive T cell assay to detect proteolytic intermediates (Paz et al., 1999). Initially, Kunisawa and Shastri utilized this approach to isolate post-proteasomal N-terminally extended peptides bound to the Group II chaperonin TRiC (Kunisawa and Shastri, 2003). Although no direct linkage between TRiC and TAP has been demonstrated, this observation indicated that N-terminally extended peptides may be protected from degradation in the cytosol to increase the efficiency of presentation. In the current manuscript (Kunisawa & Shastri, 2006), they have now expanded upon this work to isolate pre-proteasomal intermediates that are extended from the minimal SIINFEKL determinant at both the N and C termini. A substantial portion of intermediates, which associated with the cytosolic chaperone hsp90α, but not hsp90β, were degraded by the proteasome; a greater variety of pre-proteasomal intermediates were isolated after inhibition of proteasomal function (Figure 1). In addition, inhibition of hsp90α either with geldanamycin or via knockdown with interfering RNAs reduced antigen presentation to T cells. Finally, the knockdown of the co-chaperone CHIP (carboxyl terminus of Hsc70-interacting protein), a protein known to complex with hsp90 and facilitate transfer of bound substrates to the ubiquitin-proteasome-mediated degradation pathway, also reduced antigen presentation.

Taken together, these observations move us further toward the source of the MHC class I binding pathway. However, they also raise a number of questions. Are the pre-proteasomal intermediates found here DRiPs? Is degradation required to generate these intermediates, or are they premature truncation products? How prevalent is the requirement for association with hsp90α and CHIP in MHC class I-mediated antigen presentation? Molecular chaperones such as gp96 and hsp70 have been touted as players in the MHC class I presentation pathway, but primarily at the level of binding of proteasomal products. The evidence that these chaperones bind short peptides under physiological conditions, or at relevant concentrations, is controversial. However, the observation that antigen presentation in the presence of inhibitors of hsp90α and CHIP is reduced, but not ablated, indicates that other chaperones may compensate for the knockdown of these molecules.

The work of Kunisawa and Shastri has taken us further up the river toward the source of peptide in the MHC class I pathway, but has it taken us toward the only source of peptide? SIINFEKL peptide can be presented, albeit at a much reduced efficiency in comparison to endogenously synthesized antigen, after the placement of intact OVA into the cytosol of cells by electroporation or osmotic lysis of pinosomes. Thus, under some circumstances, long-lived intact protein can be made available to the MHC class I processing pathway, indicating that a number of alternate antigen depots may exist for use by the MHC class I pathway. Thus, although the source of the MHC class I pathway may be close at hand, it will be reassuring for intracellular explorers that many tributaries of the peptide river remain to be explored in the future.

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Unraveling the Pros and Cons of Interferon-γ Gene Regulation

Although transforming growth factor-β (TGF-β) inhibition of interferon-γ (IFN-γ) expression has been known for some time, in this issue of *Immunity*, Yu et al. (2006) are the first to detail the crosstalk between proinflammatory cytokine and TGF-β signaling that regulates IFN-γ expression.

The regulation of interferon-γ (IFN-γ) gene expression is a complex process because multiple signals converge to control both mRNA expression and posttranscriptional protein expression. Whereas many different cell types have been reported to express IFN-γ, the most important sources for IFN-γ are T cells (both $CD4^+$ and $CD8^+$), NKT cells, and NK cells. Regulation of IFN-γ expression in T cells has been the subject of investigation by many labs, but its expression in NK cells has been less widely studied, in large part due to the difficulty in transfecting these cells or infecting them with retroviral or lentiviral vectors. Induction of IFN-γ expression in NK cells occurs through many different types of stimulation, including cross-linking of cell-surface receptors and stimulation with cytokines, including interleukin-2 (IL-2), IL-12, and IL-18. Different regulatory regions of the IFN-γ locus have been identified and numerous transcription factors have been implicated in the activation of IFN-γ transcription, thus making the understanding of how this gene is transcriptionally regulated complex, as the model is constantly changing.

Inhibition of IFN-γ gene expression has been less widely studied. It has been known for some time that TGF-β inhibits IFN-γ expression by NK cells (Bellone et al., 1995; Hunter et al., 1995; Ye et al., 1995). Furthermore, a major phenotype resulting from the development of TGF-β-deficient mice was massive overexpression of IFN-γ (Shull et al., 1992). However, until now, the molecular mechanisms by which TGF-β inhibits IFN-γ expression have not been elucidated.

As deciphered by Yu et al. in this issue of *Immunity* (Yu et al., 2006), there is an important balance between the cytokines IL-12 and IL-18, and TGF-β in NK cells. When NK cells are treated with both IL-12 and IL-18, a tremendous synergy with respect to IFN-γ gene expression is observed (Okamura et al., 1998). A molecular basis for this synergy is that IL-12 treatment leads to the activation of the STAT4 transcription factor, which strongly increased the recruitment of the IL-18-induced transcription factor AP-1 to the IFN-γ promoter (Nakahira et al., 2002). There is also reciprocal upregulation of the receptors for both IL-12 and IL-18, thus making the cells even more responsive to these stimuli. What Yu et al. (2006) now show is that the IL-12 and IL-18 treatment also downregulates the ability of the NK cell to respond to TGF-β (Figure 1). This process occurs by both the downregulation of the mRNA and surface expression of the TGF-β type II receptor (TGF-βRII) expression over time and the downregulation of SMAD2 mRNA. The SMAD family of transcription factors is critical for TGF-β signaling, so downregulation of these factors will decrease the cells' ability to respond to TGF-β. Additionally, SMAD3 protein, but not SMAD3 mRNA, was also downregulated by the IL-12 and IL-18 treatment. Of particular interest is the observation that the overall downregulation of these signaling molecules occurs under conditions of maximum IFN-γ induction. Although TGF-βRI and SMAD4 protein levels were not affected, the inhibition of TGF-βRII and SMAD2 and SMAD3 resulted in a decreased responsiveness to TGF-β. Thus, as the cells are gearing up to maximally produce IFN-γ, their ability to respond to signals that would dampen this response is weakened.

Next, the authors directly examined how TGF-β inhibits IFN-γ mRNA expression. They focused on two targets to answer this question. The first target was T-bet, a gene shown to be required for IFN-γ expression in $CD4^+$ T cells and NK cells (Szabo et al., 2002), and the second target was the IFN-γ gene itself (Figure 1). The authors clearly demonstrate that there is a direct downregulation of T-bet expression upon TGF-β treatment and that this downregulation can be mediated through SMAD protein interaction with the T-bet promoter (Figure 1). This effect was maximized when SMAD3 and SMAD4 were used in combination, whereas SMAD2 seemed to have little impact on T-bet promoter activity. Interestingly, Yu et al. (2006) could not overexpress SMAD4 in NK cells, and although SMAD3 decreased T-bet in these cells, the effects were observed only in the presence of TGF-β, suggesting that additional TGF-β-induced factors or protein modifications (e.g., phosphorylation) are required. The role of T-bet in IFN-γ regulation has become controversial, as a recent paper (Usui et al., 2006) showed that in T cells, T-bet is not directly required for IFN-γ expression. Instead T-bet was shown to inhibit GATA-3, a transcription factor that inhibits IFN-γ expression. However, as shown in this manuscript, T-bet directly upregulates activity of a truncated IFN-γ promoter (−204), and