Targeting the human immunodeficiency virus type-1 Gag protein into the defective ribosomal product pathway enhances its MHC class I antigen presentation

Die Bedeutung fehlerhafter ribosomaler Produkte für die MHC Klasse I Antigenpräsentation des humanen Immundefizienzvirus-1 Strukturproteins Gag

Der Naturwissenschaftlichen Fakultät der Friedrich-Alexander-Universität Erlangen-Nürnberg

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1 Abstract

The major source for endogenous peptides presented via the major histocompatibility complex class-I (MHC-I) pathway are de novo synthesized, dysfunctional proteins, named defective ribosomal products (DRiPs), which are degraded in concert with or shortly after their synthesis by the ubiquitin proteasome system (UPS).

The human immunodeficiency virus type 1 (HIV-1) Gag polyprotein, a bona fide substrate of the DRiP-pathway, was chosen as a model antigen to more precisely understand the relevance of erroneous protein synthesis for the generation of MHC-I-presented peptides. To target Gag into the DRiP-pathway, various degradation signals have been introduced into Gag, and their effects on its protein synthesis, metabolic half-life, DRiP-formation as well as subcellular localization and the release of virus like particles have been investigated. As an indicator for antigen processing, the ovalbumin-derived SIINFEKL (SL) epitope was introduced into Gag expressed from a codon-optimized gag gene (syngag). It was demonstrated that exchange of the N-terminal Met residue for Arg (RGag), a destabilizing amino acid according to the N-end rule, directed Gag more efficiently into the DRiP-pathway in murine EL4 cell lines. This correlated with enhanced MHC-I antigen presentation as well as more efficient CD8\(^+\) T-cell activation in vitro and in vivo. The enhanced MHC-I presentation of SL derived from RGag in murine cells could be reproduced in a human cell line. Furthermore, stable fusion to ubiquitin (Ub), converting Gag into a substrate for the Ub fusion degradation (UFD) pathway, was even more efficient in targeting Gag into the MHC-I pathway.

The PTAP late (L)-domain motif in the p6 domain of HIV-1 Gag plays an essential role during late stages of budding and has been recently implicated in the control of Gag ubiquitination. Mutations of PTAP in the context of syngag- or HIV-1-encoded Gag increased the ubiquitination as well as the DRiP-rate of Gag and enhanced the MHC-I presentation of the Gag-derived SL epitope. This novel function of the PTAP L-domain as a naturally occurring motif that regulates the DRiP-rate of Gag might be mediated by the sequence-specific recruitment of cellular factors, most likely components of the UPS.

Altogether, the results presented in this study further underline the role of the DRiP-pathway in adaptive immunity and provide strategies to enhance the MHC-I antigen presentation of HIV-1 Gag and other antigens. It remains to be elucidated by studies performed in vivo whether such approaches may help to improve vaccination strategies.
2 Zusammenfassung

Die Hauptquelle für endogene Peptide, die von MHC Klasse I (MHC-I) Molekülen präsentiert werden sind Fehlprodukte der Proteinbiosynthese, sogenannte defekte ribosomale Produkte (DRiPs), die noch während oder kurz nach ihrer Synthese durch das Ubiquitin-Proteasom-System (UPS) abgebaut werden.


# 3 List of abbreviations

Standard three letter abbreviations are used for amino acids.

<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Term</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>AAA</td>
<td>ATPase associated with various cellular activities</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ALG2</td>
<td>Apoptosis-Linked Gene 2</td>
</tr>
<tr>
<td>ALIX</td>
<td>ALG2 interacting protein X</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
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<td>antigen presenting cell</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
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<tr>
<td>Ate</td>
<td>arginyl-tRNA-protein transferase</td>
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<tr>
<td>β2m</td>
<td>beta2-microglobulin</td>
</tr>
<tr>
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<td>bicinecinonic acid</td>
</tr>
<tr>
<td>β-Gal</td>
<td>β-Galactosidase</td>
</tr>
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<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>capsid</td>
</tr>
<tr>
<td>CCR</td>
<td>CC motif chemokine receptor</td>
</tr>
<tr>
<td>CCT</td>
<td>chaperonin containing TCP-1</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluoresceine succinimidyl ester</td>
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<tr>
<td>CHMP</td>
<td>charged MVB proteins</td>
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<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
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<td>core particle</td>
</tr>
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<td>C-X-C chemokine receptor type</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>DIAP1</td>
<td>Drosophila inhibitor of apoptosis</td>
</tr>
<tr>
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<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>deoxyribonucleic acid</td>
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<tr>
<td>dpi</td>
<td>day postinfection</td>
</tr>
<tr>
<td>DUB</td>
<td>deubiquitinating enzyme</td>
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<td>dithiothreitol</td>
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<td>Epstein-Barr virus</td>
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<td>ECL</td>
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<td>equine infectious anemia virus</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>ELISPOT</td>
<td>Enzyme linked immunospot technique</td>
</tr>
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<td>Env</td>
<td>Envelope</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAAP</td>
<td>ER aminopeptidase associated with Ag processing</td>
</tr>
<tr>
<td>ERAD</td>
<td>endoplasmic reticulum-associated degradation</td>
</tr>
<tr>
<td>ESCRT</td>
<td>endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<td>feline immunodeficiency virus</td>
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<tr>
<td>Gag</td>
<td>group specific antigen</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HAART</td>
<td>highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HECT</td>
<td>Homologous to E6-associated protein C-terminus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HTLV</td>
<td>human T cell leukemia virus</td>
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<tr>
<td>IAV</td>
<td>influenza A virus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Int</td>
<td>Integrase</td>
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<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>JAMM</td>
<td>Jab1/MPN metalloenzyme</td>
</tr>
<tr>
<td>kbp</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>LC</td>
<td>lactacystin</td>
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<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
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<tr>
<td>L-domain</td>
<td>late domain</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
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<tr>
<td>LTR</td>
<td>long terminal repeat</td>
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<tr>
<td>MA</td>
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<td>MetAP</td>
<td>Methionine aminopeptidase</td>
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<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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<tr>
<td>MHC-I</td>
<td>major histocompatibility complex class I</td>
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<tr>
<td>MHR</td>
<td>major homology region</td>
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<td>MJD</td>
<td>Machado-Joseph domain</td>
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<td>MLV</td>
<td>murine leukemia virus</td>
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<td>mouse mammary tumor virus</td>
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<tr>
<td>MoMLV</td>
<td>Moloney murine leukemia virus</td>
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<td>MPMV</td>
<td>Mason-Pfizer monkey virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>MVB</td>
<td>multivesicular body</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
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<tr>
<td>NA</td>
<td>Neuraminidase</td>
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<tr>
<td>NC</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>NME</td>
<td>N-terminal Met excision</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NTA</td>
<td>N-terminal amidases</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>OTU</td>
<td>ovarian-tumor</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>pAPC</td>
<td>professional antigen presenting cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>proteasome inhibitor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<td>-----------</td>
</tr>
<tr>
<td>PLC</td>
<td>peptide loading complex</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>pMHC</td>
<td>peptide-MHC complex</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
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<tr>
<td>POSIX</td>
<td>plenty of SH3</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>PSI</td>
<td>protein biosynthesis inhibitor</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidenefluoride</td>
</tr>
<tr>
<td>RGS</td>
<td>regulator of G-protein signaling</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radiolmmunoprecipitation assay</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RP</td>
<td>regulatory particle</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>rVV</td>
<td>recombinant vaccinia virus</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEA</td>
<td>staphylococcal enterotoxin A</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>SIV</td>
<td>simian immunodeficiency virus</td>
</tr>
<tr>
<td>SL</td>
<td>SIINFEKL</td>
</tr>
<tr>
<td>SUMO</td>
<td>small Ub-related modifier</td>
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<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
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<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>TOP</td>
<td>Thimet oligopeptidase</td>
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<td>TPPII</td>
<td>tripeptidyl peptidase II</td>
</tr>
<tr>
<td>TRiC</td>
<td>tailless complex polypeptide-1 (TCP-1) ring complex</td>
</tr>
<tr>
<td>TRIM</td>
<td>Tripartite interaction motif</td>
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<td>TRP-2</td>
<td>tyrosinase-related protein-2</td>
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<tr>
<td>Tsg101</td>
<td>tumor susceptibility gene 101</td>
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<tr>
<td>Ub</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>UBL</td>
<td>ubiquitin-like</td>
</tr>
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<td>UCH</td>
<td>ubiquitin-C-terminal hydrolase</td>
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<tr>
<td>UEV</td>
<td>ubiquitin enzyme 2 variant</td>
</tr>
<tr>
<td>UFD</td>
<td>ubiquitin fusion degradation</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin proteasome system</td>
</tr>
<tr>
<td>USP</td>
<td>ubiquitin-specific proteases</td>
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<tr>
<td>VLPs</td>
<td>virus like particles</td>
</tr>
<tr>
<td>zLLL</td>
<td>carbobenzoxyl-leucine-leucine-leucinal</td>
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</table>
4 Introduction

4.1 The human immunodeficiency virus type 1

The human immunodeficiency virus type 1 (HIV-1) is the causative agent of the acquired immunodeficiency syndrome (AIDS), first described in 1981 (1). The number of people living with an HIV-1 infection world-wide is still increasing and has reached 33.3 million in 2009, with 2.6 million people that were newly infected with HIV-1 and 1.8 million people dying from AIDS, as estimated by the World Health Organization of the United Nations. Although the introduction of highly active antiretroviral therapy (HAART) in the mid nineties has significantly reduced morbidity and mortality among AIDS patients, eradication of the virus from infected individuals has not been achieved and the disease remains incurable. With still very limited access to HIV-1 prevention and treatment in developing countries, the HIV-1 pandemic remains one of the most critical of infectious disease challenges to public health.

Fig. 4.1: Replication cycle of HIV-1 (artwork by Nadine Jänisch). Schematic representation of the major steps in HIV-1 replication. The replication cycle of HIV-1 begins with the attachment of the virus particle to CD4 and one of the coreceptors CXCR4 or CCR5, followed by membrane fusion, virus entry and uncoating. Following reverse transcription, the proviral DNA is integrated into the host cell genome. The late steps of replication start with the transcription of viral genes and the de novo synthesis of viral structural proteins which undergo assembly and budding at the plasma membrane. Following auto-catalytic activation, the viral protease processes the structural proteins resulting in the formation of a conical core that is typical for a mature, infectious virus particle.
HIV-1 belongs to the lentivirus subfamily of the *Retroviridae*, a family of enveloped RNA viruses, that, instead of using their RNA genome directly for virus replication, reversely transcribe it into proviral DNA that is integrated into the host cell genome. The retroviral life cycle, depicted in Fig. 4.1, begins with the binding of infectious viral particles to cellular receptors, in case of HIV-1 CD4 and one of the coreceptors CCR5 (CC chemokine receptor 5) or CXCR4 (C-X-C chemokine receptor type 4), followed by membrane fusion and virus entry. After reverse transcription of the RNA genome and integration of the proviral DNA into the host chromosomes, virus proteins are synthesized and assemble at the plasma membrane (PM). During budding, the Gag polyprotein precursor is processed by the viral protease (PR) after its auto-catalytic activation, resulting in the formation of a conical core which is typical for the mature and infectious HIV-1 particle.

**Fig. 4.2: Genomic organization of HIV-1.** This figure depicts the complex organization of the HIV-1 genome comprising the canonical retroviral ORFs flanked by long terminal repeat (LTR) sequences: *gag* (blue) coding for the polyprotein precursor Pr55 that is processed into the individual structural Gag proteins, *pol* (green) encoding the enzymes Protease, Reverse Transcriptase, RNaseH and Integrase and *env* (orange), coding for the glycoprotein precursor gp160 that is processed into gp120 and gp41. Six additional genes code for regulatory proteins that are classified as essential (Tat and Rev) or accessory (Vpr, Vif, Vpu, Nef). A schematic view of a mature HIV-1 virus particle with the incorporated proteins and two copies of RNA is shown below.

The HIV-1 genome is approximately 9 kbp in length and contains the three open reading frames (ORFs): *gag* encoding the structural Gag (group specific antigen) proteins, *pol* coding for the viral enzymes PR, Reverse transcriptase (RT), RNase H and Integrase (Int) and *env*, which encodes the surface glycoproteins gp 41 and gp 120. In addition to those canonical retroviral coding regions, HIV-1, as a complex retrovirus, encodes six
regulatory proteins: Tat and Rev, which are essential for efficient transcription and the nuclear export of single- or unspliced viral mRNA, respectively, and the accessory proteins Vpr, Vpu, Vif and Nef, which are dispensable for viral replication in cell culture, but play important roles during infection in vivo (2). A schematic overview of the HIV-1 genome and a virus particle is depicted in Fig. 4.2.

4.2 Gag proteins and their role in late processes of HIV-1 replication

The virus structure proteins Gag are synthesized from an unspliced RNA in the cytoplasm as a polyprotein precursor, named Pr55 according to its molecular weight (MW). During five to ten percent of all translation events, ribosomal frameshifting into the pol reading frame occurs (3), resulting in the synthesis of a Pr160 Gag-Pol fusion protein. Pr55 undergoes cotranslational myristoylation at an N-terminal Gly residue, which is crucial for binding to membranes and hence, for virus assembly and budding (4). During maturation, which occurs concomitantly with or shortly after virus release, the Gag precursor is cleaved by the viral PR into the individual structural proteins Matrix (MA, p17), Capsid (CA, p24), Nucleocapsid (NC, p7), p6 and two spacer peptides p1 and p2. However, Pr55 alone is both essential and sufficient for the assembly and release of virus like particles (VLPs; (5)). At least three conserved and interchangeable domains essential for virus assembly have been identified in Pr55: the membrane binding domain (M), the interaction domain (I) and the late domain (L). Membrane binding is mediated by the myristate group and basic residues clustered in the N-terminal region of MA (6). The I-domain is required for the assembly of particles of normal density and has been mapped to the NC domain (7). The L-domain is essential for the final abscission of the budded virion from the PM. L-domains have been identified in virtually all retroviral Gag proteins as well as other enveloped viruses like filoviruses, rhabdoviruses and arenaviruses (8, 9). The fact that, in some cases, L-domains can function independently from their position within Gag proteins and can even be switched between different viruses (10), suggests that they serve as docking sites for cellular factors to promote virus release, and several of those have been identified.

The C-terminal p6 region of HIV-1 Gag contains two distinct L-domains. The primary L-domain has been mapped to the tetrapeptide motif PTAP and promotes budding via its interaction with the UEV (Ub enzyme 2 variant) domain of Tsg101 (tumor susceptibility
gene 101), a component of the cellular endosomal sorting complex required for transport-I (ESCRT-I, (11-13)). Prevention of this interaction abolishes HIV-1 release almost completely (11, 12), as does overexpression of Tsg101 (14) or an N-terminal fragment thereof (15). PT/SAP L-domain motifs are also found in other retroviruses like HIV-2, simian immunodeficiency virus (SIV), human T cell leukemia virus (HTLV), Moloney murine leukemia virus (MoMLV), Mason-Pfizer Monkey Virus (MPMV), feline immunodeficiency virus (FIV) and Ebola virus, a filovirus (12, 16). More C-terminally within HIV-1 p6, a degenerated version (YP(X)nL) of the YPDL L-domain motif, which among others is also present in equine infectious anemia virus (EIAV), binds to the central V-domain of the ESCRT-associated adaptor protein ALIX (ALG (apoptosis linked gene)-2-interacting protein X; (17-20)). The release of PTAP L-domain mutants harboring an intact ALIX binding site can be rescued by overexpression of ALIX (18, 21, 22). While mutation of the ALIX binding site within p6 has only a mild effect on wt HIV-1 particle release, overexpression of the central V domain of ALIX severely attenuates budding in a dominant-negative manner (23) that depends on the presence of the YP(X)nL motif. Recently, ALIX was found not only to bind to HIV-1 p6, but also to the NC region of Pr55 via its N-terminal Bro1 domain (24-26), implicating a possible cooperative or alternative function of NC for the recruitment of the ESCRT to promote virus budding.

A third kind of L-domains consists of a PPxY motif and is found in Rous sarcoma virus (RSV; (27)), murine leukemia virus (MLV) and more distantly related enveloped viruses (8). The PPxY L-domain interacts with WW domains of the HECT (Homologous to E6-associated protein C-terminus) family of E3 Ub ligases (28, 29). Recently, a member of this family of Ub ligases, Nedd4-2s, has also been implicated in the egress of HIV-1 (30, 31).

In addition to the conserved retroviral assembly domains M, I and L, other regions contribute to the efficient assembly and release of viral particles (for review see (32)), amongst them the major homology region (MHR) in CA (33), the only region within Gag that shows significant sequence homology between different retroviruses (34).

### 4.3 The ubiquitin proteasome system

Besides the lysosomal system, the ubiquitin proteasome system (UPS) is the major proteolytic pathway in the cell (35). The 26S proteasome, a large, highly abundant multi-
enzyme complex distributed throughout the cytosol and nucleus, is composed of the 20S catalytic core particle (CP) and two regulatory 19S particles (RP).

The barrel-shaped CP consists of four stacked heteroheptameric rings, two outer \( \alpha \)-rings and two inner \( \beta \)-rings (\( \alpha_1-7, \beta_1-7, \beta_1-7, \alpha_1-7 \)). The N-terminal Thr residues of the \( \beta_1 \), \( \beta_2 \) and \( \beta_5 \) subunits represent the enzymatically active sites of the CP, acting as both nucleophile and proton acceptor in hydrolysis. Proteasomes can cleave after most aa residues, however, certain cleavage preferences have been attributed to the catalytic subunits: the \( \beta_1 \)-subunit cleaves after acidic residues (caspase-like activity), the \( \beta_2 \)-subunit after basic residues (tryptic activity), and the \( \beta_5 \)-subunit after hydrophobic residues (chymotryptic activity; (36)).

According to its appearance in electron micrographs, the RP is subdivided into lid and base. Within the 10-subunit base, a hexameric ring of ATPase subunits binds to the \( \alpha \)-subunits of the CP, mediates opening of the gate into the catalytic chamber, substrate unfolding and translocation. Several subunits located both within the base and lid are critical for the recognition of poly-Ub chains and substrate deubiquitination.

The immune-modulatory cytokine interferon (IFN)-\( \gamma \) induces the synthesis of the immunosubunits \( \beta_1i \) (low-molecular-weight protein 2 [LMP2]), \( \beta_2i \) (multicatalytic endopeptidase complex-like-1 [MECL1]) and \( \beta_5i \) (LMP7) that can replace the constitutive catalytic subunits in nascent 20S proteasomes to build the so-called immunoproteasome (37, 38). However, immunoproteasomes are constitutively expressed in some tissues like spleen, lymph node (LN), thymus and small intestine (39) and in antigen presenting cells (APCs) like dendritic cells (DCs; (40)). The altered cleavage specificities of immunoproteasomes is associated with differential antigen (Ag) processing and results in a modified spectrum of major histocompatibility complex class I (MHC-I)-presented epitopes (reviewed in (41)). In addition to catalytic immunosubunits, IFN-\( \gamma \) induces the 11S activator, also named PA28, a ring-like structure composed of three PA28\( \alpha \) and two PA28\( \beta \) subunits (42), which enhances the activity of the CP (43-47), probably by bringing the \( \alpha \)-subunits in an open conformation to facilitate substrate entry (42, 48).

Although Ub-independent pathways into the proteasome have been described (49), substrates are in general targeted for proteasomal degradation by the covalent conjugation of at least four Ub moieties in a process called polyubiquitinylation. After activation by the activating enzyme E1, Ub is transferred onto an E2 conjugating enzyme and, by the action of a specific E3 ligase, the C-terminus of Ub is attached via an isopeptide bond to the \( \varepsilon \)-amino group of Lys residues of the target protein. Eukaryotic E3 ligases are divided
into three classes according to their catalytic domain: HECT Ub ligases, ligases containing a RING (really interesting new gene) domain and U-box E3 ligases. In contrast to HECT E3 ligases, which form a thioester bond with the C-terminal Gly residue of Ub before transfer to the target protein (50), RING-type E3 ligases serve as platform that specifically recognizes and brings together the Ub-loaded E2 and the substrate (51). The U-box was described as a modified RING finger domain and defines the third group of E3 ligases (52, 53). In humans, two potential E1s, about 30 E2s and over 600 E3s are encoded (54), with ~95 % of the latter being of RING-type. In addition, E4 enzymes have been described, that can catalyze the extension of preexisting poly-Ub chains already attached to the target protein (55, 56).

In a process called monoubiquitination, a single Ub molecule is attached to a Lys residue of the target protein. This can also occur at several Lys acceptor sites, resulting in multiple monoubiquitination, also named multiubiquitination. Within Ub itself, seven Lys residues serve as potential acceptor sites for additional Ub moieties, resulting the formation of poly-Ub chains (57). It is well established that the linkage to at least four Lys48-linked Ub molecules (58) acts as a signal for recognition of the target protein by subunits of the 19S RP of the 26S proteasome (58) or by adaptor proteins (59). The substrate undergoes deubiquitination (60), allowing for Ub recycling, is funneled into the CP, where it is degraded into peptides ranging from 4 to 25 residues.

Mono- and multiubiquitination as well as polyubiquitination linked via Lys63 have been implicated in regulatory functions unrelated to proteasomal degradation governing for example endocytosis of cell surface receptors, DNA-repair, signal transduction, transcription and translation (61, 62). Recently, unconventional poly-Ub chains linked through K6, K11, K27, K29 or K33 have been detected. Those are either linked to the same Lys residue on each ubiquitin moiety to form a homogeneous chain, or to different Lys residues to build a heterogeneous or branched chain. K11-linked poly-Ub chains signal for proteasomal degradation, especially through ERAD (endoplasmic reticulum-associated degradation (63)), and are involved in cell cycle regulation (64).

K29/K33-linked poly-Ub chains have been described to be assembled by HECT E3 ligases (65) and have been implicated in the UFD (Ub fusion degradation) pathway (66), lysosomal degradation (67) and regulation of protein kinase activation (68). However, the relevance of those and other unconventional poly-Ub chains in vivo is not fully understood yet.
Fig. 4.3: The ubiquitination cascade and types of ubiquitination. Ubiquitination starts with ATP-dependent formation of a thioester bond between the C-terminus of Ub and the E1 ubiquitin-activating enzyme, followed by transfer of Ub to an E2 ubiquitin-conjugating enzyme. Isopeptide bond formation between the C-terminal Gly residue of Ub and the ε-amino group of a Lys residue of the target protein is mediated by the action of E3 Ub ligases. RING-finger E3 ligases act as scaffolds, whereas HECT-domain E3s bind the activated Ub to a Cys residue and transfer it to the target protein. Ubiquitination can involve a single Ub (monoubiquitination) or multiple Ubs, each conjugated to a single Lys residue (multiubiquitination), both generally regulating protein function. Ubiquitination is reversible by the action of DUBs. As any of the seven Lys residues of Ub as well as its N-terminus can be acceptor sites for Ub attachment, poly-Ub chains in which Ub itself is successively ubiquitinatated can be assembled. This might involve action of an E4 Ub chain elongation factor. Depending on the type of linkage, polyubiquitination can tag a substrate for proteasomal degradation or fulfill regulatory functions.
In addition to Ub, so called Ub-like (UBL) proteins (so far, 17 of them have been identified in mammalian cells), like SUMO (small Ub-related modifier), can also be conjugated to proteins in a way that is rather similar to ubiquitination (69). Like other processes that regulate protein function, for example phosphorylation, ubiquitination is highly dynamic and reversible. The removal of Ub moieties is catalyzed by deubiquitinating enzymes (DUBs), comprising almost 100 representatives in mammals that are grouped into five families: Ub C-terminal hydrolases (UCHs), Ub-specific proteases (USPs), ovarian-tumor (OTU) domain DUBs, Machado-Joseph domain (MJD) DUBs and Jab1/MPN metalloenzyme (JAMM) zinc-dependent metalloproteases (for review see (60, 70-72)).

Besides its numerous functions in cellular processes, the UPS plays an important role in the replication cycle of viruses (73), amongst them HIV-1 (74-77). In particular, HIV-1 takes advantage of the UPS to facilitate replication first, by targeting cellular restriction factors (78) for degradation by the proteasome through the action of accessory proteins (79-84) and second, by utilizing the cellular endosomal sorting machinery, Ub ligases and other components of the UPS for its egress (77). Moreover, Ag processing by the proteasome generates the peptides that are presented by MHC-I molecules to CD8⁺ T cells for the recognition and elimination of virus-infected cells (85, 86) and viruses have evolved efficient strategies to down-modulate Ag presentation in order to evade immune recognition (87).

4.4 Role of the UPS in late steps of HIV-1 replication

The observation that retrovirus particles are enriched in free Ub, made almost 20 years ago (88), pointed towards a function of the UPS in retrovirus budding. The role of the UPS for late steps of retrovirus replication was further emphasized by the finding that inhibition of the proteasome resulted in a defect in release and maturation of HIV-1 and other retroviruses that was highly reminiscent of the phenotype observed for L-domain mutants (89-91). Intriguingly, not all retroviruses require proteasome activity for efficient release. While the budding of HIV-1, HIV-2, MLV and MPMV, harboring PTAP- or PPPY-type L-domains has been shown to be sensitive to proteasome inhibition, mouse mammary tumor virus (MMTV) or EIAV with unknown, or YPDL L-domains, respectively, did not show reduced virus release upon treatment with proteasome inhibitors (PIs; reviewed in (9)). Although extensively studied (for review see (75, 77, 92-97)), the function of the UPS in retrovirus release is still not fully understood.
To catalyze the final abscission of the virus bud from the PM, HIV-1 and other retroviruses usurp the cellular ESCRT machinery (92), which consists of five protein complexes, ESCRT-0, -I, -II, -III, and Vps4-Vta1 as well as several ESCRT-associated proteins (98-101). The ESCRT mediates the scission of membrane necks and is, besides viral budding, involved in topologically highly similar processes, namely recognition and sorting of monoubiquitinated proteins into endosomes (101), multivesicular body (MVB) biogenesis (102, 103) and cytokinesis (104, 105).

Recent work has demonstrated that this abscission of membrane stalks is directly mediated by ESCRT-III together with the AAA ATPase (ATPase associated with various cellular activities) Vps4, both also representing the most evolutionary conserved representatives of the ESCRT (106). The ESCRT-III subunits exist as soluble monomers that polymerize into filaments, spirals or tubes that are tightly associated with membranes, thereby causing membrane deformation (107-109). Recycling of the ESCRT-III proteins back into their soluble monomeric form is accomplished by Vps4 in an ATP-dependent fashion.

As described above, HIV-1 and other retroviruses harboring a PTAP L-domain motif recruit the ESCRT machinery via binding to the ESCRT-I component Tsg101. Tsg101 in turn interacts with ALIX, an ESCRT-III associated protein. Retroviruses containing a YPDL motif on the other hand directly interact with ALIX to recruit ESCRT-III. Though the ESCRT-II complex has been proposed to play a role in HIV-1 budding (19), it seems to be dispensable for HIV-1 egress according to more recent findings (110). Thus, it appears that HIV-1 and other retroviruses containing a PTAP L-domain utilize alternative mechanisms to link ESCRT-I and III, thereby bypassing ESCRT-II.

As many components of the ESCRT bind to ubiquitinated cargo proteins (100), the ubiquitination of retroviral Gag proteins (111, 112) has been suggested to play a role in interaction with the ESCRT machinery (95). Two Lys residues within p6 have been shown to be specifically mono-ubiquitinated (111), while one of them is also sumoylated (113). The function of both modifications is, however, still unclear, as the Ub acceptor sites within p6 seem to be dispensable for virus release and replication, Gag processing, or incorporation of free Ub, at least in certain cell types (112). More recent observations indicate that, although low-level monoubiquitination most likely occurs at multiple sites within all Gag domains (114, 115), attachment of Ub to Lys-residues neighboring the PTAP L-domain seems to be important for virus release and replication (115). Moreover, there is evidence gained in vitro that monoubiquitination of p6 might influence the binding properties of Tsg101 (116). Fusion of Ub to the C-terminus of RSV Gag is able
to rescue budding from PI-treated cells (89). Recently, it has been demonstrated that fusion of Ub to the C-terminus of EIAV Gag can functionally replace the YPDL L-domain (117). Replacement of the YPDL L-domain in EIAV Gag by a PPPY or a PTAP L-domain rendered VLP release sensitive to PI treatment, while Ub fusion in this context conferred resistance to proteasome inhibition. In the absence of a functional L-domain, however, Ub fusion sensitized VLP release of EIAV to blockade of proteasome activity (117). Therefore, different models have been proposed to explain the effect of PIs on virus release. First, it has been suggested that PIs, by rapid depletion of free Ub, interfere with the monoubiquitination of Gag, which serves as a recognition signal for the ESCRT machinery. On the other hand, Ub fusion to EIAV Gag in absence of an L-domain confers sensitivity to PI treatment, supporting a model in which proteasome shutdown disturbs the cellular machinery required for particle release rather than directly affecting Gag ubiquitination (117). Moreover, when cells are treated with PIs, inhibition of virus release seems to occur faster than depletion of free Ub (Ulrich Schubert, unpublished observation and (91))(9). In a distinct, third model, it has been proposed that proteasome inhibition prevents the clearance of misfolded or otherwise damaged Gag molecules, which then interfere in a dominant-negative manner with the ordered assembly of the Gag lattice required for budding (89, 91).

While Gag ubiquitination has been shown to influence L-domain function, L-domains themselves have been shown to either positively (118, 119) or negatively regulate the ubiquitination of Gag (114, 120, 121), thus underlining the intimate relation between L-domain function and the ubiquitination machinery.

Overexpression of the UBL protein ISG (IFN-stimulated gene) 15 blocks HIV-1 and Ebola virus particle release (122-124). First reports indicated that ISG15 expression inhibited the E3 ubiquitin ligase Nedd4 that interacts with the PPEY motif of Ebola virus (124, 125) and prevented binding of Tsg101 to HIV-1 p6, though direct conjugation of ISG15 to Tsg101 or Gag has not been demonstrated (123). Interestingly, ISG15 overexpression resulted in reduced levels of Gag ubiquitination (122, 123). Recently, it has been proposed that ISG15 can additionally prevent the interaction of Vps4 with its coactivator protein LIP5 due to conjugation of ISG15 to the ESCRT-III protein CHMP5 (122).
4.5 Role of the UPS and defective ribosomal products (DRiPs) in MHC-I antigen processing

The conventional MHC-I pathway starts with the processing of endogenous Ags by the 26S proteasome in the cytosol (85, 86, 126). It has now become widely accepted that the majority of endogenous MHC-I ligands are derived from the processing of de novo synthesized, dysfunctional proteins (reviewed in (127-131)). These so-called defective ribosomal products (DRiPs; (132, 133)) fail to adopt their functional conformation and are quickly eliminated by the UPS during or following translation in order to avoid cell damage (134), regardless of the half-life of their native counterpart. Due to their rapid turnover, DRiPs can only be detected in the absence of proteasomal activity (133, 135, 136), which can be easily achieved by the addition of cell-permeable PIs, for example the irreversible and highly specific inhibitor lactacystin (137).

Endoplasmic reticulum (ER)-resident Ags can gain access to the cytosolic proteasome via ERAD (138, 139). Ags that have not been synthesized by the APC itself, but taken up via endocytosis, phagocytosis, macropinocytosis or even through gap junctions, can be presented by MHC-I in a process called cross-presentation, that is especially crucial to the priming of naïve CD8⁺ T cells by DCs (140-142). Recently, a process named autophagy, in which intracellular material such as whole organelles, cytoplasmic material or protein aggregates are sequestered into membrane-enclosed vacuoles, named autophagosomes, and destroyed by fusion with lysosomes, has gained much interest in the field of immunology. Autophagy not only provided an explanation for presentation of endogenous peptides by MHC-II, but has also been implicated in MHC-I Ag presentation (143-145).

Proteolysis by the 26S proteasome produces peptides three to 25 residues in length (146) that are transported into the ER by TAP (transporter associated with antigen processing), which is composed of TAP1 and TAP2 subunits (147) and has a specificity for peptides ranging approximately from 7 to 13 aa (148-150). In the ER, peptides undergo N-terminal trimming by luminal proteases to fit into the peptide binding groove of MHC-I molecules, which can bind, with only few exceptions, peptides 8-10 aa in length (151-153). Binding is mostly mediated by the peptide’s so called anchor residues which have to be occupied by specific residues depending on the haplotype of the MHC-I molecule. Folding of the MHC-I α-chain (heavy chain) is mediated by the chaperone calnexin and the lectin-like chaperone Calreticulin (CRT), followed by association with β2-microglobulin (β2m).
Fig. 4.4: DRiPs as main source for epitopes presented via the conventional MHC-I pathway. 1) Protein de novo synthesis and quality control. Flawless proteins enter the standard protein life cycle, whereas defective ribosomal products (DRiPs) become rapidly polyubiquitinated. 2) Ag processing by the proteasome. 3) Peptide transport and trimming. The proteasome generates mainly N-terminally extended proteolytic intermediates that can be protected by the chaperone TRiC (tailless complex polypeptide-I (TCP-1) ring complex) or CCT (chaperonin containing TCP-1), or destroyed by the protease TOP (Thimet oligopeptidase). In the ER, ERAAP (ER aminopeptidase associated with Ag processing) can N-terminally trim antigenic precursors imported by TAP. The cytosolic protease TPPII (tripeptidyl peptidase II) might contribute to Ag processing and/or trimming. 4) MHC-I folding and peptide loading. Nascent MHC-I are folded by the chaperones calnexin and CRT and undergo disulfide bond (S-S) formation and association with β2m. Fully folded MHC-I molecules are incorporated into the peptide loading complex (PLC), which catalyzes the loading with peptides imported from the cytoplasm by the heterodimeric TAP. Tapasin binds to TAP, MHC-I and the oxidoreductase ERp57. Tapasin and ERp57 are covalently linked by a disulfide bond. 5) Export, presentation and T-cell activation. MHC-I molecules loaded with high-affinity peptides are transported via the secretory pathway to the cell surface. Specific binding of the T-cell receptor (TCR) to a MHC-peptide-complex in presence of costimulatory signals (not depicted) triggers activation of CD8⁺ T cells.

The generation and stabilization of peptide-MHC-I (pMHC) complexes is accomplished by a specialized ER-resident chaperoning complex, called the peptide-loading complex (PLC), consisting of CRT, the thiol oxidoreductase ERp57, TAP and Tapasin, a chaperone-like protein (154-156). Only peptide-loaded MHC-I molecules are transported through the secretory pathway to the cell surface, where the pMHCs are presented to CD8⁺ T cells.
4.6 Regulation of UPS-mediated proteolysis by degradation signals

It has become increasingly clear over the last years that regulation of protein function and degradation by the UPS plays a key role in a number of processes like cell cycle progression, apoptosis, gene expression, stress responses, viral replication and MHC-I Ag presentation. Defects in targeted proteolysis have been implied in the pathogenesis of severe diseases, (157) including cancer (158, 159), autoimmunity (160), cardiovascular disease (161) and neurodegeneration (162-164). The PI Bortezomib (also known as PS-341 or Velcade®) has been approved in 2003 for the treatment of multiple myeloma (165-167), a plasma cell malignancy that results in the overproduction of monoclonal immunoglobulins, a feature that sensitizes multiple myeloma cells for PI-mediated induction of apoptosis (168). Next generation PIs as well as strategies aiming at upstream targets, like the development of DUB inhibitors or inhibitors of E3 ligases, are among promising candidates for the treatment of various malignancies and other diseases (159, 169).

Given the extreme importance of tight control of protein half-life in the cell (134), it is not surprising that there exist several motifs in the primary protein sequence, named degrons (170), that target a protein for degradation by the 26S proteasome causing its rapid turnover (171).

Many short-lived proteins contain regions rich in Pro (P), Glu (E), Ser (S) and Thr (T), which were identified by correlation of the metabolic half-life with the primary sequence of proteins in silico and named PEST-sequences (172). Removal of PEST sequences from short-lived proteins markedly augmented metabolic stability (173-176), thus supporting the functionality of PEST sequences in vivo. Likewise, transfer of a PEST sequence to otherwise stable proteins can induce rapid turnover (177, 178).

The so called N-end rule, which relates the metabolic half-life of a protein to the identity of its N-terminal aa has been first defined by A. Varshavsky in yeast (179) as another principle that governs protein stability. The entire degradation signal, the N-degron, consists of the destabilizing N-terminal aa, grouped into basic (Arg, Lys, His) and hydrophobic (Leu, Phe, Trp, Tyr, Ile) residues, and an internal Lys residue (180). Many components of N-end rule pathway have been identified in pioneer studies using engineered model substrates like β-Galactosidase (β-Gal; (179-182)). More recently, however, a number of physiological substrates and their functions as well as the enzymes that create and recognize N-degrons have been characterized (183-187).
The N-terminal initiator Met residue can be cotranslationally removed by Met aminopeptidases (MetAPs), but only if the following residue possesses a small side chain (Gly, Val, Pro, Ala, Ser, Thr or Cys; (188-190)). As primary destabilizing residues cannot be created this way, further modification is necessary. If the exposed residue is a Cys, however, it can be first oxidized by molecular oxygen or nitric oxide (NO) and then arginylated by an enzyme called arginyl-tRNA-protein transferase (Ate1; (191, 192)), which has been found to be important for the degradation of several regulator of G-protein signaling (RGS) proteins (183, 186, 193-196).

Arg, a primary destabilizing residue, can also be attached by Ate1 to proteins that expose Asp or Glu, which can in turn be created from Asn or Gln by the action of N-terminal amidases (NTAs; (197, 198)). N-end degrons can also be exposed by endoproteolytic cleavage. This mechanism has been demonstrated for the first physiological substrate of the N-end rule pathway in yeast, Scc1, a subunit of the cohesion complex, which is important for chromosome stability (199). Degradation by the N-end rule pathway following proteolytic cleavage has also been observed for the HIV-1 Int (200), the Sindbis virus RNA polymerase (201) and Drosophila inhibitor of apoptosis (DIAP1 (185)). In this way, substrates of the N-end rule pathway can be engineered by N-terminal in frame fusion with Ub that is co-translationally cleaved by DUBs (71, 202).

The E3 Ub ligases that recognize N-degrons, named N-recognins, have been identified as a family of proteins containing an approximately 70 aa zinc finger-like domain, called the UBR box. In mammals, at least seven UBR box proteins have been described (203), whereas in yeast, recognition of N-end rule substrates is achieved by binding of a single N-recognin, the UBR1 gene product, to primary destabilizing residues (204).

Cleavage of engineered Ub fusions by DUBs is abolished, if the C-terminal Gly residue of the Ub moiety is exchanged for a different aa, for example Ala or Val. Such fusion proteins are substrates for the so called Ub-fusion degradation (UFD) pathway (66, 205) which includes polyubiquitinylation of the Ub moiety itself at Lys48 or Lys29 (66), and proteasomal degradation of the fusion protein.

Other motifs of the protein primary sequence that govern protein stability include phosphodegrons in cyclins or an oxygen-dependent degron (ODD) in hypoxia-inducible factor-1α (HIF-1α; for review see (171)).

Clearance of damaged proteins, including misfolded or mistargeted polypeptides is one of the crucial functions of the UPS. It has been shown that mistargeting can lead to rapid degradation of a protein, thus demonstrating that the correct subcellular localization represents an essential quality criterion (206, 207), as is correct folding (208-210).
4.7 Correlation between metabolic half-life and MHC-I antigen presentation

The correlation between Ag stability and generation of MHC-I antigenic determinants has been extensively studied (207, 210-217). First, it has been demonstrated in a recombinant vaccinia virus (rVV) system that expression of an instable variant of the influenza A virus (IAV) nucleoprotein (NP) increased the efficiency in presentation of one particular epitope of NP (207), a finding that has subsequently been extended by others (211, 212). Recombinant Ub-X-Gal fusion proteins were differentially degraded according to the N-end rule in an Ub- and proteasome-dependent manner when introduced exogenously into cells and the instable counterparts of these fusion proteins evoked enhanced T-cell activation (213). A correlation between protein half-life and generation of class-I ligands has also been reported for the listeria Ag p60 (214, 215). In contrast, no such correlation has been found for generation of the SIINFEKL (SL) epitope derived from instable Ovalbumin (OVA) and β-Gal fusion proteins (217). Enhanced Ag presentation together with efficient stimulation of cytotoxic T lymphocytes (CTLs) has been shown for a metabolically instable variant of the HIV-1 accessory protein Nef (216). However, instable variants of the HIV-1 Gag polyprotein failed to elicit enhanced CTL responses although increased numbers of Gag-derived epitopes were presented on the cell surface (210).

To investigate the correlation between efficiency of protein degradation and MHC-I Ag presentation, the HIV-1 Gag polyprotein has been chosen as a model Ag for two reasons: First, it has already been demonstrated that Gag as a viral Ag can enter the DRiP-pathway (133) and, second, Gag represents a highly attractive Ag for vaccine development. Using the OVA-derived SL peptide as a model epitope, the efficiency of entry of Gag variants carrying certain degradation signals into the MHC-I pathway was investigated. Gag was expressed from a codon-optimized, synthetic gag gene, which results in high expression levels and efficient VLP release and allows studying the properties of Pr55 in the absence of other HIV-1 proteins (218). The impact of degradation signals on synthesis, metabolic stability, budding as well as localization of HIV-1 Pr55 was analysed. In addition, the PTAP L-domain motif was found not only to function in HIV-1 budding, but also in the regulation of MHC-I Ag presentation of HIV-1 Gag.
5 Results

5.1 Targeting HIV-1 Gag into the DRiP-pathway enhances MHC-I antigen presentation and CD8+ T-cell activation

5.1.1 Construction of Gag variants containing degradation signals

Although the correlation between protein half-life and MHC-I Ag presentation has been demonstrated for a number of Ags (207, 210-217), the role of protein degradation motifs in targeting Ags to the DRiP pathway is not well studied. As Gag is considered an attractive target for vaccine development (219-221), it was a central aim of this thesis to enhance the generation of Gag-derived epitopes for MHC-I presentation by targeting the Gag polyprotein Pr55 originating from the isolate HIV-1HXB10 (named Gag from hereon) for rapid degradation by the 26S proteasome. Assuming the importance of DRiPs as the major source of antigenic peptides, it was hypothesized that introduction of degradation signals attracts Gag into the DRiP-pathway.

To circumvent the necessity for co-expression of the regulatory HIV-1 tat and rev genes, which are strictly required for efficient transcription (222) and nuclear export of Gag-specific mRNA (223), respectively, a codon-optimized, synthetic gag gene was expressed under the control of the cytomegalovirus (CMV) promoter, resulting in high and Rev-independent expression levels of Gag (synGag) in various cell lines (218).

A schematic representation of the Gag variants, most of which were kindly provided by Prof. Ralf Wagner (Institute of Medical Microbiology and Hygiene, University of Regensburg) is shown in Fig. 5.1. A putative N-end rule substrate was constructed by N-terminal in frame fusion to Ub (UbRGag). This results in the expression of an Arg as destabilizing N-terminal aa (RGag) after cotranslational removal of the Ub moiety by DUBs that cleave with high efficiency between the C-terminal Gly of Ub and all aa except Pro (224). The fusion UbMGag, corresponding to the unmodified wt Gag protein upon Ub removal, was created as a control. The C-terminal PEST sequence derived from the murine ornithine decarboxylase was inserted in frame to the C-terminus of Gag. To possibly induce misfolding of Gag, single aa substitutions were introduced in the N-terminal region (P231L) as well as the MHR (L304H) of CA. Mistargeting of Gag was induced by mutation of the N-terminal myristoylation site (G2A). In addition, a putative substrate of the UFD pathway was constructed by mutation of the C-terminal Gly residue of the Ub moiety (UbG76V Gag).
5.1.2 Introduction of the OVA-derived SL epitope as indicator for Ag processing of Gag

Unfortunately, there have been no TCR-like Abs described to date which recognize MHC-I-bound Gag-derived epitopes. As isolation and handling of Gag-specific CTL clones derived from HIV-1-infected patients is rather difficult, the OVA-derived SL model epitope of was introduced as an indicator for Ag processing into the p2 spacer region localized between the CA and NC domains of Gag (Fig. 5.1 B; GagSLp2). To avoid interference with the structure and function of the p2 spacer, the SL epitope was introduced C-terminally of a putative α-helix spanning the C-terminal domain of CA and the N-terminal region of p2 (225, 226). Moreover, there is a certain length polymorphism in this region between HIV-1 isolates (www.hiv.lanl.gov). This strategy allows for the quantification of the number of SL epitopes derived from the Ag processing of Gag using the mAb 25D1.16, which specifically recognizes the SL epitope bound to the murine MHC-I molecule H2-Kb (227). As the 25D1.16 Ab selectively binds to SL in complex
with H2-K\textsuperscript{b}, it was necessary to use either H2-K\textsuperscript{b}-positive murine cell lines or H2-K\textsuperscript{b}-transgenic human cell lines for the analysis of SL-presentation. For a first set of experiments, the murine thymoma cell line EL4 that naturally expresses H2-K\textsuperscript{b} was selected to ensure that SL-presentation was analysed in the context of physiological H2-K\textsuperscript{b} expression levels.

5.1.3 Generation and characterization of GagSL-expressing EL4 cell lines

In contrast to human cell lines, in which high expression levels of Gag were obtained after transient transfection with psyngag expression plasmids (see Fig. 5.8), Gag or GagSL proteins were almost undetectable by Western blotting or flow cytometry after transient transfection of murine EL4 cells (data not shown). To obtain sufficient numbers of Gag-positive cells for FACS analysis of Ag presentation, EL4 cells were stably transfected with Gag expression constructs. When bulk EL4 cell populations were analysed for presentation of the SL epitope using the mAb 25D1.16, an enhanced SL-presentation was reproducibly detectable on the surface of cells expressing Gag variants harboring certain degradation signals (performed by Dr. A. Goldwich, data not shown). This was especially observed for the N-end rule substrate UbRGagSL, which was further compared to its counterpart UbMGagSL, expressing N-terminally Met instead of destabilizing residue Arg (see Fig. 5.1). After single cell cloning and several passaging steps, two stable clones of transgenic EL4 cells which stably express the UbMGagSL and UbRGagSL proteins, respectively, were selected.

Western blot analysis using anti-p24 and anti-p6 antibodies revealed that Gag and GagSL proteins migrated at the appropriate MW of approximately 55 kDa confirming the complete cleavage of the Ub moiety (Fig. 5.2). In addition to the 55 kDa Gag signal, a second band migrating at approximately 40 kDa was observed that possibly results from cleavage of Gag by a cellular protease. A slightly slower migration behavior of GagSL proteins reflects the presence of the SL epitope (Fig. 5.2). In addition, cell extracts standardized for protein content were analysed by p24-ELISA to determine the intracellular steady-state level of Gag (performed by Dr. A. Goldwich, data not shown). The level of Gag in the UbMGagSL/EL4 clone (4.14±2.36 ng of p24/10\textsuperscript{6} cells) was almost twice the amount of Gag determined in the UbRGagSL/EL4 clone (1.59±1.14 ng of p24/10\textsuperscript{6} cells). This slight difference in Gag expression was repeatedly observed, indicating that the lower steady-state level of Gag in the UbRGagSL/EL4 clone represents an inherent characteristic of the N-end rule substrate.
5.1.4 Half-life and DRiP-rate of UbRGagSL and UbMGagSL proteins

To determine the overall protein half-life, pulse radiolabeling of EL4 cells with $[^{35}S]$Met for 20 min followed by a 48 h long chase was performed. Gag proteins were recovered by IP using Gag-specific Abs, subjected to SDS-PAGE and visualized by autoradiography or phosphorimaging (Fig. 5.3 A). In parallel, total cell lysates were resolved by SDS-PAGE and the major band corresponding to β-actin served as an internal control (Fig. 5.3 A, lower panel). Densitometric analysis of three independently performed pulse-chase experiments revealed that both proteins displayed an overall half-life of approximately six hours in EL4 cells, though the decline of Gag expressed in the UbRGagSL/EL4 clone was somewhat faster during the first 24 h of chase when compared with the UbMGagSL/EL4 clone (Fig. 5.3 B). This slightly reduced half-life together with the generally somewhat reduced steady-state level of the UbRGag protein (Fig. 5.2) might point towards an increased turnover occurring in concert with de novo synthesis.

Due to the extremely delicate nature of DRiPs as short-lived products which are at least partially ubiquitinated and degraded by the 26S proteasome, detection can only be achieved after shutdown of proteasome activity and blocking deubiquitinating enzyme activities (133). To determine the DRiP-rate of UbM- and UbRGagSL proteins, respectively, short-term pulse-chase experiments were conducted according to previously elaborated DRiP pulse-chase protocols (133, 135). Gag-transgenic EL4 cells were treated during the final 10 min of a 30 min starvation period with a combination of distinctly acting proteasome inhibitors, the peptide aldehyde zLLL (228) and the irreversibly and highly specifically acting inhibitor lactacystin (LC; (137)).
Fig. 5.3: Pulse-chase analyses of UbMGagSL/EL4 and UbRGagSL/EL4 cells. (A+B) Half-life of UbGagSL proteins. For long-term pulse-chase experiments, UbMGagSL/EL4 and UbRGagSL/EL4 cells were radiolabelled for 20 min with $[^{35}S]$Met and chased for up to 48 h. (A) Fluorograph of Gag proteins recovered by IP using anti-p6 and anti-p24 antibodies and separated by SDS-PAGE. The band corresponding to $\beta$-actin was identified based on its MW in fluorographs of total cell lysates resolved by SDS-PAGE. (B) Densitometric quantification of $[^{35}S]$-labeled Pr55. The radioactivity of the Pr55 band was quantitated using a phosphor imager and plotted as percentage of the initial signal. Values represent the mean and standard deviation (SD) from three independent pulse-chase experiments. (C+D) DRiP-rate of UbGagSL proteins. For short-term pulse-chase experiments, UbMGagSL/EL4 and UbRGagSL/EL4 cells treated with 20 µM of zLLL/LC each during the final 10 min of a 30 min starvation were radiolabelled for 15 min and chased for up to 60 min the presence or absence of zLLL/LC. (C) Fluorograph of Gag proteins recovered by IP and resolved by SDS-PAGE as described above. (D) Densitometric quantification of $[^{35}S]$-labeled Pr55 (upper panels) and the high MW (HMW) smear recovered with anti-Gag antibodies (lower panels). The radioactivity recovered at each time point is plotted as percent of the radioactivity recovered at the time point “0” in absence of PIs. Mean values and SD from three independent pulse-chase experiments are depicted.

Cells were pulsed with $[^{35}S]$Met for 15 min and chased for up to 120 min in the presence or absence of zLLL/LC. Soluble Gag proteins in the detergent cell extracts that bound to Gag-specific Abs were subjected to SDS-PAGE and analyzed by fluorography
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(Fig. 5.3 B). The quantities of radioactivity in dried gels corresponding either to Pr55 or the total proteins migrating in the high molecular weight (HMW) range of ~60-250 kDa were quantitated using phosphorimaging instrumentation (Fig. 5.3 D). Similar to the long-term pulse-chase (Fig. 5.3 A+B), the decay of Gag was slightly faster in UbRGagSL/EL4 cells (Fig. 5.3 C+D). While data obtained in both cell lines with active proteasomes were comparable, the situation after proteasome shutdown was quite different: although addition of zLLL/LC rescued Gag proteins in both cultures from degradation, the amount of Pr55 recovered from UbRGagSL/EL4 cells increased by more than 50% immediately after the pulse, reaching maximum values of up to 80% increase within 15 min (Fig. 5.3 C, upper right panel) compared to only 30% increase of Pr55 recovered from UbMGagSL/EL4 cell extracts (Fig. 5.3 C, upper left panel). The same effect was observed for the Gag fragment migrating at ~40 kDa: the rate of recovery of which was again more pronounced in UbRGagSL/EL4 cells when compared to UbMGagSL/EL4 cells. In addition, an increase in the smear of proteins migrating in the HMW range was detected in cells treated with zLLL/LC. This smear recovered by anti-Gag antibodies was not recovered under the same conditions from non-transfected EL4 cells (data not shown), indicating that the smear represents polyubiquitinated Gag-DRiPs and not cellular proteins that bind non-specifically to beads or Gag-anti-Gag immune complexes. Similar to previous calculations in HIV-1 expressing cells (133), the radioactivity in this MW range was taken into account for the estimation of the DRiP-rates of both UbGagSL fusion proteins. Though treatment of UbMGagSL/EL4 cells with zLLL/LC resulted in modestly enhanced recovery of proteins in the HMW range, the magnitude of increase was clearly higher in UbRGagSL/EL4 cells (Fig. 5.3 D, lower panels), confirming results observed for Pr55. Similar to the kinetic of DRiP formation reported previously (133), the accumulation of labeled Gag proteins during the pulse and approximately the first 15 min of chase is of transient nature (Fig. 5.3 C+D). After reaching a certain plateau, processes like deubiquitination, proteolysis or aggregation, as well as association with membranes (114, 121), altogether continuously remove DRiPs from the pool of total Gag proteins accessible for IP. Thus, the results of the pulse-chase analyses demonstrate that the DRiP-rate of the Gag proteins clearly followed the N-end rule although the N-terminal fusion of UbR to Gag does not result in a significant reduction of the overall metabolic half-life.
5.1.5 Correlation of DRiP-rate with the MHC-I presentation of Gag-derived SL

Although the overall stability of both Gag proteins was not governed by the N-end rule, an observation which is consistent with previous reports by others using different Gag expression systems (210), an elevated DRiP-rate of the UbRGagSL variant compared to UbMGagSL was observed. To analyze whether UbRGagSL enters the MHC-I processing pathway more efficiently, the presentation of SL in complex with H2-Kb molecules was assessed by flow cytometry using the mAb 25D1.16 conjugated to Alexa Fluor 647 (25D1.16-647). In parallel, the expression of Gag was monitored by intracellular staining with a CA-specific Ab (KC57-FITC). Parental EL4 cells and a cell clone expressing relatively high levels of the SL epitope from a minigene construct, called SL/EL4, served as negative and positive controls, respectively. Similar to previous studies using rVV expression systems (211, 229, 230), a CMV-promoter construct that directs high level expression of the sequence MSIINFEKL was used to generate the stably transfected SL/EL4 clone.

Fig. 5.4: Flow cytometry analyses of SL-presentation on the surface of the UbRGagSL/EL4 and the UbMGagSL/EL4 cell lines. (A) Histogram plot of UbMGagSL/EL4 and UbRGagSL/EL4 cells double-stained for SL-H2-Kb complexes at the cell surface using the mAb 25D1.16 conjugated to AlexaFluor64 (25D) and intracellular Gag using a CA-specific mAb (KC57-FITC) (B) Quantification of six independent experiments after normalization of the mean fluorescence intensity (MFI) of the 25D1.16-647 staining to the MFI of the KC57-FITC staining. Parental EL4 cells or EL4 cells expressing the SL epitope from a minigene construct served as negative control and positive control, respectively. Bars represent mean values +/- SD (n=6; * = p<0.05).

FACS analyses revealed that the cell surface presentation of the SL epitope generated from UbRGagSL clearly surpasses the level of presentation observed for the UbMGagSL counterpart (Fig. 5.4 A). However, consistent with the ELISA and Western blot data, the
level of Gag in UbRGagSL expressing cells is about half of that observed in the UbMGagSL transgenic cells as shown by double staining with 25D1.16-647 and KC57-FITC (data not shown). To further support the notion that the increased presentation of H2-K\textsuperscript{b}-SL complexes on the surface of UbRGagSL/EL4 cells is not simply dependent on variations in expression levels of Gag, the number of H2-K\textsuperscript{b}-SL complexes presented at the cell surface was correlated with the intracellular amount of Gag in six independently performed double-staining experiments (Fig. 5.4 B). The ratio of the mean fluorescence intensity (MFI) at 647 nm (staining with 25D1.16-647) and the MFI at 488 nm (staining with KC57-FITC) was calculated, demonstrating an approximately two-fold, significant increase in SL-presentation.

Fig. 5.5: Analysis of loading of H2-K\textsuperscript{b} with SL epitopes. UbMGagSL/EL4 and UbRGagSL/EL4 cells were incubated at pH 3 for 2 min to dissociate K\textsuperscript{b}-bound peptides. After neutralization (0 min), cells were cultured for indicated time periods without inhibitors (A+B) or in the presence of PI (20 µM of zLLL) or a cocktail of protein biosynthesis inhibitors (PSIs) (C+D). The amount of surface H2-K\textsuperscript{b} (A+C) or H2-K\textsuperscript{b}-bound SL epitope (B+D) was analysed by flow cytometry. Data represent one of three independent experiments.

If the main source of SL epitopes are short lived Gag-DRiPs, the processing of UbRGagSL should lead to a higher intracellular steady-state level of the SL epitope compared to UbMGagSL. This should result in more efficient loading of empty H2-K\textsuperscript{b} molecules with SL epitopes. To challenge this assumption, cells were subjected to a standard acid wash procedure causing the dissociation of pMHC complexes and subsequent decay of MHC-I heterodimers on the cell surface (212, 231). The fate of total
H2-K\(^b\) molecules in both UbMGagSL/EL4 and UbRGagSL/EL4 cell clones after the acid wash (time point “0 min”, Fig. 5.5 A) was comparable and revealed a reduction below 50% of the original value (time point “-10 min”; Fig. 5.5 A). After the acid wash a slow, but continuous recovery of cell surface H2-K\(^b\) molecules was observed that followed comparable kinetics in both cell clones (Fig. 5.5 A). Although both, the steady-state levels and the kinetics of recovery of H2-K\(^b\), were almost identical in both cell clones, specific staining of SL-H2-K\(^b\) complexes revealed a different picture: first, and as observed already above (Fig. 5.4), the presentation of SL epitopes was approximately twofold higher in UbRGagSL processing cells (Fig. 5.5 B, time point “-10 min”). Second, after acid wash that causes a drop to almost identical baseline levels (Fig. 5.5 B, time point “0 min”), the kinetic of recovery of SL-H2-K\(^b\) complexes on the cell surface was clearly faster in the UbRGagSL/EL4 clone. According to previous studies that demonstrated the generation of epitopes from de novo synthesized Ag of different origin in a proteasome dependent manner (133, 211, 228, 232-236) control experiments using protein synthesis inhibitors (PSIs) or PIs (Fig. 5.5 C and D) revealed that both, protein biosynthesis as well as the activity of the proteasome are necessary in both cell clones for efficient MHC-I Ag presentation. This was shown for total H2-K\(^b\) (Fig. 5.5 C) as well as SL-loaded H2-K\(^b\) molecules (Fig. 5.5 D).

5.1.6  In vitro activation of the SL-H2-K\(^b\) specific T-cell hybridoma B3Z

In order to analyze whether the augmented SL-presentation on UbRGagSL/EL4 cells compared to UbMGagSL/EL4 cells results in enhanced T-cell activation, the SL-H2-K\(^b\)-specific T cell line B3Z was used. The B3Z hybridoma cell line has been generated by fusion of the NFAT-lacZ T cell clone with a SL-specific V\(\alpha\)2/V\(\beta\)5 T cell clone (237) and expresses the lacZ gene under control of the NFAT promoter. After overnight cocultivation of B3Z cells with corresponding EL4 target cells, the specific T-cell activation was detected by a colorimetric assay based on β-Gal activity, thus detecting TCR signaling.

First, we established that the experimental conditions were adequate to detect specifically differences in the presentation of H2-K\(^b\)-SL complexes. Incubation of B3Z cells with increasing concentration of the synthetic peptide SIINFEKL or a control peptide SIIKFEKL demonstrated that exogenously added epitopes are bound to cell surface H2-K\(^b\) molecules and presented to B3Z cells (Fig. 5.6 A). A linear correlation between peptide concentration and activation of B3Z cells was observed in the range of 0.3 to
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30 ng/ml, which corresponds to approximately 0.3 to 30 nM of available peptides. Complete absence of T-cell activation in the case of the control peptide confirms the high specificity of the B3Z line. In a further experiment, parental EL4 cells were incubated for 1 h with increasing concentrations of exogenously added peptides. After intensive washing, the cells were mixed with B3Z cells in an E (effector):T (target) ratio of 1:1 (Fig. 5.6 B), revealing that even a short peptide pulse can induce T-cell activation that started at 10 ng/ml of added SL. Thus, the B3Z cell line represents a very sensitive and specific indicator for SL-presentation which is able to detect subtle differences in epitope density at the level of the signaling pathway. When Gag expressing EL4 clones were co-cultivated with B3Z cells at different E:T ratios ranging from 1:10 down to 1:0.013, a significant stronger T-cell activation was observed for the UbRGagSL/EL4 clone compared to the UbMGagSL/EL4 counterpart, as shown in a representative experiment in Fig 5.6 C.

![Fig. 5.6: In vitro activation of the SL-H2-Kb-specific T cell line B3Z.](image)

(A) B3Z cells were incubated with the synthetic peptides SIINFEKL or SIIFKEKL in rising concentrations and activation of B3Z cells was assessed. (B) Parental EL4 cells were incubated for 1 h with externally added peptides in various concentrations. After intensive washing, the cells were co-cultured with B3Z cells in an E:T ratio of 1:1. (C) Transgenic EL4 cells were co-cultured with B3Z cells in an T:E ratio ranging from 10:1 to 0.013:1 in 96 well plates for 16 h. T-cell activation was analyzed by addition of 0.15 mM CPRG/0.5 % NP-40 in PBS and measuring the absorbance at 595 nm. AUFS: arbitrary units full scale.
5.1.7 In vivo activation of SL-H2-K^{b}-specific OT-1 cells and induction of SL-specific CD8^{+} T cells in naïve mice

To test whether the enhanced MHC-I presentation of SL epitopes generated from the UbRGagSL variant also augments T-cell stimulation in vivo, adoptive transfer experiments in which UbMGagSL/EL4 and UbRGagSL/EL4 cells were transferred together with OT-1 splenocytes into naïve C57BL/6 mice were performed by Dr. A. Goldwich. The OT-1 mouse is transgenic for the SL-specific TCR (V\alpha2/V\beta5), with approximately one third of all splenocytes as well as LN cells representing naïve, SL-specific CD8^{+} T cells (238). As positive controls both, the OVA-expressing EL4-derived cell line E.G7 (239) as well as the minigene-expressing SL/EL4 cell clone (see Fig. 5.4 A) were employed, while the parental EL4 cells served as negative control. To quantify proliferation of OT-1 T cells, splenocytes and LN cells derived from OT-1 mice were labeled with CFSE (Carboxyfluorescein succinimidyl ester). This method is meant to track the number cell divisions in vivo (240). In order to prevent target and effector cell encounter prior to injection and, in addition, to enhance the chance of interaction of both cell types in the recipient mouse, OT-1 cells were first injected into one tail vein and, after a delay of 5 min, the SL-presenting EL4 cells were injected into the contralateral vein. Two days following cell transfer, the spleens of the recipient C57BL/6 mice were removed and the splenocytes were analyzed by flow cytometry. Among splenocytes, the OT-1 cells were identified as V\alpha2- and CFSE-positive cells (Fig. 5.7 A, gate R3). In contrast to V\alpha2-negative cells (Fig. 5.7 A, gate R4), a certain fraction of the V\alpha2-positive cell population showed clear evidence for proliferation which was evident from a decaying CFSE fluorescence intensity. Analyzing V\alpha2- and CFSE-positive cells derived from the UbRGagSL/EL4 recipient revealed a relatively higher number of OT-1 cells that were primed and had started to proliferate compared to OT-1 cells derived from the UbMGagSL/EL4 recipient mice (Fig. 5.7 A, lower panels). This notion was further supported by the down-regulation of CD62L-expression on the surface of V\alpha2- and CFSE-positive cells which is indicative of an activated T cell phenotype (data not shown). The statistical analysis of 10 independent experiments revealed that SL-expressing cells stimulate the V\alpha2- and CFSE-positive population of the transferred spleen and LN cells to proliferate at different levels (Fig. 5.7 B). To compare differences in OT-1 T-cell stimulation, a so-called proliferation index was calculated, which takes into account the number of proliferating cells relative to the number of cell divisions.
Fig. 5.7: T-cell activation by UbMGagSL/EL4 or UbRGagSL/EL4 cells in vivo. (A+B) Activation of OT-1 cells (A) Two days after adoptive co-transfer of UbM- or UbRGagSL/EL4 cells and CFSE-labeled OT-1 cells into wt C57BL/6 mice, splenocytes were analysed by flow cytometry. The CFSE-signal of Vα2-positive OT-1 cells in the live lymphocytes-gate was analyzed. Plots represent data from one out of ten mice. (B) To calculate the proliferation stimulus of the transgenic EL4 cells, the cell numbers of primed relative to naïve Vα2-positive cells (n_{primed}/n_{naïve}) was multiplied by the number of cell divisions (log₂(MFI_{naïve}/MFI_{primed})). Values are given as mean ± SD (n=10, *=p<0.05). (C) Quantification of the SL-specific T-cell response by IFN-γ ELISPOT after immunization of naïve mice with EL4 cells. C57BL/6 mice were intravenously injected with UbMGagSL/EL4 or UbRGagSL/EL4 cells, respectively. Parental EL4 cells served as negative, synthetic SL peptide injected subcutaneously (SL s.c.) as positive control. Splenocytes were isolated 9 days post immunization and incubated with or without synthetic SL peptide. Each circle represents the frequency of IFN-γ secreting T cells from one individual mouse (mean of triplets). Mean values are depicted as bars (n = 14, *=p<0.05).
The comparison of the proliferation indices induced by UbRGagSL/EL4 cells (0.85+/-.31) and UbMGagSL/EL4 cells (0.53+/-.25) revealed that UbRGagSL induces a significantly stronger proliferation of adoptively transferred OT-1 T cells. Thus, the efficiency of SL-presentation in UbRGagSL/EL4 cells correlates with a better T-cell activation in vivo as quantified on the level of T-cell proliferation.

To further substantiate the notion that the increased DRIP-rate and MHC-I Ag presentation correlate with a more efficient induction of a T-cell response in vivo, naïve C57BL/6 mice were injected intravenously with UbMGagSL/EL4 or UbRGagSL/EL4 cells, respectively, and activation of SL-specific CD8+ T cells was quantified by IFN-γ ELISPOT. Parental EL4 cells served as negative control, while synthetic SL peptide (50 µg) was injected subcutaneously (SL s.c.) as a positive control. The results shown in Fig. 5.7 C reveal that significantly higher frequencies of SL-specific T cells were induced by immunization with UbRGagSL/EL4 cells when compared to immunization with UbRGagSL/EL4 cells. These data indicate that, in naïve mice, UbRGagSL/EL4 cells are more potent in activating not only transgenic OT-1 T cells, but also naïve SL-specific T cells.

5.1.8 In human cells, Gag is targeted into the MHC-I pathway by the N-end rule, but even more efficiently by stable N-terminal fusion to Ub

In parallel to experiments performed in murine EL4 cells, the number of SL epitopes derived from the Ag processing of UbMGagSL or UbRGagSL was assessed by flow cytometry in a human cell line. In contrast to the low expression levels obtained in murine cell lines (Fig. 5.2 and data not shown), high expression levels were achieved after transient transfection of the human cell lines HeLa or 293T with psyngag expression plasmids (Fig. 5.8 and data not shown), allowing for FACS analysis of bulk cell populations.

Following transient transfection with psyngag expression plasmids, transgenic HeLa cells that stably express high levels of H2-K\(^b\) (see Fig. 5.14), named HeLa-K\(^b\) (241), were co-stained with 25D1.16-647 specific for the H2-K\(^b\)-bound SL epitope and, intracellularly, with KC57-FITC for detection of GagSL. To normalize for possible differences in Gag expression levels, the MFI of the staining with 25D1.16-647 was divided by the MFI of the staining with KC57-FITC after gating on Gag-expressing cells. Cells expressing Gag lacking the SL sequence served as a negative control for the 25D1.16 staining. The data of four independently performed experiments are shown in Fig. 5.8 A.
Fig. 5.8: Analysis of SL-presentation in transiently transfected HeLa-Kb cells. (A) Comparison of the presentation of the SL-epitope inserted at the N-terminus of Gag (SLN-term), in the p2 spacer region (SLp2) or at the C-terminus of Gag (SLC-term). Following transfection of HeLa-Kb cells with expression plasmids coding for GagSL variants, H2-Kb-SL complexes presented on the surface of Gag-positive cells were quantified by flow cytometry using 25D1.16-647. Cells expressing wt Gag lacking the SL epitope served as a negative control. Bars represent mean values +/- SD (n=4; * = p<0.05; ns = not significant). (B) A fraction of the transfected HeLa-Kb cells analysed in A was lysed and, together with VLPs pelleted from the cell culture supernatant, subjected to SDS-PAGE and Western blotting using anti-p24 antiserum. As loading control, membranes were reprobed using an anti-β-actin Ab.

Compared to wt GagSL, higher numbers of SL-H2-Kb complexes were detected on the surface of HeLa-Kb cells expressing UbMGagSL. Expression of UbRGagSL resulted in a further increase in SL-presentation, albeit not statistically significant. Stable N-terminal in frame fusion of Gag to Ub that is achieved by mutation of the C-terminal Gly residue of the Ub moiety (UbG76V GagSL) resulted in more than threefold enhancement of SL-presentation (Fig. 5.8 A). Alternatively to introduction of the SL epitope within the p2 spacer (GagSLp2), SL was added to the C-terminus of Gag variants (GagSLC-term.). In addition, the sequence MSIINFEKL was introduced N-terminally of the wt protein (GagSLN-term.). Analysis of SL-presentation derived from the N-terminus was not possible
for Ub fusions due to the cotranslational cleavage of the Ub moiety. Comparison of SL-presentation between the different constructs by double staining with 25D1.16-647 and KC57-FITC as described above, clearly showed that only the internal position of SL resulted in efficient MHC-I presentation (Fig. 5.8 A). While SL located at the C-terminus of Gag was presented poorly by H2-K\(^b\) molecules, N-terminal SL seemed to be completely destroyed by Ag processing and was not presented above background level that was determined by staining of cells expressing Gag lacking the SL epitope (Fig. 5.8 A).

In parallel, expression of the GagSL proteins and release of VLPs was analysed by Western blotting using an anti-p24-specific Ab (Fig. 5.8 B). While Ub was efficiently cleaved from UbMGagSL and UbRGagSL fusions, Ub\(^{G76V}\)GagSL migrated as a stable fusion protein of about 100 kDa (Fig. 5.8 B). As a loading control, blots were stripped and reprobed using an anti-\(\beta\)-actin Ab. Although introduction of the SL epitope alone did not affect the release of VLPs (compare lanes 1 and 2 in Fig. 5.8 B), expression as an Ub fusion protein clearly interfered with budding. While the UbM fusion, which corresponds to \(wt\) Gag after Ub cleavage, is only mildly attenuated in terms of budding, release of VLPs is severely impaired by exchange of the N-terminal aa for Arg, both in the context of UbRGagSL\(_{p2}\) (Fig. 5.8 B, lane 4) and UbRGagSL\(_{C-term}\) (Fig. 5.8 B, lane 9), and almost completely abolished by stable fusion to Ub (Fig. 5.8 B, lane 5).

Taken together, targeting of HIV-1 Gag for degradation by the UFD pathway tremendously enhances MHC-I presentation of the Gag-derived SL epitope in HeLa-K\(^b\) cells. Targeting Gag for the N-end rule pathway has similar, but less pronounced effects compared to those observed in murine EL4 cell lines. Moreover, these results point out that the kinetics of Ub removal might be different between murine EL4 cells and human HeLa-K\(^b\) cells, as the UbMGagSL variant behaves different from the \(wt\) protein in terms of MHC-I Ag presentation and VLP-release.

### 5.1.9 N-end rule and UFD degradation signals do not influence the synthesis or metabolic half-life of Gag in HeLa cells

It was hypothesized that an increased turn-over of Gag might slow the rate of accumulation in cell when transiently expressed and, finally, lead to a diminished steady-state level. Although it was observed that all mutants reached similar steady-state protein levels after transfection of Gag-encoding expression plasmids in a human HeLa-derived cell line, we performed kinetic analyses to test if the mutants were synthesized at
comparable rates, given that *syngag* is expressed very rapidly and efficiently and, thus, saturation effects may occur in human cells. Therefore, HeLa cells were transfected with individual *syngag* expression plasmids and cell samples as well as cell culture supernatants were collected at different time points after transfection. Gag was detected by anti-p6 Western blotting following SDS-PAGE of cell lysates and VLPs. Being detectable as soon as four hours after transfection, increasing amounts of Pr55 accumulated with comparable kinetics, yielding stable protein levels approximately 12 hours after transfection (Fig. 5.9 A).

**Fig. 5.9:** N-end rule or UFD degradation signals do not influence the synthesis or the metabolic half-life of Gag. (A) HeLa cells were harvested at indicated points after transfection of *syngag* expression constructs and Gag synthesis was monitored by Western blotting using anti-p6 serum. Blots are representative of three independently performed experiments. (B) For pulse-chase experiments, HeLa cells were transfected with *syngag* expression constructs, radiolabelled for 20 min using [35S]Met and chased for up to 48 h. Pr55 was recovered from cell lysates by IP using anti-p6 and anti-p24 Abs, followed by SDS-PAGE and fluorography. (C) Densitometric quantification of [35S]-labeled Pr55. The radioactivity of the Pr55 band was quantified using a phosphor imager and plotted as percentage of the initial signal. Values represent mean +/- SD of three independent experiments.

Given the clear differences between the Gag variants in terms of MHC-I Ag processing (see Fig. 5.4 and Fig. 5.8), it was reasonable to assess the metabolic half-life of Gag by pulse-chase experiments. Following metabolic labeling of HeLa cells transiently transfected with Gag expression plasmids with [35S]Met, Pr55 was recovered from cell lysates by IP using a mixture of Gag-specific Abs. As shown in Fig. 5.9 B+C, all Gag
variants displayed a metabolic half-life of approximately 9 hours comparable to the \textit{wt} protein. In summary, none of the degron signals tested in this study significantly affected the stability of Pr55.

5.1.10 N-end rule and UFD degradation signals interfere with the release of VLPs

Although none of the degradation signals markedly affected the half-life or steady-state level of Gag, they could still impair its correct folding during or shortly after synthesis and, thus, enhance the DRiP-rate of Gag, as shown in EL4 cells (Fig. 5.3). Accumulation of Gag-DRiPs can interfere with the highly ordered processes of assembly, budding and release of VLPs. Steady-state Western blot analysis (see Fig. 5.8) already indicated a budding defect of UbRGagSL and Ub\textsuperscript{G76V}GagSL. Therefore, the efficiencies of VLP release, being regarded as indicative of the functionality of the Gag protein, were more precisely quantified by time course Western blot analyses. Equal numbers of HeLa cells were aliquoted 24 hours posttransfection after extensive washing in ice-cold PBS. VLPs released into the cell culture medium during an incubation period of up to four hours were pelleted through a sucrose cushion. Cell- and VLP-associated Gag was analyzed by SDS-PAGE followed by immunoblotting with anti-p6 antiserum (Fig. 5.10 A). Membranes were reprobed for \(\beta\)-actin as a loading control.

Following densitometric evaluation of Pr55-specific bands, release efficiencies were calculated as \([\text{Pr55 (VLPs)}*100 \%/ \text{Pr55 (VLPs + Cell)}]\) and budding of the Gag variants was compared (Fig. 5.10 B, \(n=3\)). Although it should correspond to \textit{wt} Gag after cleavage of the Ub moiety, the cleavable Ub fusion to the N-terminus (UbMGag), exhibited a release efficiency reduced by half when compared to the \textit{wt} protein (Fig. 5.10 B). However, when the N-terminal Met was replaced by an Arg residue (UbRGag) or when Ub remained attached to Gag at the N-terminus (Ub\textsuperscript{G76V}Gag), the amount of released VLPs dropped to almost undetectable levels, similar to those observed after expression of a myristoylation-deficient Gag mutant (Gag\textsuperscript{G2A}; data not shown).

The Western blot results were confirmed by thin-section electron microscopy (Fig. 5.10 C). HeLa cells were transiently transfected with psyngag and variants thereof and, 24 h posttransfection, cultivated for further 24 h in cellulose capillary tubes to prevent free diffusion of released virions, thus avoiding the need for centrifugation that could affect VLP structure. Untransfected cells served as negative control (mock). In contrast to HeLa cells expressing \textit{wt} Gag or UbMGag, from which numerous VLPs with typical immature morphology were released, only few viral structures were detected extracellularly after expression of UbRGag. Expression of Ub\textsuperscript{G76V}Gag resulted in the
formation of enlarged and deformed extracellular structures that could not be unambiguously identified as VLPs.

Fig. 5.10: N-end rule and UFD degradation signals interfere with the release of VLPs derived from synGag. (A) Time-course Western blot analysis of VLP release. Equal numbers of HeLa cells that were transiently transfected with Gag expression plasmids were aliquoted, cultured for up to 4 h while shaking and collected at the indicated time points. VLPs pelleted from the cell culture supernatant through a 20% sucrose cushion and cell lysates were subjected in parallel to SDS-PAGE and Western blotting using an anti-p6 antiserum. (B) Densitometric quantification of VLP release. Gag-specific bands in Western blots from three independent experiments were quantified using the software AIDA. VLP release calculated as the Gag fraction contained in pelleted VLPs relative to total Gag was plotted against time. Mean values +/- SD are shown. (C) Electron microscopic analysis of VLP release. Transmission EM pictures of the PM of HeLa transfected with Gag expression plasmids.
5.1.11 N-end rule and UFD degradation signals disturb the membrane localization of Gag

For targeting of HIV-1 Gag to membranes and thus, for the assembly, budding and release of virus or VLPs at the PM, myristoylation at the Gly residue at position 2 is essential. Hence, the inability of Ub$^{G76V}$Gag to form VLPs could be easily explained by the absence of an N-terminal myristoylation site in the protein. However, the negative impact of cleavable N-terminal Ub fusion on budding and release of synGag-derived VLPs could be caused by a defect in myristoylation and localization. Based on the observation, that proteasome inhibition, leading to the accumulation of DRiPs, interferes with HIV-1 budding and maturation (90), it has been proposed that Gag DRiPs might disturb budding by preventing the ordered assembly of functional Gag molecules in a prion-like manner (89, 91, 123).

To test whether Ub fusions to the N-terminus of Gag disturb the localization, immunocytochemistry as well as subcellular fractionation was performed using HeLa cells transiently transfected with psyngag expression plasmids.

Direct immunofluorescence staining using a FITC-conjugated anti-p24 Ab (KC57-FITC) revealed that, in agreement with the data obtained in EL4 cells (136), Gag wt and UbMGag are diffusely distributed in the cytoplasm and at the PM of transfected HeLa cells (Fig. 5.11 A and B). In a fraction of all cells, a more intense perinuclear staining was detected. UbRGag and Ub$^{G76V}$Gag, however, show a more punctuate staining pattern that could be the result of Gag protein aggregates. Thus, the exchange of the N-terminal Met residue for Arg affects the subcellular distribution of Gag, though not dramatically. Similar results were obtained when GagSL-GFP fusion proteins were visualized instead of Gag immunostaining following expression of psyngag constructs (data not shown).

To further substantiate this notion and to clarify if Gag can still bind to membranes, membrane flotation by density gradient centrifugation was performed. Therefore, cells were homogenized by sonification and nuclei, as well as unbroken cells were removed by centrifugation at 2000 x $g$. The cleared homogenate was subjected to membrane flotation on an Optiprep gradient and, following centrifugation for 5 h at 150,000 x $g$, eight fractions were collected from the top of the gradient, denatured and examined by SDS-PAGE and Western blotting using anti-p6 Ab. To control the fractionation, the distribution of cellular proteins localized in the membrane fraction (Transferrin receptor, TfR) or soluble fraction (ribosomal P antigen, RP0) was assessed using specific Abs. Though TfR was also found in the two bottom fractions, possibly due to solubilization, the RP0 was exclusively localized in the soluble bottom fractions.
Fig. 5.11: Analysis of Gag subcellular localization. (A-D) HeLa cells expressing Gag wt (A) or variants UbMGag (B), UbRGag (C) or UbG76VGag (D) were stained with the p24-specific KC57-FITC Ab. Nuclei were stained with DAPI. (E) Membrane flotation by density gradient centrifugation on an Optiprep gradient. HeLa cells expressing Gag variants were homogenized after removal of nuclei and unbroken cells and subjected to membrane flotation on a discontinuous Optiprep gradient. Fractions were collected from the top of the gradient, denatured and examined by SDS-PAGE and Western blotting using anti-p6 Ab. As a control, the distribution of the Transferrin receptor (TfR) and the ribosomal P Ag (RP0) was detected using specific Abs. (F) The fraction of membrane-associated Gag was calculated following densitometric quantification of anti-Gag Western blots obtained from three independent experiments. Mean values +/- SD are depicted.
As a control, the unmyristoylated Gag\textsuperscript{G2A} mutant, which is known to be unable to bind to membranes, was analysed in parallel and detected only in soluble fraction (Fig. 5.11 B), while \textit{wt} Gag was readily found in membrane fraction. Having thus confirmed that fractionation in these gradients depends on membrane localization of Gag, membrane association of the Gag Ub-fusion variants was analysed. Ub\textsuperscript{G76V}Gag, like Gag\textsuperscript{G2A} and as expected, was entirely localized in the soluble fraction. Following densitometric quantification of Gag signals in Western blots, the relative membrane association of the Gag variants was calculated based on data from three independent experiments (Fig. 5.11 C). Whereas approximately 55 % of \textit{wt} Gag and 40 % of UbMGag was localized in the membrane fraction, membrane association of UbRGag was significantly reduced to background level that was defined by Gag\textsuperscript{G2A} and Ub\textsuperscript{G76V}Gag.

Taken together, these data indicate that Ub\textsuperscript{G76V}Gag as well UbRGag fail to associate with cellular membranes which explains the budding defect observed for these variants. In agreement with an only mildly attenuated release of VLPs, UbMGag is still able to bind to membranes, though slightly less efficient when compared to the \textit{wt} protein. As the tendency of Gag to bind to membranes can be regarded as indirect evidence for its myristoylation, these results underline that processing of the N-terminal Met residue proceeding the acceptor Gly residue is a prerequisite for efficient myristoylation (242).
5.2 The PTAP Late domain regulates ubiquitination and MHC-I antigen presentation of HIV-1 Gag

5.2.1 The PTAP L-domain in the p6 region regulates budding of GagSL-derived VLPs.

As the PTAP L-domain of HIV-1 Gag has been shown by others to affect the level of Gag ubiquitination (114, 120, 121), we set out to test whether, besides its function in virus release, the PTAP motif can also regulate the MHC-I Ag processing of Gag. The PTAP motifs within the p6 region GagSL were exchanged for the sequence AIVA by site-directed mutagenesis, resulting in mutants that lack one (ΔPTAP1) or both PTAP L-domains (ΔPTAP2) as schematically depicted in Fig. 5.12 A.

![Fig. 5.12 The PTAP L-domain in the p6 region regulates budding of GagSL-derived VLPs. (A) Schematic representation of syngag-encoded wt and PTAP-deficient GagSL proteins used in this study. (B) Impaired release of PTAP-deficient GagSL as quantified by Western blot time course analyses. (C) Densitometric evaluation of the blots shown in (B). The time course of VLP release was calculated as the percentage of Gag present in the VLP fraction relative to the total amount of Gag.](image)

To test whether the PTAP L-domains are functional in terms of driving the release of VLPs derived from unprocessed GagSL, Western blot time-course analyses of budding were performed. Compared to wt GagSL, VLP-release was reduced by approximately 20 % when only one PTAP motif was mutated, and further reduced down to 60 % in the absence of both PTAP L-domains (Fig. 5.12 B and C). This result supports the functionality of the PTAP L-domain in the context of unprocessed Gag harboring the SL epitope. In further experiments, the ΔPTAP2 mutant is analyzed and will be referred to as ΔPTAP or the PTAP-mutant from hereon.
5.2.2 The PTAP L-domain regulates ubiquitination of GagSL

Next, we wanted to test whether the PTAP L-domains can regulate the ubiquitination of syngag-encoded, unprocessed GagSL. A quick cell lysis was performed under conditions where the proteasome and DUBs were inhibited, and the whole cell lysates were subjected to SDS-PAGE and Western blotting using Gag-specific Abs. A ladder of bands reminiscent of ubiquitinated Gag species migrating slower than Pr55 was detected when the PTAP-mutant was expressed. These bands were virtually absent in case of the wt GagSL protein (Fig. 5.13 A).

![Fig. 5.13 The PTAP L-domain regulates ubiquitination of GagSL.](image)

(A) Western blot (WB) of whole HeLa cell lysates expressing wt or PTAP-deficient GagSL. As a loading control, the blot was reprobed using an anti-β-actin antibody. (B) IP of ubiquitinated Gag species. Following coexpression of GagSL with HA-tagged Ub in HeLa cells, Gag was precipitated under denaturing conditions from cell lysates using anti-HIV serum prebound to protein G-sepharose. Ubiquitinated Gag was detected by anti-HA Western blotting. The membrane was stripped and reprobed with anti-HIV to demonstrate that equal amounts of Gag protein were precipitated (lower panel). (C) Reduced recovery of polyubiquitinated Gag species after coexpression of HA-UbK48R mutant. After coexpression of wt Gag or the PTAP-mutant with HA-Ub, HA-UbK48R or HA-UbK63R, Gag was immunoprecipitated and ubiquitinated species were detected by anti-HA Western blotting. The membrane was reprobed with anti-HIV (lower panels).

To confirm that mutation of PTAP augments ubiquitination of GagSL, N-terminally HA-tagged Ub (HA-Ub) was coexpressed with GagSL variants in HeLa cells. GagSL was immunoprecipitated from denatured cell lysates using a pooled serum from HIV-1 infected patients. The precipitates were subjected to SDS-PAGE, and GagSL-HA-Ub conjugates were visualized by anti-HA Western blotting. This allowed for the specific
detection of ubiquitinated Gag, as the signal is absent when either GagSL or HA-Ub were expressed alone (Fig 5.13 B, lanes 1, 2 and 5). GagSL-HA-Ub conjugates were detected as two discrete bands and a smear characteristic of polyubiquitinated protein species. Although similar quantities of GagSL were precipitated (Fig 5.13 B, lower panels), higher amounts of ubiquitinated GagSL were recovered after mutation of the PTAP motifs (Fig 5.13B, upper panels).

To investigate the type of Ub linkage, GagSL wt or ΔPTAP were coexpressed with mutant forms of HA-Ub, carrying single Lys to Arg substitutions at either Lys48 (HA-Ub\textsuperscript{K48R}) or Lys63 (HA-Ub\textsuperscript{K63R}). Overexpression of these Ub mutants has been shown to interfere with the formation of Lys48- or Lys63-linked poly-Ub chains, respectively (243, 244). Due to the abundance of wt Ub in mammalian cells (245), HeLa cells were first transfected with expression plasmids coding for Ub mutants and, 48 hours later, with Ub expression plasmids and GagSL-GFP expression plasmids together. When the PTAP-mutant was coexpressed HA-Ub\textsuperscript{K48R}, the intensity of the signal was reduced to wt levels (Fig. 5.13 C). Overexpression of the HA-Ub\textsuperscript{K63R} mutant resulted only in slightly reduced recovery of ubiquitinated GagSL\textsuperscript{ΔPTAP} (Fig. 5.13 C). These results indicate that mutation of the PTAP L-domains results in predominantly Lys48-linked polyubiquitination of GagSL.

5.2.3 The PTAP, but not the YP(X)\textsubscript{n}L L-domain regulates MHC-I antigen presentation of a Gag-derived epitope

To investigate whether the increased ubiquitination of the PTAP-mutant correlates with enhanced class-I presentation of Gag-derived SL, HeLa-K\textsuperscript{b} cells were transfected with expression plasmids that code for GagSL-GFP fusion proteins. Flow cytometry using the mAb 25D1.16 revealed that cells expressing PTAP-deficient Gag displayed approximately two- to threefold higher numbers of H2-K\textsuperscript{b}-SL complexes at the cell surface when compared to cells expressing the wt protein (Fig. 5.14 A). Indirect effects on the MHC-I pathway causing variations in the total amount of cell surface MHC-I could be excluded by staining with a mAb that specifically binds to H2-K\textsuperscript{b} molecules irrespectively of the epitope that is displayed (Fig. 5.14 B).

Evidently, the amount of protein available for Ag processing intracellularly is one determining factor for the number of epitopes derived from that Ag presented on the cell surface (246).
The PTAP, but not the YP(X)_nL L-domain, regulates MHC-I antigen presentation of a Gag-derived epitope. (A) Following transfection of HeLa-K^b cells with expression plasmids coding for GagSL-GFP wt or ΔPTAP, H2-K^b-SL complexes presented on the surface of GFP-positive cells were quantified by flow cytometry using the mAb 25D1.16 and a secondary Alexa647-conjugated anti-mouse Ab. To assess unspecific binding of the secondary Ab, cells were stained with secondary Ab only (control). A representative histogram plot is shown. (B) Samples were stained in parallel with cell culture supernatant of the hybridoma cell line B8-24-3 (B8), followed by staining with secondary Alexa647-conjugated anti-mouse Ab to assess total cell surface H2-K^b. (C) Quantification of SL-presentation including a GagSL-GFP variant with a mutated YP(X)_nL motif (ΔYP). The mean fluorescence intensity (MFI) of the 25D1.16 staining was normalized to GFP fluorescence. Bars represent mean values +/- SD. (n=7; ΔYP n=3; * = P<0.05; ** = P<0.01). (D) More efficient T-cell activation by PTAP-deficient GagSL in vitro. Activation of B3Z hybridoma T cells was assessed by a colorimetric β-Gal assay after overnight cocultivation with HeLa-K^b cells expressing GagSL wt or ΔPTAP in various effector to target ratios. (E) Western blot analysis of GagSL variants. HeLa cells expressing wt GagSL or variants ΔPTAP or ΔYP were lysed and whole cell lysates as well as VLPs pelleted from the cell culture supernatant were subjected to SDS-PAGE and Western blotting using anti-p24 antiserum.
In order to compensate for possible differences in expression levels of the GagSL-GFP variants, we normalized the MFI of the staining with 25D1.16 to the MFI of the GFP fluorescence in seven independent experiments, revealing a significant increase in SL-presentation when the ΔPTAP variant was expressed when compared to the wt (Fig. 5.14 C).

To exclude that the augmentation of SL-presentation associated with disruption of the PTAP motif results from introduction of the motif AIVA, the PTAP L-domain was alternatively exchanged for the sequence LIRL, which is commonly used in full-length HIV-1 expression systems to avoid substitutions in the overlapping pol reading frame. Intriguingly, the increase in the number of H2-K\(^b\)-SL complexes presented on the cell surface of cells expressing a PTAP-mutant was almost identical, regardless of whether PTAP was exchanged for AIVA or LIRL (data not shown).

In addition to the primary PTAP L-domain, an auxiliary YP(X)\(_n\)L L-domain motif is located more C-terminally within p6. To answer the question whether the second L-domain in HIV-1 Gag is also involved in the regulation of MHC-I Ag processing, the sequence YPLTSL was exchanged for RSLTSL in the context of GagSL, and SL-presentation of this ΔYP-mutant was analyzed. Staining with 25D1.16, as described above, revealed that the number of H2-K\(^b\)-SL complexes displayed at the surface of cells expressing the ΔYP mutant is identical to those expressing wt GagSL (Fig. 5.14 C). To assess the release of VLPs derived from the ΔYP mutant, VLPs were pelleted from the cell culture supernatant of HeLa cells transfected with expression plasmids coding for GagSL\(_{wt}\) or the variants ΔPTAP or ΔYP, respectively. Consistent with the kinetic data (Fig. 5.12 B), only minor amounts of VLPs were detected following expression of GagSLΔPTAP, whereas the ΔYP mutant was comparable to the wt protein with respect to VLP release (Fig. 5.14 D). In Western blot analyses of whole cell lysates using a Gag-specific Ab, GagSLΔPTAP displayed a ladder of ubiquitinated Pr55 (see also Fig. 5.13 A), while the ΔYP mutant shows no increased ubiquitination when compared to the wt (Fig. 5.14 D).

To confirm the results obtained by flow cytometry, we tested the ability of HeLa-K\(^b\) cells expressing GagSL either in its wt form or with a mutated PTAP motif, to activate B3Z hybridoma T cells. As a negative control, HeLa-K\(^b\) cells were transfected with an empty vector (mock). After overnight cocultivation, β-Gal activity was measured by a colorimetric assay, showing that cells expressing the PTAP-mutant more efficiently activate T cells over a broad range of effector-to-target ratios when compared to cells
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expressing the wt protein (Fig. 5.14 E). Taken together, these data indicate that the PTAP L-domain can indeed regulate the entry of Gag into the MHC-I pathway.

5.2.4  Induction of the immunoproteasome enhances presentation of the SL-epitope derived from GagSL-GFP

In vivo, naïve CD8\(^+\) T cells are predominantly primed by DCs, which express not only the constitutive subunits of the 26S proteasome, but also the immunosubunits \(\beta1\) (LMP2), \(\beta5\) (LMP7), \(\beta2\) (MECL1) and the 11S activator PA28 (40). The immunosubunits can be induced by IFN-\(\gamma\) and replace the constitutive subunits in nascent proteasomes to build the immunoproteasome (37, 38). Immunoproteasomes have been described to have altered cleavage specificities, resulting in a modified spectrum of class I-presented epitopes (41), and an enhanced cleavage capacity (43-47). Recently, IFN-\(\gamma\) has been shown to induce oxidative stress in cells, leading to the accumulation of DRiPs that are subsequently cleared by the enhanced proteolytic activity of newly formed immunoproteasomes (247). This finding points towards a more general role for IFN-\(\gamma\) in the generation class-I peptide ligands.

Fig. 5.15: Induction of the immunoproteasome enhances presentation of the SL-epitope derived from GagSL-GFP wt and ΔPTAP. (A) HeLa-K\(^b\) cells expressing GagSL-GFP wt or ΔPTAP were treated with 250 U of IFN-\(\gamma\) for 16 h or left untreated as a control. H2-K\(^b\)-SL complexes presented on the surface of GFP-positive cells were quantified by flow cytometry using the mAb 25D1.16 conjugated to APC. The MFI of the 25D1.16 staining was normalized to the MFI of the GFP fluorescence. Bars represent mean values +/- SD from three independent experiments. (B) Aliquots of the cells used in (A) were lysed, subjected to SDS-PAGE and Western blotting. GagSL-GFP was detected using a pooled serum from HIV-1-positive patients. Induction of the immunoproteasome was confirmed by staining against the inducible subunit \(\beta5\). The blot was stripped and reprobed using an antiserum directed against the constitutive proteasome subunit \(\beta5\). Staining for \(\beta\)-actin served as a loading control.
Therefore, it was tested whether induction of immunoproteasomes affects the Ag processing of GagSL. HeLa-Kb cells expressing GagSL-GFP wt or ΔPTAP were treated with IFN-γ for 16 h and SL-presentation was assessed using fluorescently labeled 25D1.16 Ab. Cells expressing GFP served as negative control. IFN-γ treatment resulted in augmentation of MHC-I presentation of the SL-epitope derived from GagSL wt and ΔPTAP (Fig. 5.15A). An increased level of β5i (LMP7) as detected by Western blotting (Fig. 5.15B), served as control for induction of immunoproteasomes by IFN-γ. This result indicates that first, IFN-γ has an overall positive effect on the presentation of the SL epitope derived from GagSL and second, that Gag-DRiPs are not only a bona fide substrate for constitutive standard proteasomes (133), but also for IFN-γ induced immunoproteasomes.

5.2.5 The PTAP L-domain regulates MHC-I antigen presentation of the SL epitope derived from processed Gag

We next asked whether the PTAP L-domain not only regulates entry of the unprocessed Pr55 polyprotein precursor, but also of processed Gag into the MHC-I pathway. To answer this question, the SL-coding sequence has been introduced into the proviral constructs pNL4-3 (248), pBRNL4-3nef-IRES-GFP, carrying an internal ribosome entry site (IRES) followed by the gfp gene (249), and the HIV-1NL4-3 subgenomic expression vector pNLenv, in which the env gene was deleted (250). Thereby, the SL epitope is located in the p2 spacer region corresponding to its position in syngag-encoded Pr55. To prevent alterations in the overlapping pol-reading frame, the PTAP-motif was exchanged for the sequence LIRL.

Following transfection of HeLa-Kb cells with pBRNL4-3nef-IRES-GFP coding for GagSL or a PTAP-mutant, H2-Kb-SL complexes presented on the surface of GFP-positive cells were analysed by flow cytometry using the mAb 25D1.16. Cells expressing pBRNL4-3nef-IRES-GFP encoding Gag wt or ΔPTAP, but lacking the SL epitope served as a negative control for SL-presentation (data not shown). In contrast to the experiments using syngag-encoded Pr55, cells expressing wt GagSL from a full-length proviral construct displayed only few H2-Kb-SL complexes at the cell surface, as evident from a weak specific staining with 25D1.16, whereas mutation of the PTAP L-domain dramatically increased SL-presentation (data not shown).
Fig. 5.16 The PTAP L-domain regulates MHC-I presentation of the SL-epitope introduced into full-length HIV-1. (A) Following transfection of HeLa-Kb cells with proviral constructs pBRNL4-3nef-IRES-GFP coding for wt Gag or a PTAP-mutant, both harboring the SL-epitope within the p2 spacer region, H2-Kb-SL complexes presented on the surface of GFP-positive cells were quantified by flow cytometry using the mAb 25D1.16. The MFI of the 25D1.16 staining was normalized to GFP
fluorescence. Bars represent mean values +/- SD (n=4; * = \( P < 0.05 \)). (B) HeLa-Kb cells were transfected with pNLenv encoding Gag \textit{wt} or \( \Delta \)PTAP followed by extracellular staining using 25D1.16-PE and intracellular staining using a FITC-labeled p24-specific mAb (KC57-FITC). Bars represent mean values +/- SD after normalization to the amount of intracellular Gag (n=7; ** = \( P < 0.01 \)). (C) Western blot analysis of Gag proteins. Cell lysates of HeLa cells transfected with pNL4-3 proviral or pNLenv constructs coding for Gag \textit{wt} or a PTAP-mutant harboring or lacking the SL-sequence, respectively, were subjected to Western blotting using anti-p24 antiserum. Membranes were reprobed with an anti-\( \beta \)-actin antibody as a loading control. Virions contained in the cell culture supernatant were pelleted through 20 \% (w/v) sucrose, lysed, and p24 was detected by Western blotting. (D) Release of infectious units from transfected cells was analysed by quantification of \( \beta \)-Gal activity after infection of HeLa-TZM-bl reporter cells.

The MFI of the 25D1.16 staining was normalized to GFP fluorescence and quantification of three independent experiments clearly revealed higher numbers of H2-K\textsuperscript{b}-SL complexes on cells expressing PTAP-deficient Gag (Fig. 5.16 A; \( \Delta \)PTAP-SL) when compared to cells expressing \textit{wt} Gag (Fig. 5.16 A; \textit{wt}-SL). The increase in SL-presentation in absence of the PTAP-motif is higher when Gag was expressed in context of full-length HIV-1 when compared to expression of Gag alone (compare Fig. 5.14 and Fig. 5.16 A). To exclude that differences in the numbers of H2-K\textsuperscript{b}-SL complexes were caused by variations in the abundance of H2-K\textsuperscript{b} molecules on the cell surface, cells were stained in parallel with a H2-K\textsuperscript{b}-specific Ab, revealing equal numbers of these MHC-I molecules on the surface of all HIV-1 expressing cells (data not shown).

Similar experiments that were conducted using the expression plasmid pNLenv coding either for \textit{wt} or PTAP-deficient GagSL (Fig. 5.16 B) confirmed that the PTAP L-domain not only regulates MHC-I Ag presentation of the SL epitope derived from unprocessed Pr55, but also of Gag that undergoes processing by the viral PR.

In order to characterize the HIV-1 variants harboring the SL epitope within the p2 spacer of Gag with respect to Gag processing and virus release, HeLa cells were transfected with pNL4-3 or pNLenv and variants thereof, followed by Western blot analysis of cell lysates and virus pelleted from the cell culture supernatant using Gag- or HIV-1-specific antibodies. For PTAP-deficient HIV\(_{\text{NL4-3}}\), the characteristic defect in Gag processing with accumulation of intermediate processing products as well as reduced virus release was observed (Fig. 5.16 C, lanes 2, 4, 6, 8). Independently of the presence of the SL-epitope, also a ladder of ubiquitinated Pr55 was detected, as already observed for \textit{syngag}-encoded GagSL. We found that introduction of the SL-sequence into the p2 spacer resulted in an altered processing of p24/p25. When SL was introduced in context of \textit{wt} Gag, the p25 band became virtually undetectable (Fig. 5.16 C, lanes 3 and 7). Introduction of SL in context of the PTAP-mutant, however, produced two Gag species that could be detected as a double-band (Fig. 5.16 C, lanes 4 and 8). In addition, a third, slower migrating band
could be detected, representing CA fused to p2 containing the SL sequence. This suggests, that introduction of the sequence SIINFEKL into the p2 spacer results either in an altered processing of p25/p24 or affects the migration behavior of the p2-containing Gag products.

In agreement with the processing defect, introduction of the SL-epitope reduced the specific infectivity of the virus by approximately 3-fold as determined by single round infection of HeLa TZM-bl reporter cells with equal amounts of virus as quantified by p24 antigen ELISA (Fig. 5.16 D). These cells harbor the lacZ and luciferase reporter genes under the control of the HIV-1 long terminal repeat (LTR) promoter. As expected, mutation of PTAP resulted in a loss of infectivity, however, even PTAP-deficient virus is further impaired with respect to infectivity in the presence of SL in the p2 spacer. Replication studies in Jurkat T cells (data not shown, performed by Christian Setz) showed an impaired replication capacity of SL-containing viruses, further substantiating the decreased infectivity observed in single-round assays. These results point out that the length and sequence of the p2 spacer peptide might play an important role for Gag processing and virus infectivity.

5.2.6 Enhanced SL-presentation of the PTAP-mutant is not a result of the budding defect and not entirely dependent on membrane association of Gag

The enhanced SL-presentation observed after expression of a GagSL-GFPΔPTAP (Fig. 5.14) could be easily explained by an increased amount of Gag protein available for Ag processing within the cell due to the budding defect of the PTAP-mutant (Fig 5.12 B, 5.14 D). To test this hypothesis, we used a Gag mutant with a single aa substitution at position 2 (G2A), which can not be myristoylated. As the cotranslational attachment of a myristate group to the N-terminal Gly residue is essential for the association of Pr55 with membranes (4, 6), this G2A mutant is, like the PTAP-mutant, incapable of budding. If the induction of a budding defect were sufficient to enhance Ag presentation, this myristoylation-deficient mutant should also display increased SL-presentation. Nevertheless, the number of SL-H2-K\(^{b}\) complexes at the surface of cells expressing the G2A-mutant did not exceed the amount displayed on cells expressing the wt protein (Fig. 5.17 A), although VLP-release was completely abolished (Fig. 5.17 B). Mutation of the myristoylation site in context of the PTAP-mutant, however, resulted in increased class I presentation of the Gag-derived SL epitope, indicating that the enhanced SL-
presentation is not caused by the budding defect and not entirely dependent on membrane association of Gag, but might be specifically regulated by the PTAP motif.

Fig. 5.17: Enhanced MHC-I antigen presentation of the PTAP-mutant is not a result of the budding defect and not entirely dependent on membrane association of Gag. (A) HeLa-K\(^b\) cells were transfected with plasmids encoding GagSL-GFP \(\text{wt}\) or \(\Delta\text{PTAP}\) and the number of H2-K\(^b\)-SL complexes on the surface of GFP-positive cells was quantified by flow cytometry. The MFI of the 25D1.16 staining was normalized to GFP fluorescence. Mean values +/- SD are shown (n=4; \(* = P<0.05\)). (B) Release of VLPs derived from GagSL-GFP variants was quantified by anti-p24 immunoblotting.

5.2.7 The interaction with Tsg101 or ALIX is not essential for the regulation of MHC-I presentation of a Gag-derived epitope by the PTAP L-domain

The PTAP motif has been shown to bind to Tsg101 (13), a component of the ESCRT-I, and this interaction is essential for the recruitment of cellular factors to support HIV-1 budding (11, 12). The disruption of this interaction by mutation of the PTAP-motif might influence the ubiquitination of Gag, possibly due to the hampered recruitment of some yet unidentified DUB, and, thus, affect the entry of Gag into the MHC-I pathway. We therefore asked whether the disturbance of the interaction between the PTAP L-domain and Tsg101 by siRNA-mediated depletion of Tsg101 also leads to increased SL-presentation as does mutation of PTAP. Although knockdown of Tsg101 with 77% efficiency could be achieved by transfection of HeLa-K\(^b\) cells with Tsg101-specific siRNA (Fig. 5.18 B), this did not lead to increased SL-presentation after coexpression of GagSL-GFP (Fig. 5.18 A).

Overexpression of ALIX, that interacts with the ESCRT-III via CHMP4B and binds to the secondary YP(X)\(_n\)L L-domain of HIV-1, can overcome the budding defect induced by mutation of the primary PTAP L-domain (22). Therefore, we tested whether restoration of the interaction with the ESCRT and rescue of the budding defect by ALIX overexpression has any influence on the presentation of the SL epitope derived from PTAP-deficient GagSL. GagSL-GFP \(\text{wt}\) or \(\Delta\text{PTAP}\) were coexpressed with Flag-tagged ALIX in HeLa-K\(^b\) cells and SL-presentation was quantified by staining with 25D1.16. Expression of ALIX
was confirmed by Western blotting (Fig. 5.18 D). Although cotransfection of increasing amounts of pFLAG-ALIX resulted in a rescue of VLP-release of PTAP-deficient GagSL-GFP as quantified by Western blotting of cell lysates and VLPs (Fig. 5.18 D), overexpression of ALIX did not affect the MHC-I presentation of the Gag-derived SL epitope (Fig. 5.18 C).

**Fig. 5.18:** The interaction with Tsg101 or ALIX is not essential for the regulation of MHC-I presentation of a Gag-derived epitope by the PTAP L-domain. (A) Following transfection of HeLa-Kb cells with Tsg101-specific or a scrambled control siRNA and, 48 h later, with siRNA and expression plasmids coding for GagSL-GFP wt or ΔPTAP, SL-presentation was assessed by flow cytometry using the mAb 25D1.16. Bars represent mean +/- SD after normalization to GFP fluorescence (n=4; * = P<0.05). (B) Knockdown of Tsg101 was confirmed by Western blotting. (C) Quantification of mean SL-presentation normalized for GFP fluorescence after coexpression of GagSL-GFP wt or ΔPTAP and ALIX or an empty vector control (mock). Bars represent mean +/- SD (n=4; * = P<0.05). (D) Rescue of VLP-release by ALIX overexpression. HeLa-Kb cells were transfected with plasmids coding for GagSL-GFP wt or ΔPTAP and increasing amounts of pCMV-Flag-ALIX (0, 0.5 or 1 µg). VLP release was assessed by staining of cell and VLP fractions with anti-p24 Ab. Expression of ALIX was confirmed by anti-Flag Western blotting.

These data point out that the disruption of the interaction between the PTAP-motif located within the p6 domain of HIV-1 Gag and the UEV domain of Tsg101, that mediates recruitment of the ESCRT-I, does not represent a likely explanation for the enhanced entry of Gag lacking a functional PTAP L-domain into the MHC-I pathway. In agreement with the results obtained for the G2A mutant (Fig. 5.17), impairment of VLP-release alone, induced here by knockdown of Tsg101, is not sufficient to increase SL-presentation. Consistently, MHC-I presentation of the Gag-derived SL epitope is not markedly influenced by the rescue of the budding defect caused by the PTAP-mutation by overexpression of ALIX.
5.2.8 Lys48-linked polyubiquitination is essential for the preferred entry of the PTAP-mutant into the MHC-I pathway

Although Ub-independent access to the proteasome has been described (reviewed in (49)), most substrates become tagged for proteasomal degradation by the attachment of at least four residues of Lys48-linked Ub (251). To test whether polyubiquitination of Gag is crucial for the better entry of the PTAP-mutant into the MHC-I pathway, GagSL-GFP expression plasmids were coexpressed with expression plasmids coding for Ub mutants Ub
\(^{K48R}\), Ub
\(^{K63R}\) or Ub
\(^{K48,63R}\) in HeLa-K\(^b\) cells. To obtain sufficiently high expression levels of Ub mutants, a consecutive transfection protocol was used as described in section 5.2.2.

SL-presentation was assessed using staining with 25D1.16 as described above. While overexpression of wt Ub, used as a control, had no effect on SL-presentation, interference with Lys48-linked poly-Ub chain formation reduced the MHC-I presentation of SL derived from both, Gag-SL-GFP wt and ΔPTAP2 to background levels (Fig. 5.19). In agreement, a reduction of SL-presentation of the same magnitude was obtained after cotransfection with the double mutant Ub
\(^{K48,63R}\), while expression of the single mutant Ub
\(^{K63R}\) had only marginal effects on SL-presentation. As massive overexpression of Ub
\(^{K48R}\) might interfere with cell viability in general, in particular with proteasome activity or MHC-I Ag presentation, the number of total H2-K\(^b\) molecules at the cell surface were monitored in parallel. However, no changes in total H2-K\(^b\) could be detected following expression of any of the Ub variants (data not shown), which indicates that the overall MHC-I Ag presentation pathway was still functional. These results point out that the attachment of Lys48-linked poly-Ub chains, the canonical signal for proteasomal degradation, regulates the entry of the PTAP-deficient Gag into the MHC-I Ag presentation pathway.

![Fig. 5.19: Lys48-linked polyubiquitination is essential for the preferred entry of the PTAP-mutant into the MHC-I pathway.](image)

Following coexpression of GagSL-GFP wt (black bars) or ΔPTAP (grey bars) with Ub or Ub mutants in HeLa-K\(^b\) cells, H2-K\(^b\)-SL complexes at the cell surface were quantified by flow cytometry using 25D1.16 and the MFI was normalized to GFP. Bars represent mean +/- SD from three independent experiments.
5.2.9 The PTAP-mutant displays a slightly decreased metabolic half-life and an increased DRiP-rate when compared to wt Gag

It has been shown here that formation of poly-Ub chains on ΔPTAP GagSL molecules is essential for increased SL-presentation (Fig. 5.19) and that the PTAP-mutant is ubiquitinated to higher levels when compared to wt GagSL (Fig. 5.13), suggesting that GagSLΔPTAP is preferentially recognized and degraded by the UPS. This led to the question if this enhanced ubiquitination correlates with a reduced half-life of the protein. Therefore, standard pulse-chase analyses were conducted using transiently transfected HeLa cells that were pulsed with [35S]Met for 15 min followed by 6 hours of chase. Gag was recovered from cell lysates by IP using a mixture of Gag-specific antibodies, subjected to SDS-PAGE and analyzed by fluorography (Fig. 5.20 A). Radioactivity in dried gels corresponding to the Pr55 band was quantified using phosphorimaging instrumentation and software. Data from four independent experiments show that the PTAP-mutant displays a slightly decreased metabolic half-life when compared to wt GagSL (Fig 5.20 B). To test whether this decreased stability is consequent to a rapid cotranslational turn-over, short-term DRiP pulse-chase experiments were performed according to previously established protocols (133, 135, 136). HeLa cells were transiently transfected with GagSL expression plasmids and treated with a combination of zLLL (228) and the highly specific PI lactacystin during the final 10 min of a 30 min starvation period, throughout a 3 min pulse with [35S]Met as well as a 60 min chase period. dimethyl sulfoxide (DMSO)-treated cells served as a solvent control. The complete shut-down of proteasomal activity at the time of metabolic labeling allows for the detection and quantification of newly synthesized proteins that would otherwise undergo cotranslational degradation and therefore escape detection. Gag proteins precipitated by specific antibodies were resolved by SDS-PAGE and analyzed by fluorography (Fig. 5.20 C). The quantities of radioactivity corresponding either to Pr55 or the total proteins migrating in the MW range of approximately 60 to 250 kDa were measured using a phosphorimager (Fig. 5.20 D). In the absence of proteasome inhibitors, the decline of Gag in cells expressing the PTAP-mutant or the wt protein was almost identical. After proteasome shutdown, there is a transient increase in recovery of Pr55 in the wt situation. In absence of a PTAP-sequence, however, the magnitude of this increase is clearly higher. The amount of Pr55 recovered from cell lysates was increased by more than 50 % immediately after the pulse and attained a maximum level of up to 80 % within 5 min (Fig. 5.20 D, left panel). After reaching a
certain plateau, the recovery of Pr55 declines during the last 30 min of chase, most probably due to processes like ubiquitination, proteolytic cleavage and membrane association.

**Fig. 5.20:** The PTAP-mutant displays a slightly decreased metabolic half-life and an increased DRiP-rate when compared to wt Gag. (A+B) For long-term pulse-chase experiments, transiently transfected HeLa cells were radiolabelled for 15 min with [35S]Met and chased for up to 6 hours. (A) Fluorograph of GagSL proteins recovered by IP using anti-p6 and anti-p24 antibodies and separated by SDS-PAGE. (B) Densitometric quantification of [35S]-labeled Pr55 using a phosphorimager. PSL: Photostimulated luminescence. Values represent mean and SD of four independent experiments. (C+D) For short-term DRiP pulse-chase experiments, transiently transfected HeLa cells treated with 20 µM of zLLL/LC or DMSO as a solvent control each during the final 10 min of a 30 min starvation period were pulsed for 3 min with [35S]Met and chased for up to 60 min the presence or absence of zLLL/LC. (C) Fluorograph of GagSL recovered by IP and resolved by SDS-PAGE. (D) Densitometric quantification of [35S]-labeled Pr55 (left panel) and the high molecular weight (HMW) smear recovered with anti-Gag antibodies (right panel) of the fluorograph shown in C.
The smear of proteins migration in the MW range of 60 kDa to 250 kDa recovered by Gag-specific antibodies is thought to represent polyubiquitinated Gag species (133, 136). Quantification of this HMW smear of proteins showed that the PTAP-mutant is ubiquitinated to higher levels and these polyubiquitinated Gag species accumulate over time (Fig. 5.20 C and 5.20 D, right panel). The accumulation of these polyubiquitinated species after proteasome shutdown was taken into account to assess the DRiP-rate of Gag. Though treatment of cells expressing \textit{wt} GagSL with zLLL/LC resulted in a minor enhancement of recovery of proteins in this MW range, the magnitude of increase was unambiguously higher in cells expressing the PTAP-mutant, reaching about 100 % after 15 min of chase (Fig. 5.20 D, right panel).

Taken together, pulse-chase analyses revealed that, although the metabolic half-life of PTAP-deficient GagSL is not considerably diminished, its DRiP-rate is clearly enhanced when compared to \textit{wt} GagSL. These data provide an explanation for the increased number of H2-K\textsuperscript{b}-SL complexes presented at the surface of cells expressing the PTAP-mutant. Nevertheless, the mechanism how the lack of a functional PTAP-motif is recognized by the cellular protein quality control system remains to be answered.
6 Discussion

In this study, the HIV-1 Gag polyprotein was chosen as a model Ag to more precisely understand the relevance of erroneous protein synthesis for the generation of MHC-I-presented peptides. To this end, the effect of degradation signals artificially fused to HIV-1 Gag on its efficiency of biogenesis, metabolic half-life, DRiP-formation as well as subcellular localization, and VLP-release has been investigated in this work. These parameters of Gag protein function have been correlated to the efficiency of MHC-I presentation of a Gag-derived SL model epitope. It was demonstrated that the exchange of the N-terminal Met residue for Arg, a destabilizing aa according to the N-end rule, directed Gag to the DRiP-pathway and resulted in enhanced MHC-I Ag presentation, as well as a better CD8+ T-cell response, both, in vitro and in vivo. Moreover, the PTAP L-domain located within the C-terminal p6 region of Pr55 was identified to be a naturally occurring sequence motif that, besides its already well characterized and essential role in virus release, also governs the DRiP formation and Ag presentation of HIV-1 Gag.

Regardless of all the efforts spent so far, there is still no protective or therapeutic vaccination against HIV-1 available. Recent vaccine trials failed to confer protection against HIV-1 infection, but may help to generate new hypotheses that can be followed in future research (252-255). Key obstacles for the development of an effective HIV-1 vaccine include, amongst others, the huge sequence variation of HIV-1. Moreover, the natural immune response to HIV-1 does not protect against superinfection (256-258). Therefore, a better understanding of what constitutes a protective immune response against HIV-1 and of the immune responses induced by vaccination is crucial. Based on this knowledge, it may be possible to enhance the immunogenicity of vaccine Ags to induce immune responses that can prevent or control HIV-1 infection.

Though it is now generally accepted that the induction of Abs that neutralize a broad spectrum of HIV-1 isolates is required to block acquisition of HIV-1 infection (219, 259), virus-specific CD8+ T cells display a key function in the immune control of virus spread (260), especially during acute viremia (261). In order to evade specific CTL responses, HIV-1 quickly mutates MHC-I-restricted epitopes, showing that those responses exert selective pressure (260-265). Moreover, certain MHC-I alleles have been correlated with viral load and disease progression (262, 266-268). Recently, a genome-wide association study suggested the binding properties of MHC-I molecules to be the major genetic factor for the control of HIV-1 infection (269). Therefore, vaccine strategies aimed at the
generation of potent virus-specific CTL responses are now generally considered as a therapeutic option to interfere with disease progression subsequent to HIV infection. In this study, HIV-1 Gag was chosen as a model protein to assess strategies to enhance MHC-I Ag presentation, since it has been demonstrated by several studies that an effective CTL response acting specifically against HIV-1 Gag, but not against other viral Ags, can be correlated with a significant reduction in viral load in HIV-1 infected patients (220, 270-272) or in SIV-1 infected Rhesus macaques (273, 274). Importantly, immune escape mutations within Gag-derived epitopes may be associated with a significant loss of viral fitness (275-277). The Gag protein can therefore be regarded as an promising vaccine Ag, and approaches that aim at optimizing the immunogenicity of HIV-1 Gag might be useful to elicit a broad and sustained cellular immune response.

In addition, Gag is exclusively present in the cytosolic compartment and thus, fully accessible to the UPS and entry into the MHC-I Ag processing pathway. Furthermore, principal biochemical procedures to measure the DRiP-rate of Gag have been established, and Gag-DRiPs have already been detected (133). Therefore, HIV-1 Gag can be considered as an interesting model Ag.

Although a number of Gag-derived MHC-I epitopes have been characterized, there is still no specific Ab available to analyze the quantity of Gag-derived epitopes in complex with MHC-I molecules at the cell surface. To circumvent this problem we found that insertion of the standard model epitope SL into the polymorphic p2 spacer region of Gag (278) resulted in efficient presentation of SL on the cell surface that did not interfere with budding and release of synGag-derived VLPs (see Fig. 5.8). However, though virus-release was not markedly affected when SL was introduced into the p2 spacer of Gag encoded by HIV-1 NL4-3, an altered migration behavior in SDS-PAGE and/or processing pattern of CA was observed (Fig. 5.15). This led us to speculate that introduction of additional eight amino acids into p2 might alter the processing of the CA-p2 protein (p25). One possible explanation is the alternative usage of a cleavage site within p2, which has been previously proposed between position Met-4 and Ser-5 of p2 (226, 279, 280). Alternatively, an additional site inserted by introduction of the SL sequence may be recognized by the PR, leading to a shortened p25 product that migrates as a double band with p24. As the C-terminal domain of CA has been shown to adopt an α-helical structure that extends into p2 (225, 226, 281), the changed migration behavior described here could also reflect structural alterations induced by the extension of the p2 linker peptide. Though this observation is not highly relevant to answer questions addressed in this work, it indicates that the length and sequence of the p2 spacer might be important for Gag
processing. However, more careful examination would be necessary to clarify this phenomenon and to provide further insight into the role of the p2 spacer peptide during the viral life cycle.

We found that the position of insertion clearly affected the efficiency of SL-presentation (Fig. 5.8). Only insertion at the internal position (p2) resulted in efficient SL-presentation. When the SL sequence was inserted at the ultimate C-terminus of Gag, the epitope was only poorly presented by H2-K\(^b\) molecules, while insertion at the N-terminus did not result in MHC-I presentation of the epitope at all. The 26S proteasome has to produce the correct C-termini of MHC-I-presented peptides, whereas N-terminally extended antigenic precursors can be trimmed by other proteases (153, 282, 283). Accordingly, proteasomal processing might not always generate the correct C-terminus of the SL-epitope. MSIINFEKL located at the N-terminus of Gag could undergo N-terminal trimming, for example by ERAAP in the ER, leading to the destruction of the epitope. Depending on the surrounding amino acids, the SL epitope may also be destroyed by cuts made within the SL sequence by the proteasome.

Several studies have established that targeting an Ag for rapid degradation by the 26S proteasome represents an effective approach to enhance MHC-I Ag presentation (206, 207, 210-214) and induction of CTL responses (216, 284-288). Motifs of the protein primary sequence that govern protein stability have been identified in cellular (171) and viral proteins (e.g. the GA-stretch of Epstein-Barr virus (EBV) that stabilizes EBNA-1 (289), an N-end rule degron in HIV-1 Integrase (200), and newly identified degradation signals within the C-terminal regions of alphavirus nsP3 (290), Influenza C virus p42 (291) and Hantavirus G1 (292).

Initially, it was attempted to target HIV-1 Gag for rapid proteasomal degradation by the introduction of various degradation signals or potentially destabilizing aa exchanges (see Fig. 5.1). However, none of these degradation signals conferred instability to Gag in terms of overall metabolic half-life, neither in murine nor in human cell lines (Fig. 5.2, Fig. 5.9 and data not shown). The validity of the N-end rule has been successfully demonstrated for a number of Ags derived from viruses and bacteria (206, 207, 211, 213, 214, 217). Nevertheless, the N-end rule does not seem to apply ubiquitously for all proteins tested so far (210, 293). Targeting to the UFD pathway reduced the half-life of most model substrates (205, 287, 294, 295), but fusion of a single copy of Ub had sometimes only modest effect on protein half-life (293, 294). Our observation that the metabolic half-life of Gag is only marginally influenced by the introduction of N-end rule or UFD degradation signals is in agreement with previous findings made by others (210).
Even addition of a short Lys-rich leader sequence to a similar UbRGag fusion protein resulted only in moderate destabilization of Gag (210).

It is shown here that targeting of Gag to the N-end rule pathway clearly increases its DRiP-rate (Fig. 5.3). This discrepancy might be explained by the possibility that N-terminal degradation signals may be buried in the context of completely folded Gag. In contrast, the destabilizing Arg-residue at the N-terminus of the nascent protein should be better accessible to cognate Ub ligases during protein translation. Moreover, its inherent capacity to self-assemble might contribute to the resistance of Gag to the N-end rule and UFD. The formation of Gag multimeric complexes during budding could obscure the recognition of degradation signals by cellular proteins. Although we can merely speculate about the underlying reasons for the variable impact of N-end rule and UFD degradation signals on protein half-life, it seems reasonable that polypeptides tagged for destruction or damaged proteins are removed as quickly as possibly, preferentially during synthesis.

The reduced Gag steady-state level in RGag expressing cells when compared to the M-Gag expressing cells might also point towards a rapid co-translational degradation of a certain proportion of total Gag. This should slightly reduce the steady-state level of RGag that otherwise exhibits the same turn-over rate during the post-translational “second” half-life. The importance of protein de novo synthesis (234, 236) for efficient MHC-I presentation, also found here for Gag-derived SL (Fig. 5.5), underlines the well established concept that DRiPs represent the main source for MHC-I-presented peptides (132, 133). Viral DRiPs have been demonstrated for HIV-1 Gag (133), IAV nucleoprotein (212, 296) and, very recently, for IAV neuraminidase (297) and EBV EBNA-1 (298).

There is only limited knowledge about naturally occurring sequences in viral Ags that regulate entry into the DRiP pathway. Results presented in the second part of this thesis point out that an intrinsic sequence of the C-terminal p6 domain, namely the PTAP late assembly domain, can govern protein stability, and, most importantly, the DRiP-rate of HIV-1 Gag. Gag-DRiPs are more efficiently processed by immunoproteasomes, leading to more efficient MHC-I presentation of Gag-derived SL (Fig. 5.15). The only marginal difference between wt and ΔPTAP in the augmentation of SL-presentation upon IFN-γ treatment (Fig. 5.15) indicates that the nature of Gag-DRiPs studied here might be different to DRiPs accumulating upon IFN-γ induced oxidative stress (247).

Beyond the function in virus release, L-domains, especially the PTAP-motif of HIV-1, have been implicated in the control of Gag ubiquitination (114, 120, 121), which could be confirmed here using PTAP-deficient GagSL (Fig. 5.13). Nevertheless, it is not clear
whether the effect of L-domains on Gag ubiquitination is related to their function to facilitate retroviral release. Gag expressed from full-length HIV-1 seemed to be more strongly ubiquitinated (Fig. 5.16 C). Consistently, the difference in SL-presentation between wt Gag and the PTAP-mutant was more pronounced (Fig. 5.16 A, B). The sensitivity of both, the increased ubiquitination (Fig. 5.13) and the enhanced SL-presentation of the PTAP-mutant (Fig. 5.19) to overexpression of Ub^K48R indicates that mutation of PTAP leads to the attachment of K48-linked poly-Ub chains to Gag. Even if complete interference with K48-linked poly-Ub chain formation was not achieved by overexpression of Ub^K48R, already a restriction in chain-length could be sufficient to significantly reduce the efficiency of Ag processing. However, we cannot exclude that other types of Gag ubiquitination, e.g. multiubiquitination or polyubiquitination linked via other Lys residues of Ub, are also regulated by PTAP. The pattern of Gag ubiquitination, also observed by others (114, 121) is consistent with multi- or polyubiquitinated Gag species. It is tempting to speculate that monoubiquitination could initiate further polyubiquitination of Gag. However, at least the Lys residues at position 27 and 33 of p6, which have been shown to be monoubiquitinated (111), are dispensable for the increased Ag presentation induced by mutation of the PTAP L-domain (data not shown). A minor fraction of GagSL_APTAP also undergoes K63-linked polyubiquitination (Fig. 5.13 and Fig. 5.19). As a recent publication described that K63-linked poly-Ub chains might also represent a signal for proteasomal degradation, at least in vitro (299), this might directly or indirectly influence the degradation of Gag by the 26S proteasome. Interestingly, K63-linked polyubiquitination of HIV-1 Gag by HECT Ub ligases has been recently shown to correlate with their ability to rescue virus budding (300).

The finding that mutations of PTAP result in increased Gag ubiquitination suggests that the PTAP L-domain recruits DUBs, possibly through its interaction with the ESCRT, and this has been proposed before (8, 114, 120, 121). The endosomal DUB AMSH interacts with components of the ESCRT-0 and -III, deubiquititates endosomal cargo prior to lysosomal degradation and specifically cleaves K63-linked poly-Ub chains in vitro (301, 302). Overexpression of AMSH has been shown to block HIV-1 release, while knockdown of AMSH had no effect on virus particle production (303, 304). However, the L-domain dependent recruitment of AMSH to sites of viral budding and its ability to deubiquitinate Gag in vivo have not been demonstrated.

In contrast, the presence of the PTAP motif might suppress ubiquitination by preventing the interaction of Gag with E3 Ub ligases. It has been shown that overexpression of Tsg101 results in enhanced ubiquitination of HIV-1 (305) and HIV-2 (306) Gag, leading
to the hypothesis that Tsg101 recruits a Gag-specific E3 Ub ligase. Controversially, disruption of the binding to Tsg101 by mutation of PTAP rather enhances the ubiquitination of HIV-1 Gag (120, 121).

It has been proposed that the increased Gag ubiquitination observed after mutation of the PTAP motif might not be due to sequence-specific interactions, but is rather an indirect result of the budding defect and caused by the prolonged association with the host cell PM (121). However, when we mutated the PTAP-motif in context of Gag\(^{G2A}\), SL-presentation was increased (Fig. 5.16). In addition, ubiquitinated Gag could be detected, though the extent of Gag ubiquitination was somewhat reduced in the absence of the myristoylation site (data not shown). These results indicate that the regulation of Gag polyubiquitination and degradation by the proteasome is not fully dependent on its membrane association.

The findings that interactions with the ESCRT components Tsg101 and ALIX are not critical for directing Gag into the MHC-I pathway (Fig. 5.18), suggest that, in addition to ESCRT interaction, the PTAP motif might regulate association of HIV-1 Gag with one or several so far unidentified cellular factors that are connected to the cellular protei...
release and Gag processing (308, 309). Moreover, misfolding of the Gag precursor could impair the accessibility of cleavage sites for the viral PR, contributing to the accumulation of processing intermediates, which have recently been shown by others (310) to trans-dominantly interfere with viral infectivity. Though it has been reported that the PTAP L-domain is dispensable for virus release in the context of PR deficient HIV-1 (118, 309), we observed that the PTAP motifs were necessary for efficient release of VLPs, a finding that is in agreement with observations made by others for a comparable Gag expression system (114).

We further wanted to evaluate the possibility that targeting HIV-1 Gag to the DRiP-pathway by replacement of the N-terminal Met with an Arg or stable N-terminal in frame fusion to Ub interferes with the ordered assembly, budding and release of synGag-derived VLPs. UbRGag or Ub\(^{G76V}\)Gag variants show little or no VLP-release, respectively, while release of UbMGag-derived VLPs is only slightly attenuated (see Fig. 5.8 and Fig. 5.10). The increased DRiP-rate of UbRGag provides a possible explanation for this effect. However, the possible lack of myristoylation provides an alternative and more simple explanation for the budding defect observed for Ub-Gag fusions. N-terminal Gly, a prerequisite for myristoylation by N-myristoyltransferase (242), is exposed by processing of the initiator Met by N-terminal Met excision (NME), catalyzed by Met aminopeptidases (311, 312). Most likely, an N-terminal Arg in exchange for Met does not represent a good substrate for this reaction, leading to a significant loss of Gag myristoylation. Both, myristoylation (313) as well as cleavage of Ub (224) have been shown to occur co-translationally. In situations where the kinetic of Ub cleavage does not sufficiently meet that of myristoylation, this need for concerted action might explain the slightly reduced release efficiency of the UbMGag variant.

As myristoylation is essential for targeting of Gag to the PM (6), the fraction of PM-associated Gag was determined by membrane flotation experiments. Approximately 50% of \( wt \) Pr55 were found to be associated with the PM (Fig. 5.11), which is in agreement with comparable studies performed in HeLa cells (314-316). However, the membrane-bound Gag fraction can differ considerably, depending on the cell type (317). In contrast to UbMGag, whose membrane association was found to be only slightly reduced, both UbRGag and Ub\(^{G76V}\)Gag failed to associate with the PM as shown by comparison to the myristoylation-deficient Gag\(^{G2A}\) mutant.

Though certain differences between Gag variants with respect to subcellular distribution could be observed by immunofluorescence analysis using a CA-specific Ab (Fig. 5.11 A-D), the overall staining pattern was quite diffuse, similar to Gag-expressing EL4 cell lines.
Discussion

(data not shown; see (136)). Such a pattern has been found in some studies for unprocessed Gag (318, 319), but is in contrast to most reports (7, 320-322), which described a more punctate pattern or increased fluorescence intensity at the PM. The discrepancies concerning Gag distribution might be explained by differences in expression levels, as noticed by others (318), or staining procedures. In addition, it has been suggested previously that the route of mRNA export from the nucleus might contribute to Gag localization (323, 324). However, to which extent this applies to codon-optimized, Rev-independent Gag expression systems is not clear.

Despite the insensitivity of HIV-1 Gag to the N-end rule in terms of overall protein half-life (210), it is demonstrated in this work that targeting of HIV-1 Gag into the DRiP-pathway, either by an N-terminal Arg, stable N-terminal fusion of Ub or by mutation of the PTAP-motif, correlates with an increase in MHC-I Ag presentation.

The MHC-I pathway is considered as a complex and multi-step process. We analyzed the initial step, the synthesis and degradation of an Ag, and the final step, the amount of pMHC-I complexes on the cell surface. Since the amount of total H2-K\textsuperscript{b}-molecules and the rate of reappearance after the acid wash procedure were similar in both EL4 cell lines, we assume that the H2-K\textsuperscript{b} expression levels as well as the intermediate steps in the MHC-I pathway do not markedly differ between the two EL4 cell lines. The differences in the proportion of H2-K\textsuperscript{b}-molecules loaded with SL epitope, however, should be consequent to disparities in the DRiP-rate. Similar to previous studies (133, 211, 228, 232-236), the generation of the SL epitope was dependent on proteasome activity and ongoing protein biosynthesis, further supporting the notion that this phenomenon studied in our system is related to the DRiP pathway.

The increased SL-presentation observed for the UbRGagSL/EL4 cell line could be reproduced in transiently transfected human HeLa-K\textsuperscript{b} cells. Interestingly, targeting Gag for degradation by the UFD pathway resulted in an even more dramatic increase in SL-presentation, again without significantly affecting the overall metabolic half-life of Gag. It may be interesting to test the immunogenicity of this Ub\textsuperscript{G76V}Gag variant in further in vivo studies.

The budding defect caused by mutation of the PTAP-motif might lead to the intracellular accumulation of Gag, thus increasing the availability of Ag for processing by the 26S proteasome. This would provide an obvious explanation for the better entry of PTAP-deficient Gag into the MHC-I pathway. However, we could exclude that there is a correlation between budding capacity and MHC-I Ag presentation. Impairment of VLP-release by mutation of the myristoylation site (G2A; Fig. 5.17), knockdown of Tsg101 or
overexpression of ALIX (Fig. 5.18), all three known to induce a budding defect (4, 11, 26), did not lead to an increase in SL-presentation. Consistently, rescue of the budding defect of GagΔPTAP by ALIX overexpression did not reduce SL-presentation (Fig. 5.18). Data presented in this thesis strongly suggests that targeting of a given Ag into the DRiP-pathway represents a suitable strategy to augment the number of pMHC complexes at the cell surface. Most importantly, the increase in MHC-I Ag presentation by N-end rule targeting of Gag was accompanied by enhanced T-cell activation, both, in vitro and in vivo (Fig 5.6 and Fig. 5.7). A correlation between the amount of TCR-ligands at the surface of professional Ag presenting cells (pAPCs) and the induction of a CTL response has been shown in other models (246, 325-327). However, in some studies, memory CTLs of lower avidity were generated (325) or progressively declined in number (246). Nevertheless, the specificity of CTLs seems to be more crucial for their ability to kill HIV-1-infected target cells compared to their functional avidity (328). Therefore, it will be important in the future to analyze not only the artificially introduced SL epitope, but also the MHC-I presentation of genuinely Gag-derived epitopes, for example the HLA-A2 restricted MA-derived SLYNTVATL.

Targeting of Ags for proteasomal degradation by the N-end rule or UFD pathway has been employed in several DNA immunization studies. N-end ruled variants of β-Gal (288) or HIV-1 Nef (206) induced more frequent CTL responses when delivered into mice using plasmids or rVV vectors. The N-end rule targeting of HIV-1 Pol increased MHC-I presentation by human DCs in vitro and CTL responses in humanized mice (329). Enhanced and protective CTL responses have been successfully induced by UbG76A fusions to the E6 protein of cottontail rabbit papillomavirus (284), to the lymphocytic choriomeningitis virus (LCMV) NP (287), to HIV-1 subgenomic sequences (330), to EBV latent membrane protein 11 (286) and Trypanosoma cruzi amastigote surface protein-2 (331).

Wong et al. reported that rVV-mediated expression of sub-genomic, altered and highly turned over fragments of Gag resulted in increased numbers of SL-H2-Kb complexes presented at the cell surface (210). However, when CTL responses were compared in DNA vaccination studies in mice, T-cell responses in vivo did not correlate with the Ag processing rates of those Gag variants. Variations between full length wt Gag studied here and fragments of Gag studied by others (210) might contribute to those differences. Consistently, in these reports, highly efficient induction of a protective CTL response was observed in the virtual absence of a humoral immune response. For example, N-end rule or UFD targeting of IAV NP induced lower Ab responses while CTL responses directed
against a specific epitope were comparable to wt NP (293). The reduced humoral immune response is thought to be a consequence of reduced availability of the full-length Ag, presumably due to increased continuous turnover and reduced steady-state levels. Rapidly degraded forms of Ag may not only fail to elicit Ab responses, but some studies find a clear correlation between metabolic stability of the vaccine Ag and induction of a CTL response (332-334). This phenomenon can be explained by the assumption that rapidly degraded forms of Ag represent poor substrates for cross-presentation, which is thought to be an important mechanism for activation of naïve CD8+ T cells in vivo (140, 335-338). This process, called cross-priming, takes place when pAPCs take up exogenous Ag transferred, for example, from virus-infected or tumor cells. Cross-priming might be especially, but not exclusively, important for induction of CTL responses against viruses that do not preferentially infect pAPCs.

Particulate Ags are introduced into pAPCs by phagocytosis, whereas soluble Ags can be taken up via receptor-mediated endocytosis or pinocytosis. Interestingly, transfer of peptides through gap junctions has also been described (339). DCs can also acquire preformed pMHC complexes by trogocytosis (340, 341). This term describes the exchange of PM patches between cells, enabling the intercellular transfer of membrane proteins (342). As ubiquitinated Gag associates with membranes (121) and proteasome inhibitors cause the accumulation of Gag-DRiPs in insoluble protein aggregates (unpublished observation), driving Gag into the DRiP-pathway might also enhance its attraction for cross-presentation.

Although CD8+ T cells generally exhibit an exceptional high sensitivity for specific pMHC-I complexes (343), enhancing Ag processing can be advantageous in certain situations. First, enhancing the MHC-I processing of viral Ags might be important for the recognition of virus-infected cells during the onset of synthesis of viral proteins, especially of stable, structural proteins like the retroviral Gag polyprotein (129, 344). During peptide loading of MHC-I molecules in the ER, a great number of peptides, derived from turnover of an estimated number of 2-3x10^9 cellular proteins compete for the available number of binding grooves (129). Thus, an enhanced DRiP-rate should result in an increased generation and steady-state level of antigenic peptides. However, an emerging concept of compartmentalization of protein synthesis and Ag processing suggests that the law of mass action could be circumvented during peptide generation and loading (reviewed in (345)). In addition, at early stages of virus infection, only few target cells may be available for recognition by CTLs. In the in vivo model of adoptive transfer, 2x10^6 of both target cell lines, UbRGagSL/EL4 and UbMGagSL/EL4, were sufficient to
induce maximal stimulation of $1 \times 10^7$ co-transferred SL-specific OT-1 T cells. However, when the number of target cells was limited to $2 \times 10^5$, a clear difference in OT-1 T-cell proliferation was observed. This indicates that for efficient stimulation of CD8$^+$ T cells in vivo, limited availability of target cells can be compensated by a higher presentation rate of specific pMHC-I complexes per target cell.

Second, CD8$^+$ T cells compete with each other for pMHC-I complexes at the surface of target cells in a process called cross-competition, which is especially important to shape the hierarchy of T-cell responses (346). Increased numbers of specific pMHC-I complexes might therefore help to induce CTLs of the desired specificity.

Third, enhanced MHC-I presentation of epitopes derived from tumor Ags seems favorable to break tolerance and can initiate an anti-tumor CTL response (347-349).

An elegant study conducted by the group of Nilabh Shastri recently characterized DRiPs derived from EBV EBNA-1 as truncated polypeptides, whose synthesis is governed by the GA-stretch of the protein (298). The authors speculated that herpesviral proteins involved in episome maintenance might have evolved to interfere with the formation of DRiPs and, thus, with MHC-I Ag presentation. The novel function of PTAP in regulation of DRiP-formation might also decrease MHC-I presentation of Gag-derived epitopes in vivo and, thus, contribute to immune escape during HIV-1 infection. Cryptic epitopes derived from HIV frameshift sequences are presented to CTLs in infected individuals (350). Those polypeptides might be regarded as nonsense products of incorrect reading frame selection by the ribosome and could therefore be considered as DRiPs. In addition, APOBEC3 editing of HIV, which is known to produce truncated and misfolded viral proteins, enhances CTL recognition of infected cells (351). Further research is needed to elucidate the nature of DRiPs derived from HIV-1 proteins, their contribution to the pool of MHC-I-presented epitopes and countermeasures that HIV-1 might have evolved.

Taken together, data presented in this thesis support the hypothesis that DRiPs constitute the main source for endogenous peptides presented by the MHC-I pathway. Moreover, it was shown that HIV-1 Gag can be targeted into the DRiP-pathway either by artificial introduction of degradation signals or by mutation of the PTAP L-domain motif within the p6 domain. These findings could be interesting with respect to new ideas for the design of optimized vaccine Ags.
Material and methods

Expression plasmids, siRNA and molecular cloning
The expression plasmid psyngag encoding the entire Pr55 Gag polyprotein originating from the isolate HIV-1HX10 as well as the construction of the plasmids pUbMsyngag and pUbRsyngag have been described elsewhere (136, 218). For pUbG76Vsyngag, the C-terminal Gly residue of the Ub-fusion part was mutated to Val using the QuikChange site directed mutagenesis kit (Stratagene) and oligonucleotides (Biomers) G76V-fw (5´-TCC TGC GCT TGA GGG GGG TGA TGG GCG C-3´) and G76V-rc (5´-GCG CCC ATC ACC CCC CTC AAG CGC AGG A-3´).

The introduction of the SL-coding sequence into psyngag as well as generation of a minigene expression construct encoding MSIINFEKL have been described before (136). Both copies of the PTAP motif have been exchanged for the sequence AIVA by site-directed mutagenesis using the QuikChange kit (Stratagene) and oligonucleotides PTAP1-fw (5´-GCA GGC CCG AGG CCA TCG TCG CCC CCT TCC TGC-3´) and PTAP1-rc (5´-GCA GGA AGG GGG CGA CGA TGG CCT CGG GCC TGC-3´) as well as PTAP2-fw (5´-GGC CCG AGG CCA TCG TCG CCC CCG AGG AGA-3´) and PTAP2-rc (5´-TCT CCT CGG GGG CGA CGA TGG CCT CGG GCC-3´). To introduce the SL-coding sequence into the proviral constructs pNL4-3 (248), pBRNL4-3nef-IRES-GFP (249) and the HIV-1 NL4-3 subgenomic expression vector pNLenv1, in which the env gene is deleted (250, 352), as well as mutants thereof (ΔPTAP) in which the PTAP-motif has been exchanged for LIRL without affecting the overlapping pol reading frame (309), the gag gene was subcloned into the pGEM-T vector (Promega) via Sph I and Sbf I. A BstE II site was introduced by site-directed mutagenesis using oligonucleotides 5´-GAA GCA ATG AGC CAG GTG ACC AAT CCA GCT ACC-3´ and 5´-GGT AGC TGG ATT GGT CAC CTG GCT CAT TGC TTC-3´. Oligonucleotides BstEII-SL-fw (5´-GTG ACC TCG ATC ATC AAC TTC GAA AA G CTA-3´) and BstEII-SL-rv (5´-GTC ACT AGC TTT TCG AAG TTG ATG ATC GAG-3´) were used to introduce the SL epitope and gag was cloned back using Sph I and Sbf I. All sequences were confirmed by DNA sequencing using the Big Dye v3.1 sequencing kit (Applied Biosystems) on an ABI PRISM 3100 sequencing instrument (Applied Biosystems).

The expression vector pHA-Ub has been obtained from H.-G. Kräusslich and is described elsewhere (114). Construction of pCMV-FLAG-ALIX is described elsewhere (353).
For knockdown of Tsg101, synthetic siRNA (Integrated DNA technologies) was used as described (11). The sequence was sense 5’-CCU CCA GUC UUC UCU CGU CTT-3’ and antisense 5’-GAC GAG AGA AGA CUG GAG GTT-3’.

**Cell culture, transfection procedure and generation of stable cell lines**

EL4, B3Z and Jurkat T cells, all growing in suspension were maintained in RPMI 1640 medium supplemented with 10 % (v/v) heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.01 % sodium pyruvate and 0.1 % nonessential amino acids. EL4 is a thymoma cell line derived from the C57BL/6 mouse (H2-K b). The EL4-derived cell line E.G7 synthesizes and secretes OVA (239). The SL-H2-K b-specific, murine CD8 + hybridoma T cells B3Z express the lacZ reporter gene under the control of the NFAT enhancer (354). The Jurkat T cell leukemia line was isolated from blood and was initially called JM. (355). The generation of stable gag-expressing EL4 cell lines is described elsewhere (136).

HeLaSS6, HeLa-TZM-bl (356) and 293T cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10 % (v/v) inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. For HeLa-K b cells (241), which express high levels of H2-K b and were obtained with permission of Ian York, Michigan State University, 1 mg/ml of G418 were added. All media and compounds were purchased from Gibco. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

**p24 antigen ELISA**

Quantification of p24 Ag was performed by enzyme-linked immunosorbent assay (ELISA; Aalto) according to the manufacturer’s instructions. Deviations from the standard protocol for determination of Gag expression in EL4 cells are described elsewhere (136).

**Single round infection assay**

In 96 well plates, HeLa TZM-bl indicator cells were infected in triplicates (4000 cells/well) with 2 ng of p24 in a volume of 100 µl medium containing 10 µg/ml polybrene. To prevent further spread of infection, fresh medium containing 100 µg/ml dextran sulphate was added after overnight incubation. 48 h postinfection, cells were washed with PBS and lysed by addition of Tropix® Gal-Screen® substrate (Applied Biosystems, buffer B). Lysates were transferred into opaque 96 well plates (Corning
Costar) and incubated at 28 °C for 1 h. Luminescence was measured in an ELISA reader (Bio-Tek).

**Viruses**

Cell culture supernatant was harvested 48 h after transfection of 293T cells with proviral constructs and passed through a 0.45 µm pore-size filter. Virus was pelleted through 20 % (w/v) sucrose (16000 x g, 4 °C, 90 min) and stocks were normalized for p24 as quantified by p24 ELISA. Aliquots were stored at -80°C.

**Infection of T cell lines**

For infection of T cell cultures, 1×10^5 Jurkat T cells were incubated overnight with 20 ng of p24, and cell culture supernatant was collected every third day postinfection (dpi). Virus replication was assessed by quantification of the virus-associated RT activity by [^32P]-TTP incorporation using an oligo(dT)-poly(A) template as described elsewhere (357).

**Flow cytometry**

For detection of H2-K^b-bound SL-epitope or H2-K^b molecules, cells were incubated with hybridoma cell culture supernatant containing the monoclonal antibodies (mAbs) 25D1.16 (227) or B8-24-3 (358), respectively, followed by staining with secondary chicken anti-mouse-AlexaFluor647 Ab (Invitrogen). Alternatively, 25D1.16 mAb was purified from hybridoma cell culture supernatant and labeled with an AlexaFluor647 labeling kit as described in (136), or obtained as an allophycocyanin (APC)-conjugate from a commercial source (25D1.16-APC, eBioscience). H2-K^b-SL complexes derived from NL4-3Δenv were detected using Phycoerythrin-conjugated 25D1.16 (25D1.16-PE; eBioscience) diluted 1:100 in FACS buffer (5 % (v/v) FCS, 0.02 % (v/v) NaN₃ in PBS). Intracellular Gag was detected by staining with a FITC-conjugated anti-p24 Ab (KC57-FITC; Beckman Coulter) diluted 1:100 in Perm/Wash™ buffer (BD Biosciences) after permeabilization of cells using Cytofix/Cytoperm™ (BD Biosciences). Flow cytometry was performed on a FACSCalibur using CellQuest software (BD Biosciences). Data analysis was performed using the FCS Express V3 software (De Novo).

**Acid wash**

SL-H2-K^b complex formation at the cell surface was followed by flow cytometry after an acid wash procedure. Cells were incubated for 2 min at pH 3 and 4 °C in buffer
containing 131 mM sodium citrate and 66 mM NaH$_2$PO$_4$. After neutralization in PBS, cells were incubated in complete RPMI medium in the presence or absence of 20 µM carbobenzoxy-leucine-leucine-leuinal (zLLL; Sigma) or protein synthesis inhibitors (PSIs; 6.3 µM emetine, 8.3 µM cycloheximide and 1.7 µM puromycin; all purchased from Sigma) for desired periods at 37 °C. At least 500,000 cells were incubated with supernatants of hybridomas 25D1.16, B8-24-3 or medium alone as a control for 1 h on ice, followed by two washing steps in PBS and staining with Cy2-conjugated anti-mouse IgG (Rockland) diluted 1:200 in FACS buffer for 30 min on ice. Cells were fixed and analysed by flow cytometry as described above.

**Immunocytochemistry**

For immunofluorescence analysis, cells were seeded onto coverslips (Superfrost, Roth), transiently transfected and fixed the next day for 30 min at room temperature (RT) in 3 % paraformaldehyde (PFA). Following permeabilization in 0.1 % (v/v) Triton X-100 in PBS, cells were washed twice and blocked for 30 min in 1 % (w/v) BSA (bovine serum albumin) in PBS. Staining was performed for 20 min at RT using FITC-conjugated anti-p24 Ab (KC57; Beckman-Coulter) diluted 1:200 in 1 % BSA in PBS and 1 µg/ml of DAPI (4’6-Diamidin-2’-phenylindoldihydrochlorid, Pierce) for 10 min to counterstain nuclei, followed by three washing steps in PBS. Immunofluorescence was visualized using a confocal Leica TCSSP5 microscope DMI 6000.

**Time course analysis of Gag synthesis and budding**

For analysis of Gag synthesis, 0, 4, 6, 8 and 12 h after transfection of HeLa cells with various psyngag expression plasmids, cells were harvested and lysed in CHAPS-deoxycholate lysis buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5 % (w/v) CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), 0.3 % (w/v) sodium deoxycholate) or RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % (v/v) Nonidet P-40, 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 5 mM EDTA) supplemented with 1 mM phenylmethylsulphonylfluoride (PMSF), 5 mM N-ethylmaleimide (NEM), 20 µM zLLL and complete protease inhibitor cocktail (Boehringer, Mannheim). Equal amounts of soluble protein as quantified by bicinchoninic acid (BCA) assay (Pierce) were subjected to SDS-PAGE and Western blotting.

For analysis of VLP release, cells were aliquoted 24 h after transfection and, after 0, 0.5, 1, 2 and 4 h, cells and supernatants were collected by centrifugation. VLPs released into the cell culture supernatant were pelleted through 20 % (w/v) sucrose in PBS by
centrifugation and directly lysed in 2x SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) β-mercaptoethanol or 100 mM dithiothreitol (DTT), 0.02 % (w/v) bromphenol blue).

**Western blotting and antibodies**

Proteins separated by SDS-PAGE were blotted onto polyvinylidene fluoride (PVDF) membranes (Hybond™, GE healthcare) and incubated with a specific primary Ab followed by incubation with horseradish peroxidase (HRP)-conjugated secondary Abs (Dianova) if required and standard enhanced chemiluminescence (ECL) procedure. The following antibodies or antisera were used at the indicated dilutions in low fat milk solution or 1 % BSA in PBS/0.1 % Tween: rabbit anti-p6 antiserum (1:10,000; (359), rabbit anti-p24 antiserum (1:10,000; Seramun), monoclonal anti-β-actin Ab (1:10,000; Sigma), monoclonal anti-Transferrin receptor Ab (TfR; 1:1000, Zymed), pooled serum of 20 HIV-1 positive patients (PKT, 1:5,000, NIH AIDS Research Reference Reagent Program, USA, Cat.Nr. 3975 or pooled sera obtained from the Nationales Referenzzentrum für Retroviren, Institute for Clinical and Molecular Virology, Universitätsklinikum Erlangen, Germany), monoclonal rat anti-Hemagglutinin Ab (HA; clone 3F10, HRP-conjugated, 1:10,000; Roche), ribosomal P antigen (RP0)-specific antiserum (1:5,000; Immunovision Inc.), monoclonal anti-β5i (LMP7) Ab (1:1,000; Enzo Life Sciences), polyclonal rabbit anti-β5 (1:1,000; Enzo Life Sciences).

**Pulse-chase analysis**

Pulse-chase experiments were basically performed as described elsewhere (133, 136). For detection of Gag-DRiPs, short-term pulse-chase experiments were performed, whereas long-term pulse-chase experiments were used to analyze protein stability. Briefly, for short-term pulse-chase experiments, transgenic EL4 cells or HeLa cells were washed in PBS and treated with 20 µM of each zLLL and LC or DMSO as solvent control during the last 10 min of a 30 min starvation period in Met-free, serum-free RPMI (Invitrogen). Cells were radiolabelled for 15 min (EL4 cells) or 3 min (HeLa cells) with 3 mCi/ml [³⁵S]Met (Amersham Life Sciences or Hartmann Analytic) and chased for up to 120 min while shaking at 37 °C in DMEM supplemented with 10 % fetal calf serum and 10 mM Met in the presence or absence of PIs. Cells were harvested and lysed in 200 µl CHAPS-Doc or RIPA buffer supplemented with 1 mM PMSF, 5 mM NEM and complete protease inhibitor cocktail for 5 min on ice and separated from the insoluble fraction by centrifugation at 20,000x g for 10 min. Gag was recovered by immunoprecipitation (IP)
using a mixture of polyclonal rabbit anti-p6 and anti-p24 antibodies or human PKT prebound to Protein G-Sepharose (GE Healthcare). Samples were separated by SDS-PAGE on a 10 % (w/v) ProSieve gel (Cambrex Bioscience) backed with Gel Bond film (FMC Bioproducts). Following fixation for 1 h in 40 % methanol, 10 % acetic acid, gels were rinsed with water, soaked in 1 M sodium salicylic acid solution for 5 h, and dried. Radioactivity in gels was analyzed using phosphorimaging instrumentation (Fujifilm BAS-2000, Fujifilm) or fluorography using BioMax MR films (Kodak) and quantified by AIDA imaging software (Raytest).

For long-term pulse-chase experiments, after a 30 min starvation period, cells were radiolabelled with 3 mCi/ml [35S]Met for 30 min (EL4 cells) or 10 min (HeLa cells), plated in DMEM containing an excess of unlabeled Met and chased for up 48 h. Analyses of radioactivity in cell lysates were performed as described above.

**Adoptive transfer of OT-1 T cells and INF-γ ELISPOT**

Adoptive transfer of OT-1 T cells and INF-γ ELISPOT after immunization of naïve C57BL/6 mice with GagSL expressing were performed by Dr. A. Goldwich as described in (136). Briefly, spleen and LN cells from OT-1 mice (360), were labeled with CFSE (Carboxyfluorescein succinimidyl ester, Vybrant CFDA SE Kit; Molecular Probes) and transferred i.v. into the tail vein of naïve C57BL/6 recipient mice (10 x 10^6 cells/mouse, Charles River Laboratories). Parental or transgenic EL4 cells (0.2 x 10^6 cells/mouse) were transferred 5 min later into the contralateral tail vein. Animals were sacrificed two or three days after adoptive transfer, spleen cells were harvested and analysed by flow cytometry. In order to analyze the OT-1 cell proliferation, OT-1 cells were identified within the live lymphocyte (FSC/SSC) gate by staining for the transgenic T cell receptor (TCR) α-chain Vα2, and CFSE levels were quantified.

Naïve C57BL/6 mice were injected into the tail vein with parental EL4 or UbGagSL/EL4 cells. As a positive control, 50 µg of synthetic SL-peptide dissolved in 50 µl incomplete Freund's adjuvant were subcutaneously injected near the tail root. After nine days splenocytes were collected and an ELISPOT assay was performed. Briefly, in 96-well ELISPOT plates precoated with rat anti-mouse IFN-γ antibody (MabTech). Following washing, splenocytes (10x10^6 cells/ml) were added in triplicates and cells were pulsed with SL peptide (100 ng/ml) or phytohemagglutinin (PHA) and staphylococcal enterotoxin A (SEA; 5 µg/ml each) in 100 µl RPMI-1640 medium supplemented with 10 % (v/v) FCS. To control for unspecific reaction, cells were treated in parallel without peptide. After incubation at 37 °C in a 5 % CO₂ atmosphere for 20 h, cytokine production
was detected by using biotinylated Ab against IFN-γ (5 μg/ml, MabTech, Hamburg) and alkaline phosphatase-streptavidin (0.2 U/ml). The IFN-γ spots were developed by addition of 100 μl of BCIP/NBT solution. Spots in dried plates were counted using computer-assisted image analysis with a Carl Zeiss Axioplan 2 and VisionKS ELISPOT version 4.9.15. For analysis, the number of spots without peptide (unspecific reaction) were subtracted from the number of spots with peptide (specific reaction). To control for variations in the frequencies of CD8⁺ cells in the spleens of individual mice, FACS analyses using anti-CD8a-FITC (Beckmann Coulter) were performed.

**Transmission electron microscopy (TEM)**

For TEM, HeLa cells were transfected with individual psyngag expression plasmids and, 24 h posttransfection, transferred into cellulose capillary tubes (361) and grown for another 24 h. Following fixation in 3 % paraformaldehyde for 1 h at 37 °C, capillaries were stored until further preparation at 4°C. Tubes were collected by centrifugation and sealed by immersion in low-melting-point agarose. The samples were post fixed with OsO₄ (1% in distilled water, 1h), tannic acid (0.1 % in Hepes 0.05 M, 30 min) and uranyl acetate (1 % in distilled water, 2h) followed by stepwise dehydration in graded ethanol and embedding in epon resin, which was subsequently polymerized. Thin sections were prepared with an ultramicrotome (Ultracut S; Leica,) and counterstained with uranyl acetate and lead citrate. The sections were examined using a TEM 902 (Carl Zeiss SMT AG) at 80 kV, and the images were digitized using a slow-scan charge-coupled-device camera (Pro Scan).

**Membrane flotation by Optiprep density gradient centrifugation**

HeLa cells were transiently transfected with psyngag expression plasmids, washed twice in PBS and subsequently detached in ice-cold PBS containing 10 mM EDTA. Following one more washing step in PBS, cells were washed in homogenization buffer (0.25 M sucrose, 1 mM EDTA, 2 mM MgCl₂, 20 mM Hepes-NaOH, pH 7.4), centrifuged and resuspended in 500 μl of homogenization buffer. Cells were disrupted by sonfication in ice water and the homogenate was centrifuged at 2000 x g for 12 min to remove unbroken cells and nuclei. The supernatant was adjusted with Optiprep (Progen) to final concentration of 30 % iodixanol.

In SW41 centrifuge tubes, a discontinuous Optiprep gradient, containing, from top to bottom, 2.5 %, 10 %, 17.5 % and 25 % of iodixanol diluted in 0.25 M sucrose, 6 mM
EDTA, 12 mM MgCl₂, 120 mM Hepes-NaOH, pH 7.4 was prepared and finally underlayered with 600 µl of the sample.

Following centrifugation for 5 h at 151,000 × gav in a SW41 rotor (Beckman Coulter), material above the visible membrane-containing fraction was displaced, fractions à 0.5 ml were collected from top of the gradient, boiled in 2x SDS sample buffer and subjected to SDS-PAGE and Western blotting using Abs specific for p6, TfR and RP0. Localization of Gag in membrane fractions was calculated after densitometric quantification as the amount of Gag present in membrane fractions (containing TfR, but not RP0) divided by the total amount of Gag.

**Statistical analysis**

Mean fluorescence intensities (MFIs; geometric mean) obtained in flow cytometry experiments were compared using the Mann-Whitney U test (two-tailed, $P \leq 0.05$). Data obtained in individual pulse-chase experiments were subjected to regression analysis, from which the mean metabolic half-lives of GagSL variants were calculated and compared by a two-tailed t-test.
8 References


144. Munz C. Antigen processing via autophagy--not only for MHC class II presentation anymore? Curr Opin Immunol 22: 89-93.


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