Establishment of an *in vitro* vesicle generation assay
to investigate intracellular trafficking of MHC class I molecules

by

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ABSTRACT

Antigen presentation by major histocompatibility complex (MHC) class I molecules is a central step in the cellular immune response of vertebrates. Antigens are presented in the form of peptides, the product of proteasomal degradation in the cytosol. They are then transported into the endoplasmic reticulum (ER) to bind to freshly synthesized class I. The complex of class I molecule and peptide only is able to reach the Golgi apparatus and the plasma membrane where it is presented to cytotoxic T cells (CTL). If a specific CTL recognizes a certain MHC class I/peptide combination, it will induce apoptosis of the target cell. This allows the immune system to detect aberrations in a cell’s proteome (e.g. upon viral infections or cancerogenesis) and to eliminate their further spread in the body.

Due to the fatal potential of CTL recognition, peptide presentation at the cell surface is strictly regulated. Peptide-receptive MHC class I molecules are therefore kept inside the cell under normal conditions to prevent binding of extracellular peptides and bystander killing. It is unknown how the cell distinguishes between peptide-loaded and peptide-receptive forms of class I and where this differentiation takes place. Peptide-receptive class I molecules could be strictly retained inside the ER or they could be able to cycle between the ER and the ER-Golgi-intermediate compartment (ERGIC).

To address these questions, a new experimental system, which enables the generation and isolation of ER-derived coat protein complex II (COPII) vesicles in vitro from isolated ER membranes or perforated cells, was first established in yeast and further adopted to mammalian cells. Analysis of vesicle proteins by immunoblot revealed that overexpressed as well as endogenous class I molecules could leave the ER of transporter associated with antigen presentation (TAP)-deficient cells to similar extents as they did in the TAP-proficient counterparts. Though overexpression resulted in more efficient class I packaging into the generated vesicles, these data suggest that peptide-receptive forms are able to cycle between the compartments of the early secretory pathway. ER escape and retrieval mechanisms may not solely be responsible but at least they contribute to intracellular retention and transport regulation of peptide-receptive class I molecules. Thus, MHC class I quality control and peptide loading may even take place in compartments beyond the ER.
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Many thanks to Ute for protein purification, preparation of yeast semi-intact cells, and a lot of happy, non-scientific conversations. Additionally, I want to thank my group, especially Mohammed and Praveen, for providing a nice working atmosphere.

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ABBREVIATIONS

Ampere
ATP binding cassette transporter
ammonium persulfate
adenosine triphosphate
β2-microglobulin
bovine serum albumine
degrees Celsius
Chinese hamster ovary
H-2Db-transfected CHO cells
TAP2-deficient H-2Db-transfected CHO cells
calnexin
concanavalin A
coat protein complex
cytotoxic T lymphocyte
diethylaminoethyl
deoxyribonucleic acid
dithiotreitol
enzyme-catalyzed fluorescence
ethylene glycol bis(2-aminoethyl ether)-tetraacetic acid
endoglycosidase H
endoplasmic reticulum
ER associated degradation
ER exit sites
ER-Golgi-intermediate compartment
fetal calf serum
gauge (needle size)
gram
acceleration of gravity
GTPase-activating protein
guanosine diphosphate
guanine nucleotide exchange factor
<table>
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<th>Description</th>
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<tr>
<td>GMP-PNP</td>
<td>guanylimide diphosphate</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>H-2</td>
<td>histocompatibility-2</td>
</tr>
<tr>
<td>HC</td>
<td>heavy chain of the MHC class I molecule</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HSLB</td>
<td>high salt lysis buffer</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-beta-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KDEL</td>
<td>KDEL receptor</td>
</tr>
<tr>
<td>l</td>
<td>liter</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani bacterial medium</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
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<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NTA</td>
<td>nitriloacetic acid</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ODU</td>
<td>optical density unit</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDB</td>
<td>protein data bank</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulfide oxidoreductase</td>
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<tr>
<td>PLC</td>
<td>peptide loading complex</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>ppαF</td>
<td>prepro α-factor</td>
</tr>
<tr>
<td>gpαF</td>
<td>glycosylated pro α-factor</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
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<tr>
<td>RMA</td>
<td>mouse lymphoid cell line, parental of RMA-S</td>
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<tr>
<td>RMA-S</td>
<td>TAP2-deficient mouse lymphoid cell line, derivative of RMA</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute mammalian cell medium</td>
</tr>
<tr>
<td>SC</td>
<td>selective synthetic defined yeast medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SIC</td>
<td>semi-intact cells</td>
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<tr>
<td>SNARE</td>
<td>N-ethylmaleimide-sensitive factor attachment protein receptors</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
</tr>
<tr>
<td>wt</td>
<td>wildtype</td>
</tr>
<tr>
<td>T1</td>
<td>human lymphoid cell line, parental of T2</td>
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<td>T2</td>
<td>TAP2-deficient human lymphoid cell line, derivative of T1</td>
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<td>TAP</td>
<td>transporter associated with antigen presentation</td>
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<tr>
<td>TAP2&lt;sub&gt;def.&lt;/sub&gt;</td>
<td>TAP2 deficiency</td>
</tr>
<tr>
<td>tapasin</td>
<td>TAP-associated glycoprotein</td>
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<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline supplemented with Triton</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
<tr>
<td>YPD</td>
<td>yeast extract peptone dextrose medium</td>
</tr>
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</table>

Abbreviations for amino acids and chemical elements follow standard nomenclature.
INTRODUCTION

1.1 PROTEIN SORTING AND TRANSPORT FROM THE ER TO THE GOLGI APPARATUS

All membrane proteins as well as soluble proteins destined for secretion are synthesized into the Endoplasmic reticulum (ER). Here, they fold and become further trimmed before allowed to pass to the next compartment. As soon as the proteins have reached their native conformations, they exit the ER in coat protein II (COPII) vesicles which mediate transport to the Golgi apparatus. In this chapter, the mechanisms involved in ER quality control and exit of proteins will be explained in detail.

1.1.1 ER PROTEIN QUALITY CONTROL AND SORTING

During or shortly after their synthesis, secretory and membrane proteins are translocated into the Endoplasmic reticulum (ER) via the Sec61 translocon channel. Usually, translocation occurs co-translationally though post-translational translocation of completed polypeptide chains has been observed in yeast [Rothblatt and Meyer 1986]. In the ER, folding and maturation takes place before the protein can reach its final destination. The addition of asparagine-linked (N-linked) oligosaccharides to proteins in the ER provides a binding site for lectin chaperones like calnexin and its soluble homologue calreticulin that aid in protein folding [for review see Williams 2005]. The trimming of N-linked sugars also serves as indicator for the success of protein folding. Protein disulfide oxidoreductases like PDI and ERP57 are directly engaged in protein folding by catalyzing correct disulfide bond formation [Ellgaard and Helenius 2003]. Correctly folded proteins are then exported. If a protein cannot reach a native conformation and is terminally misfolded, its forward transport to the Golgi or later compartments of the secretory pathway is denied in order to prevent nonfunctional proteins being present in the cell. Instead, such proteins get retrotranslocated from the ER into the cytosol where ubiquitination and proteasomal degradation takes place (ER associated degradation, ERAD; for review see McCracken and Brodsky 1999).
Folded proteins can be distinguished from misfolded proteins by the ER quality control system. Protein sorting mechanisms in the ER further differentiate between cargo proteins that should advance to the next compartment and ER resident ones that should stay in place [Kaiser 2000]. Failure of these mechanisms to successfully act would result in disturbed protein delivery and depletion of essential resident proteins (e.g. ER chaperones). The cellular mechanisms to destine proteins to their correct compartments are extremely complex and not yet fully elucidated.

In both yeast and mammals, some membrane and soluble cargo proteins are concentrated at certain areas of the ER before being exported [Minzuno and Singer 1993, Balch et al. 1994, Malkus et al. 2002]. These ribosome-free ER exit sites (ERES) are characterized by the presence of coat protein II (COPII) markers [Hammond and Glick 2000], which provide the machinery for vesicular transport from the ER to the Golgi apparatus (see chapter 1.1.2). Cargo proteins can specifically interact with COPII to induce their uptake into anterograde transport vesicles. Several yeast and mammalian membrane proteins have been identified to bind to the COPII coat via their cytosolic tails including the yeast soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) Bet1p and Sec22p involved in vesicle targeting and fusion [Mossessova et al. 2003, Springer and Schekman 1998], the mammalian lectin-binding protein ERGIC-53 (also called p58) [Kappeler et al. 1997, Nufer et al. 2003], and the vesicular stomatitis virus glycoprotein (VSV-G) [Nishimura and Balch 1997].

Soluble and membrane cargo proteins that do not possess such export signals can leave the ER specifically by binding to cargo receptor proteins. These cargo receptors in turn bind to the COPII coat by means of their cytosolic export signals thereby mediating the inclusion of cargo proteins into the vesicles (receptor-mediated transport). Proteins with cargo receptor function providing indirect interaction with the COPII machinery are yeast Erv29p [Belden and Barlowe 2001] and mammalian ERGIC-53 (p58) [Appenzeller et al. 1999]. Export from the ER, especially for soluble cargo proteins, can apparently also occur randomly without further concentration at ERES by bulk flow [Wieland 1987, Martinez-Menarguez 1999, Schülein 2004]. However, the soluble cargo protein pro α-factor (pαF), a precursor of a secreted yeast mating pheromone, is highly concentrated in COPII vesicles dependent on the cargo receptor Erv29p [Herrmann et al. 1999, Malkus et al. 2002]. Thus, sorting
mechanisms providing the requirements for ER exit seem to be diverse and may depend on the protein examined. Interestingly, ERES are enriched in cargo proteins while ER-resident proteins like calnexin and yeast Sec61p have no access and are excluded from COPII vesicles [Kuehn et al. 1998, Belden and Barlowe 2001, Mezzacasa and Helenius 2002]. An active retention mechanism could also be responsible for keeping misfolded proteins in the ER [Hendershot 2000, Nehls et al. 2000]. ER rentention can also be achieved by retrieving accidentally “escaped” proteins from post-ER compartments (ERGIC, Golgi apparatus) back to the ER via retrograde COPI vesicles. This is the function of the mammalian KDEL receptor (KDELR), a retrograde cargo receptor responsible for retrieval of ER-resident proteins bearing a KDEL sequence. Anterograde cargo receptors like ERGIC-53 (p58) and SNARE proteins are also able to cycle between ER and Golgi to ensure several rounds of forward cargo transport and to maintain the vesicle targeting and fusion machinery [Klumperman et al. 1998, Ellgaard and Helenius 2003].

1.1.2 PROTEIN EXPORT FROM THE ER: THE COPII COAT
In eukaryotic cells, intracellular protein transport is mediated by membrane-enclosed vesicular carriers that deliver their contents from a donor to a target organelle. As mentioned before, it is a highly selective process that results in protein sorting. This selection is partly achieved by the protein coats surrounding the emerging vesicle and selectively incorporating cargo molecules. Vesicular carriers can be clearly identified by their coats which assemble on specific donor membranes only, i.e. clathrin coats mediate vesicular transport between the Golgi apparatus and the plasma membrane while coat protein complex I (COPI) coats drive retrograde transport from the Golgi back to the endoplasmic reticulum (ER) as well as intra-Golgi traffic. Anterograde transport of freshly synthesized membrane and secretory proteins from the ER to the Golgi apparatus is driven by the COPII protein coat. Even though the various coats are made up of different proteins, the mechanisms of vesicle formation are very similar consisting of initiation at the donor membrane, coat assembly, vesicle budding, uncoating, and finally fusion with the target membrane [for reviews see Schekman and Orci 1996, Kirchhausen 2000, Bonifacino and Lippincott-Schwartz 2003].
**INTRODUCTION**

Figure 1. COPII vesicle formation at the ER membrane. Exchange of GDP for GTP by Sec12 triggers Sar1 membrane insertion (1). Membrane-bound Sar1 attracts Sec23/24 and Sec13/31 complexes to polymerize at the ER membrane which is subsequently deformed into a bud at the site of polymerization. Cargo protein become incorporated into the forming vesicle by direct or indirect interaction with the COPII coat (2 and 3). GTP hydrolysis results in dissociation of Sar1 from the vesicle membrane and finally in uncoating (4). See text for detailed information.

The COPII coat mediates protein export form the ER to the Golgi apparatus. Both the components of the coat as well as the process of vesicle formation are preserved in yeast and vertebrates. The COPII coat consists of the small GTPase Sar1 and the heterodimeric protein complexes Sec23/24 and Sec13/31. COPII vesicle generation is initiated by binding of Sar1 to the ER membrane (*Figure 1*, step 1). Initially, the cytosolic form of this GTPase is associated with GDP. Binding of GTP is stimulated by the guanine nucleotide exchange factor (GEF) Sec12, an ER transmembrane protein [Springer et al. 1999]. The exchange of GDP to GTP by Sec12 triggers the exposure of a hydrophobic tail at the N-terminus of the protein that inserts into the ER membrane. Structural and biochemical studies have revealed a switching mechanism, by which the anchor is retracted into a surface groove on the GDP-bound form and exposed for membrane binding on GTP-bound Sar1 [Huang et al. 2001, Bi et al. 2002, Bickford et al. 2004].
Upon Sar1 activation, the heterodimeric Sec23/24 complex is recruited to the membrane where it is able to interact with various membrane cargo proteins (Figure 1, step 2). So, Sec23/24 exhibits a dual mode of binding by recognizing membrane-bound Sar1 as well as cytosolic cargo protein domains [Springer and Schekman 1998, Springer et al. 1999]. The subsequent association of Sec13/31 with the Sar1/Sec23/24 pre-budding complex forms a large, polymeric coat that imparts curvature on the donor membrane and finally the budding of a vesicle [Bickford et al. 2004] (Figure 1, step 2 and 3).

To allow the later fusion of the vesicle with the target membrane, the COPII coat must be shed. GTP hydrolysis hides the hydrophobic tail of Sar1 and causes its dissociation from the membrane with the destabilisation of the coat [Bickford et al. 2004] (Figure 1, step 4). Sec23 is the GTPase-activating protein (GAP) for Sar1, such that, as the pre-budding complex forms, it may only have some seconds before GTP hydrolysis triggers disassembly [Antonny et al. 2001]. Subsequent binding of Sec13/31 additionally accelerates the GTPase rate, so that the coat has probably only a brief opportunity to select cargo and polymerize before uncoating begins [Bickford et al. 2004]. GTP hydrolysis is necessary for the release of Sar1 from the vesicular membrane and the subsequent uncoating, but it could occur even before the vesicle separates from the donor membrane. Thus, Sar1 is required to assemble but not to maintain the COPII coat [Barlowe et al. 1994, Springer et al. 1999].

Docking and fusion events of the uncoated vesicles are driven by SNARE proteins like Sec22 and rBet1, which mechanically tether carriers to target membranes [for review see Jahn and Scheller 2006]. In yeast, COPII generated vesicles fuse with the Golgi apparatus while in mammalian cells, an additional organelle is present, the ER-Golgi intermediate compartment (ERGIC) [Schweizer et al. 1990]. COPII originated vesicles are targeted to this organelle, which is eventually moved towards the Golgi apparatus on microtubule tracks [Bonifacino and Lippincott-Schwartz 2003]. From here, the routes of different proteins diverge depending on their final destination; e.g. cell surface proteins are directed to the plasma membrane for exposition.
1.2 FUNCTION, STRUCTURE AND TRANSPORT OF MHC CLASS I MOLECULES

Major histocompatibility complex (MHC) class I molecules are membrane-bound cell surface proteins. They play a central role in the immune defense of vertebrates by presenting antigenic peptides to cytotoxic T lymphocytes (CTL). Their sorting and forward transport is strictly dependent on the presence of bound antigen. They can therefore be used as exemplary proteins to study protein quality control and ER export processes. On the other hand, class I investigation may also give further insights to the regulation of the cellular immune response.

1.2.1 FUNCTION AND STRUCTURE OF MHC CLASS I MOLECULES

The highly polymorphic major histocompatibility complex (MHC) class I molecules were originally discovered as the main cause for allogeneic transplant rejections, hence their name. These cell surface protein complexes present intracellularly derived antigens to cytotoxic T lymphocytes (CTL). The antigens are presented at the cell surface in the form of peptides that are the result of intracellular protein degradation. If a specific CTL recognizes the complementary class I/peptide complex with its T cell receptor (TCR), it induces apoptosis of the target cell. This way, the immune system monitors the intracellular proteome in order to control viral infection and replication as well as cancer initiation and development. Three MHC class I gene loci exist in the mammalian genome, which are called human leukocyte antigen (HLA-A, -B, -C) in humans and histocompatibility-2 (H-2D, K, and L) in mice. In both humans and mice, the gene products are highly polymorphic resulting in allelic variations (e.g. HLA-A2 or H-2D\textsuperscript{b}) [Janeway et al. 2001]. Class I polymorphism diversifies the populational immune response to a certain antigen and thereby guarantees the survival of the species.

The trimeric MHC class I molecule consists of the polymorphic membrane-bound heavy chain (HC, α chain), the soluble subunit β-2-microglobulin (β2m), and the antigenic peptide (Figure 2). The HC is a glycoprotein made up of three luminal domains (α\(_1\), α\(_2\), α\(_3\)), a transmembrane segment, and a C-terminal cytosolic tail. The α\(_3\) domain is non-covalently bound to the small β2m protein; both adopting the typical
**Figure 2. Crystal structure of an MHC class I molecule.** The panel shows the computer graphic representation of human HLA-A2 complexed with an epitope from the HIV-1 Gag-protein (PDB ID code: 2C7U). The protein consists of two chains. Two domains of the heavy or α chain (blue) form the peptide-binding groove (α₁ and α₂) where the peptide (red) is buried. The α₃ domain as well as the β₂m chain (yellow) show immunoglobulin fold. The transmembrane domain and the cytoplasmic tail of the α chain are omitted in this figure.

immunoglobulin fold (Figure 2) [Bjorkman et al. 1987]. The peptide binding region is exclusively located on the heavy chain. Two α-helices in the α₁ and α₂ domains sit on top of a β-sheet to form a peptide binding groove [Bjorkman et al. 1987]. The peptide is buried in this groove with its N- and C-termini tightly bound to conserved HC residues via hydrogen bonds [Bouvier and Wiley 1994]. Therefore, an optimal class I-binding peptide has a restricted length of 8-10 amino acids for every allele. Peptide binding is further supported by anchor residues, peptide side chains, that contact polymorphic residues in the peptide binding groove of the heavy chain (binding pockets) [Garrett et al. 1989, Matsumura et al. 1992]. The anchor residues define selective peptide binding to a certain class I allele. A peptide just needs the right length and the correct anchors to fit into the groove while the remaining positions are less restrictive in this respect. They extend upwards and contact the TCR, which recognizes a specific peptide in context with a self MHC I molecule [Garcia et al. 1996, for review see Rudolph et al. 2006]. Since a defined class I allele can bind a broad spectrum of peptides with variable sequences, the repertoire of the immune response of an individual is extremely large.
1.2.2 ANTIGEN PROCESSING AND ASSEMBLY OF MHC CLASS I COMPLEXES IN THE ER

The generation of antigenic peptides starts in the cytosol where substrate proteins are tagged for degradation by polyubiquitination and subsequently become degraded by the proteasome, a multicatalytic protease complex [for review see Hershko and Ciechanover 1998] (Figure 3, step 3). Those can be specific proteins, which become degraded at defined moments (i.e. cell cycle proteins) or when they approach the end of their natural life [Groothuis et al. 2005]. Another pool of proteasome substrates for antigen generation are freshly synthesized proteins [Reits et al. 2000, Schubert et al. 2000], which could either be degraded co-translationally or shortly after translation, possibly as the result of protein misfolding, misassembly or mistranslation [Groothuis et al. 2005]. In case of a viral infection, this would speed up an antiviral immune response.

During proteasomal degradation, the cell does not distinguish between self and foreign proteins so that self peptides also become presented at the cell surface in context with MHC class I molecules. Elimination of self-reactive CTL usually avoids autoimmune reactions. The constitutive proteasome can be upregulated upon interferon-\(\gamma\) (IFN-\(\gamma\)) treatment (immunoproteasome) [Hisamatsu et al. 1996]. The different digestion patterns of constitutive and immunoproteasome together probably increase the variety of class I-presented peptides [Toes et al. 2001]. However, there is also evidence that non-proteasomal means of MHC I peptide generation and trimming exist [Vinitsky et al. 1997, Luckey et al. 1998, Reits et al. 2004].

Peptides are generated in the cytosol but MHC class I molecules are synthesized and assembled in the ER. Class I assembly has much in common with the folding and assembly of other multimeric glycoproteins. It is a complicated and defined process that involves a number of accessory molecules including specialized components as well as common chaperones [Cresswell 2000, Williams et al. 2002]. Shortly after synthesis, the heavy chain associates with calnexin and ERp57 (Figure 3, step 1). These general chaperones (see chapter 1.1.1) bind to many newly synthesized proteins in the ER and also promote stabilization and folding of the HC [Ellgaard and Helenius 2003, Bouvier 2003]. In this stage, ERp57 may promote appropriate disulfide bond formation in the heavy chain of class I molecules [Cresswell 2000, Lindquist et al. 2001].
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Figure 3. MHC class I assembly in the ER. Shortly after synthesis, the heavy chain (HC) associates with calnexin and ERp57 (1). After binding of β2m, calnexin is replaced by calreticulin (in humans) (2). Cytosolic peptides are generated by proteasomal degradation and translocated into the ER by TAP, which is linked to the HC via tapasin. The PLC supports peptide loading into the binding groove (3). Peptide-loaded class I molecules may dissociate from the PLC and enter COPII vesicles for further transport to the Golgi apparatus (4 and 5). Protein disulfide isomerase (PDI), which is also part of the PLC, is not shown in this scheme. See text for detailed information.

Following this, the HC becomes competent to bind to β2m, which triggers the release of human HC/β2m dimers from calnexin and the binding of calreticulin (Figure 3, step 2) [Sugita and Brenner 1994]. This is different for mouse alleles, which remain associated with calnexin [Nößner and Parham 1995]. Subsequently, the HC/β2m dimer binds to the accessory proteins called the MHC class I peptide loading complex (PLC). The PLC includes the dimer, calreticulin (in humans), and ERp57 as well as the transporter associated with antigen presentation (TAP), and the TAP-associated glycoprotein (tapasin) (Figure 3, step 3). In contrast to calreticulin and ERp57, the functions of TAP and tapasin are exclusively restricted to MHC class I peptide loading. TAP is a heterodimer made of the TAP1 and TAP2 subunits. It is a member of the ATP binding cassette transporter family (ABC transporters) and translocates peptides from the cytosol into the ER in an ATP-dependent manner [Neefjes et al. 1993, Lankat-Buttgereit and Tampe 2002]. It exhibits a certain degree of peptide size
and and sequence specificity preferentially transporting peptides of 9-12 amino acids size (like MHC class I), although longer peptides may be transported [Koopmann et al. 1996]. In the ER, the peptides can be further trimmed prior to class I association [Paz et al. 1999, Yan et al. 2006]. Tapasin is a transmembrane glycoprotein and belongs to the immunoglobulin superfamily. It links the HC/β2m dimer to TAP and may therefore be responsible for the loading of optimal peptide ligands [Grandea et al. 1995, Ortmann et al. 1997, Wright et al. 2004]. Notably, ERp57 is linked to the loading complex via an intermolecular disulfide bond with tapasin [Dick et al. 2002]. Recent findings have also identified protein disulfide isomerase (PDI) as component of the class I peptide loading complex regulating the oxidation state of the disulfide-bond in the peptide-binding groove and hence influencing peptide binding [Park et al. 2006]. The highly co-operative interactions of all members in the PLC aim to optimize peptide loading to MHC class I molecules by improved peptide binding selection; a process that is also called peptide editing. Binding of an optimal peptide with prolonged dissociation rate guarantees that the ligand cannot be lost on the way to or during the exposition time at the cell surface. Thus, peptide binding and class I assembly are linked phenomena. Once an optimal peptide has bound, the trimeric HC/β2m/peptide complex may change conformation to gain a native state [Elliott et al. 1991, Springer et al. 1998]. After dissociation from the PLC, the trimer gets access to ER exit sites to enter COPII vesicles for export [Spiliotis et al. 2000] (Figure 3, step 4 and 5).

**1.2.3 EXPORT OF MHC CLASS I MOLECULES FROM THE ER**

The presentation of peptide-receptive MHC class I molecules at the cell surface is mostly avoided under physiological conditions. A peptide-receptive class I molecule – HC/β2m dimers as well as monomeric heavy chains – can either be devoid of any peptide or loaded with low affinity peptide, which can easily be lost (e.g. by temperature increase or presence of high affinity peptides). Therefore, exposure of peptide-receptive MHC I molecules at the cell surface could lead to the binding of extracellular peptides from the environment and “innocent bystander” killing of a healthy cell. The mechanisms by which those class I molecules are kept away from the cell surface are still unknown. It is clear that under physiological conditions, only fully assembled trimeric complexes can reach the trans Golgi and finally the cell
surface whereas peptide-receptive forms are retained intracellularly [Salter and Cresswell 1986, Jackson et al. 1994]. Since the release of secreted or transmembrane proteins from the ER depends on acquiring their native conformation, peptide-receptive MHC class I molecules probably do not fulfill these criteria. This is at least true for really empty, peptide-free class I molecules, which are too unstable to crystallize. Furthermore, the $\alpha_1$ and $\alpha_2$ domains seem to be more flexible without peptide bound, indicating a non-native conformation [Springer et al. 1998, Bouvier and Wiley 1998, Zacharias and Springer 2004].

Intracellular restriction of probably non-native, peptide-receptive class I molecules could be achieved in two ways: either by an active retention inside the ER or by retrieval processes from the ERGIC/cis Golgi back to the ER. In the first case, peptide-receptive class I forms may be actively kept inside the ER or selectively excluded from entering COPII vesicles. Interactions with members of the PLC could be responsible for such an ER retention as many of them contain ER retention motifs (i.e. calreticulin, ERp57, and tapasin) [Cresswell 2000]. The latter model would postulate that peptide-receptive class I molecules could be exported from the ER in COPII vesicles but would subsequently recognized and retrieved back to the ER in COPI vesicles. In fact, heavy chains as well as peptide-free dimers have been detected in the ERGIC of $\beta_2$m- and TAP-deficient cells [Hsu et al. 1991, Raposo et al. 1995]. This would imply permanent cycling of peptide-receptive class I forms between the ER and the ERGIC/cis Golgi. Thus, peptide-receptive class I molecules should or should not be found in ER-derived COPII vesicles depending on which mechanism is responsible for their intracellular retention.

1.3 **AN IN VITRO VESICLE GENERATION ASSAY (BUDDING ASSAY) TO INVESTIGATE CLASS I TRANSPORT**

To elucidate whether peptide-receptive class I molecules are retained inside the cell either by their inability to be exported from the ER or by cycling between ER and Golgi, we thought to investigate their packaging into ER-derived COPII vesicles. Transport vesicles could have not been isolated from cells so far as they are extremely short-lived *in vivo*. However, it is possible to generate them *in vitro* from isolated donor membranes in order to examine their protein contents. In *Saccharomyces*
*cerevisiae*, the five COPII components Sar1p, Sec23/24p, and Sec13/31p (see chapter 1.1.2) are necessary and sufficient to produce and isolate COPII vesicles containing cargo proteins from ER membranes *in vitro* [Barlowe et al. 1994, Bednarek et al. 1995, Kuehn et al. 1998]. A comparable system for *in vitro* vesicle generation and isolation from mammalian ER membranes is available [Nohturfft et al. 2000, Espenshade et al. 2002, Kim et al. 2005].

**Figure 4.** The COPII vesicle generation assay (= budding assay). To obtain intact portions of the ER, the plasma membrane has to be permeabilized to generate semi-intact cells (SIC) or cells have to be destroyed to isolate ER membranes (= microsomes) (1). Donor membranes (SIC or microsomes) are incubated with nucleotides and a COPII source to induce COPII vesicle formation (2). Generated vesicles are isolated (3), lysed, and their protein contents are analyzed by SDS-PAGE with subsequent detection methods (4). Detail (d): Use of GTP results in the generation of vesicles without a protein coat while the use of a non-hydrolyzable GTP analogue (e.g., GMP-PNP) gives vesicles that are still associated with the COPII proteins. See text for detailed information.

The first step of such an *in vitro* vesicle generation assay is to obtain intact portions of the ER. For this, one might either isolate ER membranes (enriched ER membranes = microsomes) from the remaining cellular compartments or permeabilize the plasma membrane to generate semi-intact cells (SIC) (*Figure 4*, step 1). The washed membrane preparation (ER membranes or semi-intact cells) is then reconstituted with purified COPII components or cytosol as a COPII source, ATP, and GTP for Sar1.
membrane binding (see chapter 1.1.2) to promote vesicle budding (therefore also referred to as “budding assay”) (Figure 4, step 2). While fast GTP hydrolysis results in naked vesicles devoid of a protein coat, the use of non-hydrolyzable GTP analogues like GMP-PNP prevent loss of the COPII proteins from the membranes [Barlowe et al. 1994] (Figure 4, detail). The generated vesicles are first separated from the donor membranes by a medium-speed spin that settles the heavier membranes and leaves the lighter vesicles in the supernatant (Figure 4, step 3). The vesicles are then pelleted by a high-speed spin and solubilized to analyze their protein cargo by electrophoresis with subsequent immunoblot or autoradiographic detection (Figure 4, step 4).

1.4 GOAL OF THIS STUDY

The aim of this project is to investigate the intracellular retention mechanism for peptide-receptive class I molecules by means of the in vitro COPII vesicle generation assay. The absence or presence of peptide-receptive class I molecules inside the generated COPII vesicles should help to understand whether ER retention or ER-Golgi-ER retrieval processes were responsible to keep these class I forms away from the cell surface.

In a first step, the COPII budding assay should be established in yeast where the system is easier to handle than in mammalian cells. In vitro translated and post-translationally translocated yeast α-factor precursor as well as endogenous yeast proteins should be used as model proteins for in vitro packaging experiments with yeast membranes. In a second step, the experience obtained from yeast budding is thought to help adopting a similar system with mammalian membranes isolated from Chinese hamster ovary (CHO) cells. Two endogenous proteins, one efficiently exported from the ER and the other one strictly retained inside the ER, should be identified and further used as positive and negative controls, respectively, to get the mammalian in vitro budding assay to work reliably. As cell surface proteins like MHC class I molecules are thought to be packaged with only medium efficiency (compared to a cycling cargo receptor protein like ERGIC-53), the assay has to be refined to detect even low protein amounts in the vesicle fractions.

To compare the uptake into COPII vesicles of peptide-loaded and peptide-receptive class I forms, TAP-defective cell lines and their wildtype counterparts should be
examined. In cells lacking the peptide transporter, peptide supply is inhibited, class I molecules are trapped in a peptide-receptive state, and cannot proceed to the cell surface under physiological conditions [for review see Lankat-Buttgereit and Tampe 1999, Lankat-Buttgereit and Tampe 2002]. *In vitro* packaging of the mouse class I allele H-2D\(^b\) should be investigated in (CHO) cells and the corresponding TAP-defective cell line as well as in the lymphoid cell line pair T1 (TAP-proficient) and T2 (TAP-deficient). The latter cell lines endogenously express HLA-A2 and -B5 and the results could be compared to those obtained from overexpressed H-2D\(^b\). Lymphoid cell lines are thought to be better candidates to follow budding of endogenous class I as they are antigen presenting cells and therefore traffick class I molecules quite efficiently.

Since maturation and transport studies on MHC class I molecules could help to model general processes involved in protein quality control and sorting, this project combines immunological and cell biological questions centered around an immunologically relevant protein.
2 MATERIALS AND METHODS

All chemicals were purchased from Karl Roth GmbH & Co. KG (Karlsruhe, Germany) and AppliChem GmbH (Darmstadt, Germany) unless indicated otherwise. The methods given here reflect their state at the time of writing.

2.1 PROTEINS: ANALYTICAL AND PREPARATIVE TECHNIQUES

2.1.1 BRADFORD ASSAY FOR PROTEIN CONCENTRATION
This assay is based on the colour change of Coomassie blue G-250 upon binding to proteins [Bradford 1976, van Kley 1977] and is used to estimate protein concentrations between approximately 20 and 500 µg/ml.

A constant volume of Bradford reagent (600 mg Coomassie blue G-250 in 1 l of 2% perchloric acid) was mixed with different sample dilutions and absorbance at 495 nm was measured. Bovine serum albumin (BSA) was used as a standard.

2.1.2 ENDO-H TREATMENT AND CON-A PRECIPITATION OF GLYCOSYLATED PROTEINS
Endoglycosidase H (EndoH) cleaves high mannose oligosaccharides from N-linked glycoproteins only that have not yet achieved complex glycosylations in the median Golgi. Therefore, EndoH resistance can be used as marker for glycoprotein localization inside the cell. Samples were denatured in 1× glycoprotein denaturing buffer at 100°C for 10 minutes. After addition of 1/10 volume of 10× G5 buffer and 1-2 µl of EndoH, the reaction was incubated at 37°C for 1 hour. Loading buffer (6× concentrated buffer contains 350 mM Tris-Cl, pH 6.8, 10% sodium dodecyl sulfate (SDS), 36% glycerol, 0.6 M dithiotreitol (DTT), 0.05% bromophenol blue) was added to a final concentration of 2× and samples were subjected to SDS-PAGE (polyacrylamide gel electrophoresis) and immunoblot. EndoH, was purchased from New England Biolabs Inc. (Ipswich, Massachusetts, USA). Glycoprotein denaturing and G5 buffers are included.
The lectin Concanavalin A (ConA) recognizes commonly occurring sugar structures. ConA conjugates can therefore be used to examine or purify glycoproteins. Samples were denatured in 2% SDS at 95°C for 5 minutes. Then, 9/10 volume ConA buffer (500 mM NaCl, 1% Triton X-100, 20 mM Tris-Cl, pH 7.6, 2 mM NaN3) was added to wash out SDS. Samples were centrifuged for 5 minutes at 14,000× g. To the supernatant 1/30 volume of 20% (v/v) ConA agarose (Vector Laboratories through Linaris GmbH (Wertheim-Bettingen, Germany) was added and the suspension was incubated for 1 hour at room temperature. ConA agarose beads were spun down for 2 minutes at maximum speed and washed twice in 500 µl ConA buffer. Glycosylated proteins were analyzed by SDS-PAGE and immunoblot.

2.1.3 CHLOROFORM PRECIPITATION OF PROTEINS
Cells were resuspended in 150 µl lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100 plus protease inhibitors, which had been 1 mM PMSF (phenylmethylsulphonylfluoride), 10 µM pepstatin A, 10 µM leupeptin), vortexed, and incubated for 10 minutes on ice. The maximal lysis concentration of the buffer is 2×10^7 cells/ml. After incubation, cell debris was pelleted at 14,000× g for 5 minutes at 4°C and the supernatant was transferred to a new tube. Four volumes of methanol and one volume of chloroform were added to the supernatant, the solution was vortexed, three more volumes of water were added, and vortexed again. After a 14,000× g spin (5 minutes, 4°C), the upper phase was carefully removed, three volumes of methanol were added, and the solution was vortexed. Protein precipitates were spun out (15 minutes, 14,000× g, 4°C), dried, and redissolved in 20 µl 1× loading buffer. Protein analysis was performed by SDS-PAGE and immunoblot.

2.1.4 SDS-PAGE OF PROTEINS
Proteins were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) in the discontinuous buffer system of Laemmli [Laemmli, 1970]. Acrylamide concentrations were 5% in the stacking and 8-12.5% in the resolving gels. For improved protein separation, 4 M urea gels containing 18% acrylamide were used. Detailed gel contents are given in Table 1A and 1B. Gel preparation and gel electrophoresis were performed using the equipment for the Hoefer Electrophoresis.
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Unit SE600 series and the Hoefer Mighty Small dual gel caster (GE Healthcare Europe GmbH, Munich, Germany).

Protein containing samples were mixed with loading buffer to reach a final buffer concentration of 1×, vortexed and heated to 95°C for 5 min. After cooling down and spinning briefly, samples were ready for application. Gels were run in 1× running buffer (3 g Tris-base, 14.4 g glycine, 1 g SDS ad 1 l with water) at 20-40 mA for 1-5 hours depending on the gel size.

Table 1A: SDS acrylamide and urea gel preparation for 30 ml resolving gels

<table>
<thead>
<tr>
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<th>8%</th>
<th>10%</th>
<th>12.5%</th>
<th>18%</th>
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<tbody>
<tr>
<td>Acrylamide</td>
<td>8 ml</td>
<td>10 ml</td>
<td>12.5 ml</td>
<td>18 ml</td>
</tr>
<tr>
<td>Water</td>
<td>14 ml</td>
<td>12 ml</td>
<td>9.6 ml</td>
<td>to 30 ml</td>
</tr>
<tr>
<td>1.5 M Tris-Cl, pH 8.8</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
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<tr>
<td>20% SDS</td>
<td>150 µl</td>
<td>150 µl</td>
<td>150 µl</td>
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<tr>
<td>TEMED</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
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<tr>
<td>10% APS</td>
<td>200 µl</td>
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<td>200 µl</td>
<td>200 µl</td>
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<td>Urea</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.2 g</td>
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Table 1B: SDS acrylamide and urea gel preparation for 20 ml stacking gels

<table>
<thead>
<tr>
<th></th>
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<th>5%</th>
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<tbody>
<tr>
<td>Acrylamide</td>
<td>3.3 ml</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>Water</td>
<td>11.4 ml</td>
<td>to 20 ml</td>
</tr>
<tr>
<td>0.5 M Tris-Cl, pH 6.8</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>24 µl</td>
<td>24 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>160 µl</td>
<td>160 µl</td>
</tr>
<tr>
<td>Urea</td>
<td>-</td>
<td>5 g</td>
</tr>
</tbody>
</table>

2.1.5 ANALYSIS OF RADIONLABELLED PROTEINS

For autoradiographic analysis of radiolabelled protein, resolving gels were fixed, stained in Coomassie blue R-250, dried, and exposed on a Fujifilm Imaging Plate (Fujifilm Photo Film Europe GmbH, Duesseldorf, Germany) for at least 12 hours. During that time, radioactivity generates fluorescent signals on the plate coating, which were read out using the Phosphoimager FLA 3000 (Fujifilm Photo Film
MATERIALS AND METHODS

Europe GmbH). As radioactive protein marker, \(^{14}\)C Methylated Protein Molecular Weight Marker (GE Healthcare Europe GmbH) was used.

### 2.1.6 IMMUNOBLOT ANALYSIS OF NON-LABELLED PROTEINS

Non-labelled proteins were further subjected to immunoblot transfer onto polyvinylidene fluoride (PVDF) carrier membranes. For this, gels and membranes were placed on top of each other and protein transfer took place in 1× transfer buffer (3 g Tris-base, 14.8 g glycine, 150 ml methanol to 1 l with water) for at least 2 hours at 80-150 mA in the TE 22 or TE 42 tank transfer units (GE Healthcare Europe GmbH). As protein molecular mass marker, SeeBlue Plus2 Pre-Stained Standard (Invitrogen GmbH, Karlsruhe, Germany) was used.

After transfer, membranes were blocked for 1 hour in 5% milk in 1× TBST (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween). Incubation with primary antibodies was carried out overnight at 4°C. To detect proteins of different sizes in the same lane, the PVDF membrane was cut and the different stripes were incubated separately. The next day, membranes were washed 3× in 1× TBST for 8 minutes and subjected to incubation with secondary antibodies conjugated with the enzyme alkaline phosphatase for 1 hour. After washing 6× in 1× TBST for 5 minutes, membranes were incubated in enzyme-catalyzed fluorescence (ECF) substrate (GE Healthcare Europe GmbH), which becomes fluorescent after conversion by alkaline phosphatase. Fluorescent signals were scanned using the Phosphoimager FLA 3000.

### 2.1.7 PROTEIN EXPRESSION AND PURIFICATION

Yeast and mammalian COPII constructs were kindly provided by Jinoh Kim and Randy Schekman (Berkeley, USA). Hamster \textit{sar1Awt} and mutant \textit{sar1AT39N} genes in pJK1 and pJK3, respectively, as well as yeast wild-type \textit{SARI} in pTY40 were cloned in a pGEX-2T background. Thus, the corresponding proteins were expressed as glutathione-S-transferase (GST)-fusions under control of the \textit{lac} promoter. Yeast \textit{Sec13/31} was expressed and purified as complex in pNS3141 with a 6-his-tag on the Sec31 protein. It was cloned in a YEp352 background with \textit{URA3} auxotrophy as selection marker. Yeast \textit{SEC23} in PTYY117 (YEp352-based, \textit{URA3}) and \textit{SEC24} in pTYY121 (YEp351-based, \textit{LEU2}) were expressed simultaneously in one strain. Sec23/24p complexes were purified on the basis of 6-his-tagged Sec24p [Kim et al.
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2004, Barlowe et al. 1994, Pryer et al. 1993]. In this study, yeast COPII components purified by Crystal Chan, Howard Hughes Medical Institute, Berkeley, were used. The procedure of purification is described for completeness.

For purification of mammalian Sar1A and yeast Sar1p GST-fusion proteins, chemically competent *Escherichia coli* BL21(DE3)pLysS cells (Novagen, Darmstadt, Germany) were transformed by heat shock and plated onto selective Luria Bertani (LB) agar (1% bacto-tryptone, 0.5% bacto yeast extract, 1% NaCl, 2% agar, pH 7.0) containing ampicillin and chloramphenicol. Overnight cultures (SPB533 expressing hamster wild type Sar1Aw t, SPB535 expressing hamster Sar1AT39N, and SPB87 for yeast Sar1p) were resuspended in 1 l fresh selective LB medium and grown at 37°C until OD_{600} had reached a value of around 0.6. Protein expression was induced by addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). After an incubation of 3 hours at 30°C, cells were harvested at 5,000× g at 4°C for 10 minutes. The pellet was resuspended in 10 ml 1× TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing PMSF and frozen in liquid nitrogen for up to one week.

Cells were thawed in a 30°C waterbath and lysis was performed in 1% Triton X-100 followed by sonication in a Sonorex UW70 (Bandelin electronic GmbH & Co. KG, Berlin, Germany). The suspension was spun at 16,000× g at 4°C for 20 minutes and the supernatant was applied to a column containing glutathione agarose beads allowing GST-fusions to bind. After sequential washing steps in 1× TBST, 1× TBS, and 1× TCB2 (50 mM Tris, pH 8.4, 5 mM CaCl2, 150 mM KAc), Sar1 proteins were released from bound GST by addition of 400 units of thrombin. Columns were drained, washed with 1× TCB2 and fractions were collected. Protein content of the fractions was determined by Bradford assay and desired fractions were pooled. Thrombin precipitates were removed by a 15,000× g spin for 5 minutes and the final protein concentration of the solution was determined. Aliquots were frozen in liquid nitrogen and stored at –80°C.

Expression of his-tagged yeast Sec complexes was performed in different strains of *Saccharomyces cerevisiae*. Sec13/31p was expressed in SPY371 (-ura3) and Sec23/24p in SPY370 (-ura3, -leu2). Transformed cells were plated onto selective synthetic defined medium (SC (-ura) and SC (-ura, -leu), respectively) (1.7 g/l minimal medium, 20 mg/l selective amino acid mix minus uracil/leucine, 2% glucose) and overnight cultures were resuspended in 50 ml of liquid yeast
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extract/peptone/dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose). Resuspended cells were transferred to 1 l YPD medium and incubated at 30°C until OD<sub>600</sub> had reached about 1.5. Cells were spun down at 3000× g for 5 minutes and weight out. The following buffers all include protease inhibitors (1 mM PMSF, 10 µM pepstatin A, 10 µM leupeptin). Eight grams of cells were resuspended in 50 ml buffer A (10 mM DTT, 100 mM Tris, pH 9.4) and left at room temperature for 10 minutes. Cells were spun down at at 3000× g for 5 minutes and resuspended in 50 ml lyticase buffer (700 mM sorbitol, 25 mM Tris-Cl, pH 7.4, 1 mM DTT, 0.5% glucose in YPB medium) plus 1.5 ml lyticase solution. Lysis of the yeast cell wall was for 30 minutes in a 30°C waterbath.

Yeast spheroplasts were centrifuged at 3200× g for 20 minutes at 4°C. The pellet was resuspended gently in 100 ml of high salt lysis buffer (HSLB) (750 mM KAc, 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 6.8, 0.1 mM EGTA, 10% glycerol, pH was adjusted to 7.0 with 5 M KOH). Spheroplasts were frozen in liquid nitrogen and stored at −80°C or directly homogenized in a blender (Waring Commercial, Torrington, USA). Cell debris was pelleted at 13,000× g for 75 minutes at 4°C, the supernatant was transferred to ultracentrifuge tubes (rotor 45Ti, Beckman Coulter GmbH, Krefeld, Germany), further cleaned at 185,000× g (75 minutes, 4°C), and the high-speed supernatant was carefully collected. In the meantime, fresh nickel nitrilotriaceticacid (Ni-NTA) agarose beads (Invitrogen GmbH) were washed twice in 4 ml water by inverting the tube several times and spinning down the beads at 2000× g for 1 minute at 4°C. After washing in 4 ml 0.2 M acetic acid and 10% glycerol and subsequently in 6 ml water, the beads were equilibrated in 8 ml (HSLB) (750 mM KAc, 50 mM HEPES, pH 6.8, 0.1 mM EGTA, 10% glycerol, 40 mM imidazole).

To purify his-tagged Sec13/31 and Sec23/24 protein complexes, equilibrated Ni-NTA beads and the high-speed supernatant were combined and rotated for 1 hour at 4°C to allow binding of his-tagged proteins. Beads were spun down at 2000× g for 2 minutes at 4°C and washed in 3× 10 ml wash buffer I (750 mM KAc, 50 mM MES, pH 6.3, 0.1 mM EGTA, 10% glycerol, 40 mM imidazole). The beads were loaded into a column (Sarstedt AG & Co., Nümbrecht, Germany) and washed 10× with 1 ml of wash buffer I and 8× with 1 ml wash buffer II (500 mM KAc, 50 mM HEPES, pH 7.0, 0.1 mM EGTA, 10% glycerol, 40 mM imidazole). Elution of his-tagged Sec
complexes from the column was in 10 ml elution buffer 500 mM KAc, 50 mM HEPES, pH 7.0, 0.1 mM EGTA, 10% glycerol, 400 mM imidazole).

To get rid of high imidazole concentrations in the Sec13/31p purifications, collected fractions were loaded onto diethylaminoethyl (DEAE) cellulose columns (Sigma-Aldrich Chemie GmbH, Munich, Germany), which had been equilibrated with 8 ml buffer A (500 mM KAc, 50 mM HEPES, pH 7.0, 0.1 mM EGTA, 10% glycerol), the flowthrough was collected, the column was washed 10× with 1 ml buffer A, and 1 ml fractions were collected. Elution was performed 10× with 0.5 ml buffer B (800 mM KAc, 50 mM HEPES, pH 7.0, 0.1 mM EGTA, 10% glycerol, 50 mM imidazole) and 0.5 ml fractions were collected.

Sec23/24p complexes were separated from free Sec24-his in a Sephadex G-25 column (Sigma-Aldrich Chemie GmbH) equilibrated with desalting buffer (500 mM KAc, 50 mM HEPES, pH 7.0, 0.1 mM EGTA, 10% glycerol, 100 mM imidazole). The sample was collected in 3.5 ml desalting buffer. Location peaks of preparations were verified by Bradford assay and SDS-PAGE and protein-containing fractions were pooled, frozen, and stored at –80°C.

2.2 ANTIBODIES

All antibodies were used for immunoblotting and are summarized in Table 2.

2.2.1 FOR DETECTION OF YEAST PROTEINS

The SNARE protein Sec22p was detected using a rabbit antiserum, which was a kind gift from Randy Schekman (Berkeley, USA).

2.2.2 FOR DETECTION OF MAMMALIAN PROTEINS

If not otherwise stated, all antibodies had been rabbit antisera. The antibody recognizing the ER chaperone calnexin in all tested cell species was purchased from Stressgen Bioreagents (Ann Arbor, USA). In chinese hamster ovary (CHO) and RMA cells, the ERGIC-marker p58 (ERGIC-53 in humans) was detected with an antibody kindly provided by Jaakko Saraste (Bergen, Norway). The same protein in T1 cells was recognized by an antibody donated by Ralf Pettersson (Stockholm, Sweden). The serum detecting mouse H-2D\(^b\) (T18) was a kind gift from Tim Elliott (Southampton,
England). The rabbit polyclonal antibody against human HLA (H-300) was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). For detection of the TAP2-transporter, the purified mouse monoclonal antibody clone 435.3 was used. The latter was kindly provided by Peter van Endert (Paris, France). Further information to the cell lines are given in Table 3.

Table 2: Overview of antibodies used for immunoblotting

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Company/cooperator</th>
<th>Anti-species</th>
<th>Dilution</th>
<th>Secondary antibody</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit serum Anti-Sec22p</td>
<td>R. Schekman (Berkeley, San Francisco, USA)</td>
<td>Yeast</td>
<td>1:1,000</td>
<td>Alkaline phosphatase conjugated goat anti-rabbit</td>
<td>Dianova (Hamburg, Germany)</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Rabbit serum Anti-calnexin</td>
<td>Stressgen Bioreagents (Michigan, USA)</td>
<td>All species tested</td>
<td>1:10,000</td>
<td>Alkaline phosphatase conjugated goat anti-rabbit</td>
<td>Dianova (Hamburg, Germany)</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Rabbit serum Anti-p58</td>
<td>J. Saraste (Bergen, Norway)</td>
<td>Hamster Mouse</td>
<td>1:2,000</td>
<td>Alkaline phosphatase conjugated goat anti-rabbit</td>
<td>Dianova (Hamburg, Germany)</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Rabbit serum Anti-p58</td>
<td>R. Pettersson (Stockholm, Sweden)</td>
<td>Human</td>
<td>1:4,000</td>
<td>Alkaline phosphatase conjugated goat anti-rabbit</td>
<td>Dianova (Hamburg, Germany)</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Rabbit serum Anti-H-2D&lt;sup&gt;b&lt;/sup&gt; and K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>T. Elliott (Southampton England)</td>
<td>Mouse</td>
<td>1:500</td>
<td>Alkaline phosphatase conjugated goat anti-rabbit</td>
<td>Dianova (Hamburg, Germany)</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Rabbit polyclonal Anti-HLA</td>
<td>Santa Cruz Biotechnology Inc. (Heidelberg, Germany)</td>
<td>Human</td>
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<td>Alkaline phosphatase conjugated goat anti-rabbit</td>
<td>Dianova (Hamburg, Germany)</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Mouse monoclonal Anti-TAP2</td>
<td>P. van Endert (Paris, France)</td>
<td>Human Mouse</td>
<td>1:500</td>
<td>Alkaline phosphatase conjugated goat anti-mouse</td>
<td>Dianova (Hamburg, Germany)</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>
2.3 IN VITRO BUDDING ASSAY USING YEAST MEMBRANES

2.3.1 PREPARATION OF YEAST SEMI-INTACT CELLS

For preparation of yeast semi-intact cells (SIC), the *Saccharomyces cerevisiae* protease-reduced strain SPY 245 was grown in yeast extract/peptone/dextrose (YPD) medium (1% yeast extract, 2% bacto peptone, 2% dextrose in water) to an optical density at 600 nm (OD$_{600}$) of 0.4 and overnight culture were inoculated 1/200. The next day, cells were spun at 5,000×g for 5 min and washed in water once. The cell pellet was resuspended in 25 OD units/ml (one OD unit is the concentration giving an OD of 1.0 for a 1 ml sample) buffer A (100 mM Tris-Cl, pH 9.4, 10 mM DTT, 20 mM NaN3, 10 mM NaF) and incubated at room temperature for 10 minutes. After a spin at 5,000×g for 5 minutes, cells were resuspended in spheroplasting buffer (700 mM sorbitol, 10 mM Tris-Cl, pH 7.5, 1 mM DTT, 20 mM NaN3, 10 mM NaF) to 25 OD units/ml. Digestion of the yeast cell wall was performed by addition of 1-3 µl lyticase solution per OD unit of cells and incubation at 30°C for 30 minutes. Spheroplasts were spun at 5,000×g for 3 minutes, washed in 10 ml yeast lysis buffer (400 mM sorbitol, 150 mM KAc, 20 mM HEPES, pH 6.8, 2 mM MgAc, 0.5 mM EGTA (ethylene glycol tetraacetic acid) once, and resuspended in lysis buffer at 125 OD units/ml. The yield was determined by measuring OD$_{250}$ of an 1/500 dilution in 2% SDS in lysis buffer. The concentration of the final suspension was adjusted to an OD$_{250}$ of 0.2 at 1/500 dilution. Yeast semi-intact cells were aliquoted and frozen at −80°C using a cell freezer (Nalge Nunc International, Rochester, USA).

2.3.2 IN VITRO TRANSCRIPTION, TRANSLATION, AND TRANSLOCATION OF YEAST ALPHA FACTOR INTO YEAST SEMI-INTACT CELLS

For *in vitro* transcription/translation of the yeast pheromone precursor prepro α-factor (ppαF), TNT SP6 coupled reticulocyte systems (Promega GmbH, Mannheim, Germany) was used. The respective gene construct was a kind gift from Randy Schekman (Berkeley, USA) and was cloned in pDJ100 (pSP64 Poly(A) background) suitable for *in vitro* transcription from the SP6 promoter. The template DNA was purified using the QIaprep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany)
and had to be free of ethanol, calcium, RNase, and salt. All the following steps were performed under RNase-free conditions.

The reticulocyte lysate was thawed at 37°C and all components were placed on ice immediately. One transcription/translation reaction contained 12.5 µl rabbit reticulocyte lysate, 1 µl TNT reaction buffer, 0.5 µl TNT SP6 RNA polymerase, 0.5 µl amino acid mixture minus methionine, 1 µl \(^{35}\)S methionine (Redivue™ PROMIX, GE Healthcare Europe GmbH), 1 µl template DNA, 8.5 µl nuclease-free water in a final volume of 25 µl. The reaction was gently mixed and incubated for 90 minutes in a 30°C waterbath. Translation efficiency was determined by SDS-PAGE and radioactive analysis. Freshly synthesized \(^{35}\)S-ppαF was either used directly or frozen at –20°C for up to four weeks.

For post-translational translocation of \(^{35}\)S-ppαF into the ER of yeast semi-intact cells (strain SPY245), 100 µl of membrane suspension was spun at 14,000×g for 4 minutes at 4°C. The pellet was washed with 500 µl buffer 88 (20 mM HEPES, pH 7.2, 250 mM sorbitol, 150 mM KAc, 5 mM MgAc) and semi-intact cells were resuspended in 100 µl buffer 88. One translocation reaction contained 30 µl yeast SIC, 3 µl translation reaction, 5 µl 10× ATP regeneration mix (55 mg ATP, 1.3 g creatine phosphate, 20 mg creatine phosphokinase in 10 ml buffer 88, pH 6.8 with KOH), 12 ml buffer 88 in a total volume of 50 µl. The reaction was mixed and incubated for 30 minutes in a 10°C waterbath. After a spin at 14,000×g for 4 minutes at 4°C, supernatants were removed and membranes were washed twice in 1 ml buffer 88. Finally, the pellet was resuspended in 2× loading buffer and subjected to SDS-PAGE analysis. Translocation of \(^{35}\)S-ppαF resulted in cleavage of the signal sequence (pro alpha factor [pαF]) and addition of N-linked carbohydrates (gpαF), which was verified by EndoH treatment, ConA precipitation, and protease protection.

2.3.3 PROTEASE PROTECTION ASSAY OF TRANSLOCATED YEAST ALPHA FACTOR

Membrane-protection of translocated \(^{35}\)S-gpαF from proteases was shown by treating 30 µl translocation yeast semi-intact cells with 3 and 6 µl of trypsin (1 mg/ml) or proteinase K (1 mg/ml) in a final volume of 40 µl in buffer 88. To lyse the protecting ER membrane, 4 µl of 10% Triton X-100 was added to the respective samples and mixed. Incubation was for 10 minutes on ice. To stop further protease action, trypsin
and proteinase K were inhibited by addition of 3 and 6 μl trypsin inhibitor (2 mg/ml) and 3 μl of PMSF (250 mM), respectively. After addition of loading buffer, samples were subjected to urea SDS-PAGE and digest of α-factor was monitored by autoradiography.

2.3.4 IN VITRO BUDDING ASSAY OF YEAST PROTEINS INTO COPPI VESICLES

Prior to each yeast in vitro budding assay, α-factor was synthesized and post-translationally translocated into yeast semi-intact cells as described before. Translocated $^{35}$S-gpαF was then packaged into COPII vesicles in vitro. Each 50 μl budding reaction contained 5 μl $^{35}$S-gpαF-containing yeast SIC, 5 μl 10× ATP regeneration mix, 170 μM guanosine triphosphate (GTP) or its non-hydrolyzable analogue guanylyl imidodiphosphate (GMP-PNP), and the following amounts of COPII components: 0.3 μg Sar1p, 0.5 μg Sec23/24p complex, 0.5 μg Sec13/31p complex in buffer 88 in siliconized tubes (Biozym Scientific GmbH, Oldendorf, Germany). The reaction was mixed and incubated for 30 minutes in a 25°C waterbath.

To separate generated vesicles from donor membranes, samples were spun at 14,000× g (4 minutes, 4°C, medium-speed spin) except for that one representing the total membrane fraction. From the latter, 40 μl were transferred directly (100% total), from the remaining samples 40 μl of the supernatant was transferred to ultracentrifuge tubes (rotor TLA-55, Beckman Coulter GmbH). Vesicles and total membranes were pelleted by spinning at 100,000× g (high-speed spin) for 25 minutes at 4°C. After resuspending in 20 μl 1× loading buffer, the total sample was diluted to 20%, and all samples were run out on SDS acrylamide gels followed by autoradiographic detection of $^{35}$S-gpαF.

For budding analysis of endogenous Sec22p, SDS gels were subjected to immunoblot transfer prior to fixation. Post-transfer gels and PVDF-membranes were both analysed for the presence of $^{35}$S-gpαF. Additionally, membranes were further used for immunoblot analysis of endogenous protein.
2.4 IN VITRO BUDDING ASSAY USING MAMMALIAN MEMBRANES

2.4.1 MAMMALIAN CELL CULTURE

Frozen cells were kept in liquid nitrogen in freezing medium containing fetal calf serum (FCS (Perbio Science Deutschland GmbH, Bonn, Germany), 10% glycerol). For seeding, cells were thawed in a 37°C waterbath and subsequently transferred to 75 ml flasks (Sarstedt AG & Co., Nümbrecht, Germany) containing 15-30 ml cell culture medium. All cell lines were grown at standard conditions at 37°C in an atmosphere of 5% CO₂ and fed every 3-4 days depending on cell proliferation. Adherent fibroblasts were cultured in Ham’s F12 medium (Sigma-Aldrich Chemie GmbH, Munich, Germany), supplemented with 5% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. The cells were splitted using a trypsin/EDTA mixture (Cambrex Bioscience, Verviers, Belgium). Prior to an experiment, the cells were seeded in 10 cm cell culture dishes (Sarstedt AG & Co.) and harvested at a confluency of 80-90%. Lymphoid suspension cells were maintained in RPMI 1640 medium (Sigma-Aldrich Chemie GmbH) supplemented with 10% FCS and the same amounts of penicillin/streptomycin and glutamine.

Table 3: Overview of used cell lines

<table>
<thead>
<tr>
<th>Name</th>
<th>Cell type</th>
<th>Species</th>
<th>Original tissue</th>
<th>TAP2 deficiency</th>
<th>Endogenous MHC class I background</th>
<th>Stably transfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-K1</td>
<td>Fibroblast</td>
<td>Hamster</td>
<td>Ovary</td>
<td>-</td>
<td>Not known</td>
<td>-</td>
</tr>
<tr>
<td>CHO/Dᵇ</td>
<td>Fibroblast</td>
<td>Hamster</td>
<td>Ovary</td>
<td>-</td>
<td>Not known</td>
<td>Mouse H-2Dᵇ</td>
</tr>
<tr>
<td>CHO/Dᵇ/TAP2ᵇ⁻</td>
<td>Fibroblast</td>
<td>Hamster</td>
<td>Ovary</td>
<td>+</td>
<td>Not known</td>
<td>Mouse H-2Dᵇ</td>
</tr>
<tr>
<td>T1</td>
<td>Lymphoid</td>
<td>Human</td>
<td>T/B lymphocyte hydrids</td>
<td>-</td>
<td>HLA-A2 HLA-B5</td>
<td>-</td>
</tr>
<tr>
<td>T2</td>
<td>Lymphoid</td>
<td>Human</td>
<td>T/B lymphocyte hydrids</td>
<td>+</td>
<td>HLA-A2 HLA-B5</td>
<td>-</td>
</tr>
</tbody>
</table>
Chinese hamster ovary fibroblasts (CHO-K1) were purchased from the European Collection of Cell Cultures (ECACC). CHO cells stably transfected with mouse H-2D\textsuperscript{b} as well as their TAP2-deficient counterpart were a kind gift from Keith Gould (London, England). The human cell lines T1 and T2 were kindly provided by Alain Townsend (Oxford, England). Detailed informations about the used cell lines are summarized in Table 3.

2.4.2 PREPARATION OF MAMMALIAN MICROSOMES

Adherent cells were grown in ∅ 10 cm cell culture dishes (Sarstedt AG & Co.) up to 80-90% confluency (corresponding to approximately 2 million cells). Suspension cells were counted and 2 million cells per sample were used. The procedure was adopted from published protocols [Nohturfft et al. 2000, Kim et al. 2005].

Cells were put on ice, washed once with 2 ml ice-cold 1× PBS (phosphate buffered saline), pH 7.4 (1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 2.5 mM KCl, 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 136 mM NaCl), spun at 1,000× g (5 minutes, 4°C), and resuspended in 5 ml 1× PBS. Fibroblasts were scraped in the second PBS wash and suspensions from all examined dishes were pooled. After another spin (1,000× g, 5 minutes, 4°C), cell pellets were resuspended in 0.2 ml buffer F (10 mM HEPES-KOH, pH 7.2, 250 mM sorbitol, 10 mM KAc, 1.5 mM MgAc\textsubscript{2}) plus protease inhibitors and passed through a 22 gauge (22G) needle 20 times. The suspension was spun again (1,000× g, 5 minutes, 4°C) to sediment crude cell debris and the supernatant was transferred to siliconized tubes (Biozym Scientific GmbH). Enriched ER membranes (microsomes) were pelleted by a 6,000× g for 10 minutes at 4°C. The membrane pellet was washed twice in 0.1 ml buffer G (20 mM HEPES-KOH pH 7.2, 250 mM sorbitol, 150 mM KAc, MgAc\textsubscript{2}) containing protease inhibitors and was finally resuspended in 30 µl buffer G. OD\textsubscript{600} of the suspension was between 0.05 and 0.5 (30 µl of membrane suspension in 470 µl water, 500 µl water as blank). To each budding reaction, 30 µl of membrane suspension was added.

2.4.3 PREPARATION OF MAMMALIAN SEMI-INTEGRAL CELLS

Two million cells were washed 3× with 5 ml ice-cold 1× PBS, adherent cells were scraped in the third wash. Cells were spun at 800× g for 5 minutes at 4°C. The pellet was resuspended in 200 µl buffer G, spun at 800× g for 5 minutes at 4°C, and
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resuspended in buffer G containing protease inhibitors. The cell suspension was
shock-frozen in liquid nitrogen for 1 minute and rewarmed in a 40°C waterbath until
totally thawed. The freeze-thaw cycle was repeated twice. Semi-intact cells were
pelleted at 800× g for 5 minutes at 4°C, washed once in 200 µl buffer G, and
transferred to siliconized tubes. After another 800× g spin for 5 minutes at 4°C, the
final pellet was resuspended in 30 µl buffer G and included in one budding reaction.

2.4.4 PREPARATION OF MAMMALIAN TISSUE CYTOSOL

Cytosol preparation was adopted from published protocols [Nohturfft et al., 2000].
Freshly excised livers from rats or Djungarian hamsters were washed three times with
ice-cold 1× PBS and cut in small pieces. The following steps were carried out in the
cold room at 4°C. The tissue was disrupted in buffer E (50 mM HEPES-KOH,
ph 7.2, 250 mM sorbitol, 70 mM KAc, 5 mM potassium EGTA, 2.5 mM MgAc2)
plus protease inhibitors (1-2 ml buffer E/g liver) using mortar and pestle.
Homogenisation was performed by 10 strokes in a Dounce homogenizer with loose
pestle. The crude extract was spun in Oakridge tubes (Nalge Nunc International) at
25,000× g for 15 minutes at 4°C and the supernatant was filtered through one layer of
gauze. After centrifugation at 186,000× g for 1 hour (ultracentrifugation tubes, rotor
TLA55, Beckman Coulter GmbH), the aqueous supernatant was carefully transferred
to fresh tubes and the centrifugation was repeated. The final supernatant was
designated cytosol with concentrations of 15-25 µg/ml determined by Bradford.
Cytosol aliquots were frozen in liquid nitrogen and stored at –80°C.

2.4.5 IN VITRO BUDDING ASSAY USING MAMMALIAN MEMBRANES

Each reaction contained 20 mM HEPES-KOH, pH 7.2, 250 mM sorbitol, 150 mM
KAc, and 0.5 mM MgAc2 (buffer G), 0.2 mM GTP or GMP-PNP, an ATP
regenerating system composed of 1 mM ATP, 40 mM creatine phosphate, and 0.2 mg
creatine phosphokinase, 30 µl of mammalian membrane suspension (microsomes or
SIC), 4 mg/ml of cytosol or 25 µg/ml yeast Sar1p, 15 µg/ml Sec23/24p, 20 µg/ml
Sec13/31p, and protease inhibitors in 80 µl final volume in siliconized tubes. When
mentioned, the phosphohydrolase apyrase (New England Biolabs Inc., stock: 1 mg/ml
in water) was included to a final concentration of 10 µg/ml, purified mammalian
wildtype or mutant Sar1A to a final of 500 nM. After 5 minutes preincubation on ice,
the budding reaction was carried out for 30 minutes at 25°C (waterbath) and terminated by transferring tubes on ice.

Except for the 100% (total) sample, fractions were spun at 14,000× g (medium-speed spin) for 20 minutes at 4°C to separate the generated vesicles from the donor membranes. From the 100% total sample as well as from the medium-speed supernatant, 60 µl were transferred to ultracentrifugation tubes (rotor TLA55, Beckman Coulter GmbH). Vesicles and total membranes were collected at 100,000× g (high-speed spin) for 25 minutes at 4°C. High-speed pellets were washed once with 100 µl buffer G and the 100,000× g spin was repeated. Pellets were resuspended in 1× loading buffer, the total fraction was diluted to different ratios, and samples were heated to 95°C for 5 minutes. Samples were frozen at –20°C or directly loaded onto small 8-10% SDS acrylamide gels, transferred to PVDF membranes and analyzed by immunoblotting with the indicated antibodies (Table 2).

**2.5 SIGNAL QUANTIFICATION**

Radioactive yeast 35S-gpαF budding signals converted to fluorescent signals on the Imaging Plate (chapter 2.1, Analysis of radiolabelled proteins) were quantified using AIDA software, version 4.0. Protein content of the total membrane fraction was set to 100% and budding efficiencies were calculated relative to that number. Intensity and appearance of immunoblot signals are strongly dependent on concentration, affinity, and background binding of both primary and secondary antibodies. Thus, immunoblot data are not linear making quantifications less reliable. They were therefore analyzed “by eye”.
3 RESULTS

3.1 YEAST ALPHA FACTOR: IN VITRO SYNTHESIS AND TRANSLOCATION

Yeast in vitro trafficking systems are much better established than mammalian ones. They are more easily to control because of the availability of yeast alpha factor (α-factor, αF), a well understood model protein for in vitro trafficking studies. The advantage is that α-factor can be labelled during in vitro synthesis and afterwards post-translationally translocated into yeast membranes. Being the only radioactive protein in the membranes after translocation, α-factor detection is very convenient since it is independent of antibodies. Furthermore, freshly synthesized and translocated proteins are known to be packaged into COPIII vesicles with high efficiency. So far, such a system does not exist for mammalian trafficking studies and so we decided to first develop a yeast budding assay with α-factor as a positive budding control. In a second step, the yeast budding system was adopted to mammalian cells (chapter 3.3).

3.1.1 IN VITRO TRANSLATION AND TRANSLOCATION INTO THE ER OF YEAST SEMI-INTACT CELLS

The TNT quick coupled transcription/translation systems (Promega GmbH) are convenient single-tube, coupled transcription/translation reactions for eukaryotic in vitro translation. They further simplify the process by combining RNA polymerase, nucleotides, amino acids (minus methionine), salts and RNase inhibitor with reticulocyte lysate in a single Master Mix [Promega website, www.promega.com]. The reaction had to be free of endogenous mRNA as well as of active RNase to avoid generation of unspecific translation products or digestion of freshly synthesized α-factor mRNA, respectively. In vitro synthesis of yeast prepro-α-factor ($^{35}$S-ppαF) was performed in the presence of radioactive methionine. Three microliters of $^{35}$S-ppαF translation product were run on a 12.5% acrylamide gel and analyzed by autoradiography. As a negative control, a reaction without ppαF template DNA was included. Figure 5 shows in vitro synthesized $^{35}$S-ppαF at a position of approximately 20 kDa (lane 1). The translation product was clean without background translation.
from contamination with unspecific mRNA. The reaction was specific for α-factor since no protein was generated when template DNA was omitted (Figure 5, lane 2). Marker protein sizes are indicated.

<table>
<thead>
<tr>
<th>template</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 kDa</td>
<td></td>
<td></td>
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<tr>
<td>14.3 kDa</td>
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</tr>
<tr>
<td>lane 1</td>
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**Figure 5. In vitro translation of ^35^S-ppαF.** The product of the coupled *in vitro* transcription/translation reaction was analyzed by autoradiographic SDS-PAGE. The arrow points to freshly synthesized ^35^S-ppαF with an apparent molecular weight of 20 kDa (lane 1). Positive reaction including (+), negative reaction without template DNA (-), molecular weight markers are indicated.

In the next step, ^35^S-ppαF was post-translationally translocated into the ER of yeast semi-intact cells. It is important to keep a constant translocation temperature of 10°C. Below that, translocation would not occur whereas above 15°C, budding into COPII vesicles could already take place. For better separation, translation and translocation reactions were run on 4 M urea, 18% acrylamide gels and proteins were detected by autoradiography.

Upon ER translocation of ^35^S-ppαF, its signal sequence is cleaved off and glycosylation occurs resulting in pro α-factor (^35^S-gpαF) which runs more slowly on the gel compared to untranslocated ^35^S-ppαF (28 kDa and 20 kDa, respectively, Figure 6, lanes 2 and 4). Incomplete translocation resulted in unglycosylated ^35^S-ppαF sticking to the ER membrane from the outside. Since translocation into the ER by the Sec61p complex is an energy-dependent process, glycosylation did not take place when ATP was omitted (Figure 6, lanes 3 and 5). Notably, untranslocated ^35^S-ppαF showed the tendency to stick to the ER membrane from the outside (Figure 6, lanes 2-5).
RESULTS

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Figure 6. *In vitro* translocation of $^{35}$S-ppαF into the ER of yeast semi-intact cells results in glycosylation of $^{35}$S-gpαF. *In vitro* synthesized $^{35}$S-ppαF became glycosylated upon post-translational translocation into the ER of yeast SIC resulting in a band shift on the gel (from 20 kDa to 28 kDa for $^{35}$S-gpαF). Translocation and glycosylation are ATP-dependent events. Untranslocated $^{35}$S-ppαF (∗), translocated, glycosylated $^{35}$S-gpαF (∗∗), translation product (lane 1), translocation reaction in the presence (lane 2, 4), and in the absence of ATP (lane 3, 5), molecular weight markers are indicated.

3.3.2 PROTEASE PROTECTION OF TRANSLOCATED ALPHA FACTOR

A protease protection assay was performed to prove whether the 28 kDa $^{35}$S-gpαF has been transported into the ER lumen, where it is protected from environmental influences. Yeast SIC containing $^{35}$S-gpαF were treated with trypsin (*Figure 7A*) or proteinase K (*Figure 7B*) and incubated for 10 minutes on ice. Trypsin activity was tested on $^{35}$S-ppαF translation reaction (*Figure 7A*, lanes 1 and 2). When protease was added to the translocation reaction, only untranslocated $^{35}$S-ppαF was digested whereas translocated $^{35}$S-gpαF was protected from the proteases by the ER membrane (*Figure 7A and B*, lanes 4, 6 and 2, respectively). Membrane-protection of $^{35}$S-gpαF was broken up by simultaneous addition of the detergent Triton X-100. Upon lysis of the ER membrane, also $^{35}$S-gpαF could be digested by trypsin (*Figure 7A*, lane 5 and 7). Furthermore, proteinase K digested untranslocated $^{35}$S-ppαF sticking to the outside of the ER membrane more efficiently than the same amount of trypsin did (*Figure 7B*, lane 2 and *Figure 7A*, lane 4). This assay confirmed that the upper $^{35}$S-gpαF band appearing after translocation, indeed resembled $^{35}$S-gpαF inside the ER lumen protected from external proteases.
Figure 7. Protease protection of $^{35}$S-gpαF following \textit{in vitro} translocation into the ER of yeast SIC. \textit{In vitro} synthesized and post-translationally translocated $^{35}$S-gpαF is protected from protease digestion by the ER membrane. Untranslocated $^{35}$S-ppαF (*), translocated, glycosylated $^{35}$S-gpαF (**), molecular weight markers are indicated. A. Protease protection using trypsin. Translation reaction without (lane 1), and with trypsin (lane 2), translocation reaction (lane 3), translocation reaction with 3 and 6 µl trypsin (lane 4, 6, respectively), with trypsin and Triton (lane 5, 7). B. Protease protection using proteinase K. Translocation reaction without (lane 1) and with proteinase K (lane 2).
3.3.3 ENDO-H TREATMENT AND CON-A PRECIPITATION OF GLYCOSYLATED ALPHA FACTOR

Yeast SIC containing post-translationally translocated α-factor were lysed and
\(^{35}\)S-gpαF was subjected to EndoH digest. Samples were run on 4 M urea, 18% acrylamide gels and autoradiographically analyzed. EndoH cleaves N-linked high mannose residues from glycosylated proteins only that have not yet reached the median Golgi where EndoH resistance is acquired by addition of complex oligosaccharides. When \(^{35}\)S-gpαF was treated with this glycosidase, its sugar residues, which had been attached to the protein upon ER translocation were cut off resulting in a downshift of the band from 28 kDa to 20 kDa. Without sugar attachments, the size of \(^{35}\)S-gpαF corresponded to that of untranslocated and unglycosylated \(^{35}\)S-ppαF (Figure 8A, lanes 2 and 3).

Figure 8. Translocated \(^{35}\)S-gpαF is sensitive to EndoH and precipitates with ConA. Yeast semi-intact cells containing \(^{35}\)S-gpαF were lysed and subjected to EndoH treatment or ConA precipitation. Untranslocated \(^{35}\)S-ppαF (*), translocated, glycosylated \(^{35}\)S-gpαF (**), molecular weight markers are indicated. A. EndoH treatment. Translation reaction (lane 1), untreated translocation reaction (lane 2), EndoH-treated translocation reaction (lane 3). B. ConA precipitation. Translation reaction (lane 1), untreated translocation reaction (lane 2), ConA-precipitated translocation reaction (lane 3).
For ConA precipitation, yeast SIC containing $^{35}$S-gpaF were lysed and glycosylated proteins were precipitated from the post-lysis supernatant using ConA agarose. Alpha factor was detected by autoradiography. ConA is a lectin that specifically binds to sugar residues. Only translocated and glycosylated $^{35}$S-gpaF was precipitated by ConA agarose whereas untranslocated, unglycosylated $^{35}$S-pppF was not (Figure 8B, lanes 2 and 3).

Both experiments confirmed glycosylation of $^{35}$S-gpaF upon translocation into the ER of yeast semi-intact cells. Furthermore, the total amount of $^{35}$S-gpaF was EndoH-sensitive showing that no transport of the protein to further compartments than the cis Golgi had taken place (Figure 8A, lane 3).

### 3.2 IN VITRO COPII BUDDING ASSAY USING YEAST SEMI-INTACT CELLS

The next step was to take advantage of an in vitro budding assay to investigate the trafficking of yeast α-factor and endogenous yeast proteins. Using yeast semi-intact cells containing $^{35}$S-gpaF, COPII budding from the ER was reconstituted in vitro by addition of purified COPII components originally prepared by Crystal Chan, Howard Hughes Medical Institute, Berkeley. By this, the possibility of generating other vesicles than COPII was excluded. After separation of donor membranes from generated vesicles, vesicular proteins were either analyzed by autoradiography (α-factor) or immunoblot for endogenous protein (Sec22p).

#### 3.2.1 IN VITRO BUDDING OF YEAST ALPHA FACTOR

Yeast α-factor was translated, post-translationally translocated into yeast semi-intact cells and in vitro COPII budding was performed using purified COPII components, an ATP regenerating system and GTP or its non-hydrolyzable analogue GMP-PNP. Generated COPII vesicles were separated from donor membranes by centrifugation, lysed in loading buffer, and samples were subjected to urea SDS-PAGE. Vesicular and total α-factor were detected by autoradiography. Figure 9 shows specific budding of $^{35}$S-gpaF into COPII vesicles whereas the lower $^{35}$S-pppF band was excluded being outside of the ER (Figure 9, lanes 2 and 3). Packaging of translocated $^{35}$S-gpaF was slightly more efficient when using GMP-
PNP rather than GTP (35 and 22%, lanes 3 and 2, respectively). Since GMP-PNP cannot be hydrolyzed, the COPII protein coat stays on the vesicular membrane whereas the coat is cast off after GTP hydrolysis. Probably, the more intense \(^{35}\)S-gpaF band in the GMP-PNP sample was simply due to better pelleting during high-speed centrifugation (vesicles with protein coat are heavier than naked ones) rather than more efficient budding of \(^{35}\)S-gpaF. Budding into COPII vesicles was specific, since no packaging of \(^{35}\)S-gpaF was observed without inclusion of either nucleotides or COPII proteins (Figure 9, lanes 4 and 5).

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**Figure 9. Packaging of \(^{35}\)S-gpaF into COPII vesicles in vitro.** Post-translationally translocated \(^{35}\)S-gpaF can leave the ER of yeast semi-intact cells in COPII vesicles generated in vitro. Untranslocated \(^{35}\)S-ppaF (*), translocated, glycosylated \(^{35}\)S-gpaF (**), translation reaction (lane 1), in vitro budding reactions with GTP (lane 2) or GMP-PNP (lane 3), negative control reactions without nucleotides (lane 4), without COPII proteins (lane 5), total translocation reaction (lane 6), molecular weight markers are indicated.

### 3.2.2 IN VITRO BUDDING OF ENDOGENOUS YEAST SEC22

So far, in vitro budding of post-translationally translocated \(^{35}\)S-gpaF into COPII vesicles was shown. Since packaging efficiency of freshly synthesized \(^{35}\)S-paF was very high, the question arises whether COPII budding of endogenous yeast SIC proteins is as efficient, too.
RESULTS

Alpha factor served as budding control and was translocated into yeast semi-intact cells after in vitro translation. An in vitro budding assay was performed in the presence of purified COPII components, an ATP regenerating system and GMP-PNP. Generated vesicles were separated from donor membranes, lysed in loading buffer and samples were run on 4 M urea, 18% acrylamide gels. Proteins were transferred onto PVDF membranes. Post-transfer gels were dried and both, gel and PVDF membranes, were exposed on Imaging Plates to detect insufficiently transferred 35S-gpαF bands. Budding of endogenous yeast Sec22p was detected by immunoblot using a Sec22p anti-serum (Table 2).

COPII budding of glycosylated 35S-gpαF could be detected (Figure 10, lane 2, upper panel). Interestingly, transfer of the different α-factor bands (35S-ppαF and 35S-gpαF) was very heterogenous, e.g. no vesicular 35S-gpαF transferred to the PVDF membrane but was instead left entirely on post-transfer gel (Figure 10, lane 2, middle and upper panel). On the other hand, 35S-gpαF in the total translocation sample transferred very efficiently (Figure 10, lane 5, middle panel). The transfer problems were observed several times when using 4 M urea, 18% acrylamide gels (data not shown). In the negative control samples without COPII components or ATP/GMP-PNP, no budded 35S-gpαF could be detected neither on the gel nor on the membrane (Figure 10, lanes 3 and 4, upper and middle panel).

COPII budding of the SNARE protein Sec22p corresponded to 35S-gpαF budding: A strong band could be detected by immunoblot only when both ATP and COPII components were included in the reaction (Figure 10, lane 2, lower panel). Packaging efficiency of Sec22p into COPII vesicles was 20-30% of total Sec22p in the translocation reaction. Due to the transfer problems, the budding efficiency of 35S-gpαF was not evaluated. Furthermore, yeast Sec61p, which is part of the translocon complex and stringently retained in the ER of yeast cells [Belden and Barlowe 2001], could not be found in the vesicle fractions (data not shown). These results confirmed that in vitro COPII packaging could not only be reconstituted for post-translationally translocated 35S-gpαF but also for endogenous cycling yeast proteins like Sec22p.
Figure 10. In vitro COPII packaging of $^{35}$S-gpaF and Sec22p. Yeast SIC containing post-translationally translocated $^{35}$S-gpaF were subjected to in vitro COPII budding. $^{35}$S-gpaF as well as endogenous Sec22p was found in COPII vesicles. $^{35}$S-gpaF was detected autoradiographically, Sec22p was detected by immunoblot. Post-transfer gel (upper panel), autoradiographic analysis of PVDF membrane (middle panel), immunoblot analysis of PVDF membrane (lower panel). Untranslocated $^{35}$S-gpaF (*), translocated, glycosylated $^{35}$S-gpaF (**), translation reaction (lane 1), in vitro budding reaction (lane 2), negative control reactions without COPII proteins (lane 3), without nucleotides (lane 4), total translation reaction (lane 5), molecular weight markers are indicated.

3.3 ESTABLISHING A MAMMALIAN IN VITRO BUDDING ASSAY

After the successful establishment in yeast, it was time to adopt a mammalian in vitro budding system. In principle, yeast and mammalian in vitro budding reactions are performed in a similar way even though a mammalian system is more difficult to handle due to the more complex cellular organization. Furthermore, a convenient budding control as in vitro translocated yeast α-factor does not yet exist for a
mammalian assay and makes the system dependent on antibodies for protein detection. So, the first step in developing a mammalian budding assay was to identify antibodies against cycling and ER-resident proteins that could serve as positive and negative budding controls. Second, the in vitro budding system was to be established with the control proteins.

### 3.3.1 FINDING CONTROL PROTEINS FOR MAMMALIAN IN VITRO BUDDING

To minimize problems associated with transfection and protein overexpression, we decided to establish endogenous proteins as negative and positive budding controls. Proteins suitable to serve as negative budding control are ER-resident ones that cannot escape the ER. However, a positive control protein must be able to leave the ER quite efficiently. Since mammalian COPII in vitro budding has not yet been shown for other proteins than cycling ones, we decided to find a candidate located in ER, ERGIC, and Golgi complex with probable high budding efficiency (like the SNARE Sec22p in yeast, see chapter 3.2.2).

CHO cells were lysed and proteins were chloroform precipitated. Antibodies against putative control proteins were tested on lysate titrations by immunoblot. The ER-chaperone calnexin is supposed to be ER-resident [Ellgaard and Helenius 2003] and was therefore selected as putative negative control. A commercially available rabbit calnexin anti-serum (Table 2) showed high reactivity against the 90 kDa antigen even at low cell numbers (Figure 11, calnexin). For immunoblot detection of protein packaging, highly reactive antibodies are extremely important. Since packaging of efficiently exported endogenous proteins is maximal 20-30% of total (e.g. for p58, Kim et al. 2005), a useful antibody must still be reactive against very low amounts of antigen.

On the same PVDF membrane, a p58 antiserum from Jaakko Saraste (Bergen, Norway) was tested (Table 2). The putative cargo receptor p58 is called ERGIC-53 in human cells due to the slightly different molecular weights (58 kDa in rodents and 53 kDa in humans). It is a cycling protein mainly located in the ERGIC [Schweizer et al. 1990] and already established as positive control for mammalian in vitro budding studies [Kim et al 2005]. The p58 antiserum reacted to its antigen with similar intensities as the calnexin antibody on the similar membrane did (Figure 11, p58).
Antibodies against the mammalian SNARE rBet1 and the KDEL receptor did not show reactivity on CHO lysates by immunoblot analysis (data not shown). Thus, calnexin and p58 as negative and positive control proteins, respectively, were used in the following to establish the mammalian in vitro budding assay.

Figure 11. Cell lysate titration for testing of anti-calnexin and anti-p58 in immunoblots. Calnexin (clx) and p58 could serve as mammalian budding control proteins (apparent molecular weights are 90 and 58 kDa, respectively). The tested antibodies are sufficiently reactive to detect even low protein amounts. Lysates from $2 \times 10^6$ (lane 1) to $1 \times 10^4$ CHO cells (lane 8) were used for titration. Molecular weight markers are indicated.

3.3.2 DEVELOPMENT OF AN IN VITRO BUDDING ASSAY PROTOCOL USING MAMMALIAN MICROSOMES

3.3.2.1 ISOLATED MAMMALIAN ER MEMBRANES ARE SENSITIVE TO FRAGMENTATION

In contrast to the yeast system, in which semi-intact cells as well as yeast microsomes can be used for in vitro budding from the ER, for mammalian systems only the use of enriched ER membranes has been described [Nohturfft et al. 2000, Kim et al. 2005]. A protocol published by Nohturfft et al. (2000) was used as a starting point to establish a mammalian in vitro budding assay. It is very similar though not identical to the protocol described in chapter 2.4.2. CHO microsomes were prepared by passing the cells through a 22G needle and pelleted at 16,000× g for 3 minutes. In vitro budding was performed at 37°C in the presence of an ATP regenerating system, GMP-PNP and yeast COPII components. Purified yeast COPII components have
been described to promote *in vitro* vesicle generation from mammalian membranes [Espenshade et al. 2002]. Vesicle fractions were separated from donor membranes at 16,000× g for 3 minutes and pelleted at 100,000× g for 25 minutes.

Figure 12. Mammalian microsomes are sensitive to fragmentation. A. *In vitro* budding was performed with CHO microsomes as source of donor membranes. Both control proteins, cycling p58 as well as ER-resident calnexin (clx) can be found in the vesicle fractions. Addition of nucleotides has no influence on signal intensities. This speaks against vesicle generation and specific protein packaging but implies fragmentation of the donor membranes. Budding reactions (lanes 1, 2), control reaction without nucleotides (lane 3), empty lane (lane 4), 10% total microsomes (lane 5).

B. *In vitro* budding reactions were incubated at the indicated temperatures showing that fragmentation of CHO microsomes is temperature-dependent. Reactions were incubated at a temperature range between 37°C (lane 1) and 4°C (lane 4), empty lane (lane 5), 10% total microsomes (lane 6). Molecular weight markers are indicated.

Using the Nohturfft et al. (2000) protocol, the positive control protein p58 as well as the negative one calnexin were detected in the budding fractions by immunoblot (*Figure 12A*, lanes 1 and 2). There was no difference in the band pattern whether ATP and GMP-PNP were omitted (*Figure 12A*, lane 3). This contrasts to the findings
in yeast budding where the minus ATP/GMP-PNP control was very stringent and did not show budding of \(^{35}\)S-pαF or Sec22p at all (Figures 9 and 10). These data suggested fragmentation of the isolated ER membranes. Since the ER fragments could not be pelleted by a 16,000× g spin but at 100,000× g, they were supposed to have the size of vesicles so that separation and differentiation from the generated vesicles would be impossible. As a result, any real budding signal would be obscured by unspecific fragment signals (Figure 12A, p58 in lanes 1 and 2) and a control reaction in which budding is inhibited due to the lack of nucleotides would show false budding signals (Figure 12A, p58 in lane 3). Also, an ER-resident protein like calnexin could be found in the vesicle fractions without being able to bud into COP II vesicles (Figure 12A, calnexin in lanes 1 and 2).

The reason for fragmentation of the isolated microsomes was unclear. The attempt to improve separation of vesicles from donor membranes by prolonged centrifugation (16,000× g for 20 instead of 3 minutes) was unsuccessful (data not shown). To get rid of the ER fragments by extensively washing the microsomes did not prevent fragmentation either (data not shown). Though incubation of the budding reactions at lower temperatures (30 and 25°C instead of 37°C) decreased ER fragmentation, it could not be avoided (Figure 12B, lanes 1-3). Only if the samples were incubated on ice, when vesicle generation cannot take place, ER fragmentation did not occur (Figure 12B, lane 4).

Thus, we changed the protocol according to Kim et al. (2005), which is described in chapter 2.4.2, and managed to eliminate fragmentation of the ER (Figure 13). The main differences compared to the assay from Nothurf et al. (2000) are variations in the centrifugation steps. After needle passage, CHO microsomes were pelleted at 6,000× g for 10 minutes (instead of 16,000× g for 3 minutes). The decrease of the centrifugation speed probably prevented smaller ER fragments from pelleting and thus contaminating the budding reaction. Furthermore, vesicle fractions were separated from donor membranes at 14,000× g for 20 minutes (instead of 16,000× g for 3 minutes). Also, in vitro budding from the ER was performed at 25°C (instead of 37°C) to minimize the possibility of ER fragmentation (Figure 12B). Unfortunately, the budding reactions did not differ from the negative control reactions (minus COP II components or minus nucleotides) and did not show a positive signal for p58-budding (Figure 13, p58, lanes 1, 2 and 3, 4, respectively).
RESULTS

<table>
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Figure 13. Fragmentation can be avoided when the preparation protocol is slightly changed. CHO microsomes were prepared differently and in vitro budding was performed. No specific packaging can be observed. In vitro budding reactions with GTP (lane 1) or GMP-PNP (lane 2), negative control reactions without COPII proteins (lane 3), without nucleotides (lane 4), empty lane (lane 5), 10% total microsomes (lane 6), molecular weight markers are indicated, clx (calnexin).

3.3.2.2 THE COPII SOURCE: CYTOSOL VERSUS PURIFIED COPII COMPONENTS

Using yeast membranes, in vitro vesicle generation can be performed with either purified yeast COPII components or yeast cytosol as a COPII source [Shimoni and Schekman 2002]. For mammalian membranes, usually cytosol from mammalian tissue is used to promote vesicle budding [Nohturfft et al. 2000, Kim et al. 2005]. The generation of COPII vesicles from mammalian ER membranes in the presence of yeast purified COPII components has been reported once [Espenshade et al. 2002]. However, COPII vesicles apparently cannot be made from mammalian membranes in the presence of purified mammalian COPII components when mammalian cytosol is absent [Kim et al. 2005]. The major disadvantage of using cytosol instead of purified COPII components, is the likely generation of different kinds of vesicles (COPI, clathrin) rather than COPII vesicles only.

To compare vesicle generation using purified yeast COPII proteins or mammalian cytosol as COPII protein source, microsomes were prepared from CHO cells according to the Kim et al. (2005) protocol (see chapter 2.4.2), and budding reactions
RESULTS

were carried out in the presence of either purified yeast COPII components or hamster liver cytosol. No p58-budding could be observed when purified yeast COPII components were used (Figure 14, p58, lane 1). The reason could not be insufficient reactivity of the respective antibody against low antigen concentrations, since 20% of total p58 could still be detected (Figure 14, lane 7 and 8, respectively). The reactions, in which cytosol was included, showed many unspecific background signals using the p58-antibody (Figure 14, lanes 2 and 3), so that an eventual budding signal for p58 could not be seen. The cytosol was therefore high-speed spun and the post-spin supernatant was used for in vitro budding. However, this did not diminish the cytosol background (Figure 14, lane 3).

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Figure 14. Yeast COPII components and mammalian cytosol for in vitro budding. Reactions with CHO microsomes as membrane source were incubated with yeast COPII components (lane 1), cytosol (lane 2), high-speed spun cytosol (lane 3), or no COPII source as control reaction (lane 4). Yeast COPII components do not promote vesicle generation from mammalian microsomes in vitro. The use of cytosol results in strong background signals on immunoblots obscuring eventual budding signals. Microsomes are shown as reference in a titration range between 100% and 5% of total (lane 5-8), molecular weight markers are indicated, clx (calnexin).

Since vesicle generation from mammalian membranes using purified yeast COPII components did neither work sufficiently for others [Jinoh Kim, personal conversation], we decided to focus on cytosol to establish a mammalian in vitro budding assay. For this, it was necessary to get rid of the crossreactive signals when incubating with anti-p58 (Figure 14, p58, lane 2 and 3). The cytosol background could be drastically reduced when vesicle pellets were washed in buffer G after the
100,000× g spin; this revealed a budding signal for p58 (Figure 15, p58, lane 2). This band represented true p58-packaging, since a control reaction lacking cytosol (and therefore COPII proteins) did not promote generation of p58-containing vesicles (Figure 15, p58, lane 3). Budding efficiency was approximately 30% of total p58. Calnexin reactivity was less compared to the corresponding p58-signal in the same lane (Figure 15, 100% microsomal membranes, lane 5) and calnexin could not be detected in the vesicle fractions (Figure 15, calnexin, lanes 1-4).

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Figure 15. The ERGIC-marker p58 can be packaged from CHO microsomes into vesicles in vitro using mammalian cytosol. Washing the high-speed vesicle pellet before the gel run minimizes cytosol background signals and reveals p58-packaging. ER-resident calnexin (clx) cannot be detected in the generated vesicles. In vitro budding reaction unwashed (lane 1) and washed (lane 2), negative control reactions without cytosol (lane 3), without nucleotides (lane 4), Microsomes are shown as reference in a titration range between 100% and 20% of total (lanes 5-7), molecular weight markers are indicated.

3.3.2.3 INFLUENCE OF APYRASE ON PROTEIN PACKAGING FROM MICROSONAL MEMBRANES

The generation of vesicles from the ER membrane is an energy-consuming process and dependent on the presence of the nucleotides ATP and GTP or a GTP analogue (e.g. GMP-PNP) (see chapter 1.1.2 and chapter 1.3). As in vitro budding efficiency is limited by the concentrations of COPII proteins in the supplied cytosol, the addition of non-hydrolyzable GMP-PNP can potentially deplete the cytosol of free COPII components due to failed coat disassembly and failed liberation of COPII proteins. The loss of available COPII components could eventually result in budding decrease
or inhibition [Jinoh Kim, personal conversation]. To avoid, all the following in vitro budding reactions were carried out using GTP to allow coat disassembly upon GTP hydrolysis and recycling of cytosolic COPII proteins.

Budding reactions using ER membranes from CHO cells were performed as described in chapter 2.4.2 in the presence or in the absence of apyrase. Apyrase is a phosphohydrolase catalyzing nucleotide hydrolysis. It can therefore be used to specifically inhibit the budding process. The packaging efficiency of p58 was reduced from approximately 30% to 10% when apyrase was added (Figure 16, p58, lanes 1 and 4, 6). When ATP/GTP were omitted, p58-budding was less than 5% no matter whether apyrase was present or not (lanes 2 and 5). This indicated that the cytosol used to promote the budding reaction did not contain much endogenous ATP. Calnexin was a suitable negative control protein since it was not detectably packaged into the generated vesicles (Figure 16, calnexin, lanes 1-6). As reactivities of both the p58 and the calnexin antibodies were comparable (Figure 16, lane 7), the exclusion of calnexin from the vesicles must have been specific rather than being a result of insufficient detection. An unknown non-specific signal can be seen below the p58-band at roughly 50 kDa.

<table>
<thead>
<tr>
<th>budding reactions</th>
<th>100% total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>+ - + + - +</td>
</tr>
<tr>
<td>GTP</td>
<td>+ - + + - +</td>
</tr>
<tr>
<td>cytosol</td>
<td>+ + - + + +</td>
</tr>
<tr>
<td>apyrase</td>
<td>- - - + + +</td>
</tr>
</tbody>
</table>

Figure 16. The phosphohydrolase apyrase destroys nucleotides and diminishes p58-packaging from microsomes in vitro. CHO microsomes were subjected to in vitro budding in the absence (lanes 1-3) or presence of apyrase (lanes 4-6). If this enzyme is present, nucleotides become limited in the reaction and packaging is impaired. Calnexin (clx) is not packaged in detectable amounts in vitro. The signal below p58 is a crossreaction against an unknown protein. Budding reactions (lanes 1, 4, 6), negative control reactions without nucleotides (lanes 2, 5), without cytosol (lane 3), 100% total microsomes (lane 7), molecular weight markers are indicated.
3.3.3 IN VITRO BUDDING ASSAY USING MAMMALIAN SEMI-INTACT CELLS

3.3.3.1 COMPARISON OF PROTEIN CONTENTS AND IN VITRO BUDDING FROM MAMMALIAN MICROSOMES AND SEMI-INTACT CELLS

Proteins that cycle between ER, ERGIC, and Golgi apparatus are packaged very efficiently in vitro like the putative cargo receptor p58 (20-30% budding efficiency, Figure 15 and 16) [Kim et al. 2005] whereas budding of a cell surface protein like MHC class I may be very low at steady-state. For a class I budding experiment it is therefore very important to begin with a large amount of starting material. An attempt to increase membrane and thus protein yield by preparing microsomes with smaller gauge needles (26G instead of 22G) remained unsuccessful (data not shown). Besides, it is possible that during isolation of microsomes a lot of original ER material gets lost. So, we decided to develop an in vitro budding assay using entire mammalian semi-intact cells (SIC) as a membrane source. As semi-intact cells resemble whole cells with a permeabilized plasma membrane only, all interior membranes stay untouched and loss of ER material can be minimized.

To compare total protein contents of enriched ER membranes and semi-intact cells and to furthermore test in vitro budding competency of SIC, in vitro budding assays using both membrane sources were performed in parallel. Semi-intact cells were prepared by repeated freeze-thaw cycles (see chapter 2.4.3). For both preparation methods, the same amount of starting material was used (two million CHO cells), so that a direct comparison of the protein yields by immunoblot was possible. The total amount of microsomal calnexin is approximately 10fold less compared the total calnexin in semi-intact CHO cells (Figure 17, calnexin, lanes 1 and 5). As calnexin showed ER-restriction in our assay (Figures 15 and 16), one can conclude that the majority of ER membranes (90%) is lost during microsome preparations. Also, the amount of p58 is significantly lower in isolated ER membranes than in semi-intact cells (Figure 17, p58, lanes 1 and 5).

Furthermore, SIC were fully competent for in vitro packaging of p58 (Figure 17, p58, lane 2). Budding efficiencies from both semi-intact cells and microsomes were comparable (about 30%) though with much stronger intensities for SIC p58 (p58, lanes 2 and 6). The budding signal for p58 generated from SIC membranes, was even more intense than the p58 signal present in the total microsome preparation (p58,
RESULTS

lanes 2 and 5). The calnexin signals observed in the vesicle fractions from semi-intact cells are independent of the presence of cytosol and therefore most probably resemble unspecific background (Figure 17, calnexin, lanes 2 and 3). These data indicate that in vitro budding from mammalian semi-intact cells as well as from microsomal membranes underlies the same mechanisms.

<table>
<thead>
<tr>
<th>ATP</th>
<th>100% total</th>
<th>semi-intact cells</th>
<th>GTP</th>
<th>100% total</th>
<th>microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>cytosol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

Figure 17. Comparison of microsomes and semi-intact cells as membrane source for in vitro budding. CHO semi-intact cells (lanes 1-3) or CHO microsomes (lanes 4-7) were used for vesicle generation and protein packaging in vitro. Whole semi-intacts contain approximately 10-fold more protein than microsomal preparations and are as competent for in vitro budding. Budding reactions (lanes 2, 6), negative control reactions without cytosol (lane 3, 7), empty lane (lane 4), 100% total SIC (lane 1) or 100% total microsomes (lane 5), molecular weight markers are indicated, calnexin (clx).

3.3.3.2 INFLUENCE OF APYRASE ON PROTEIN PACKAGING FROM SEMI-INTEGRANT CELLS

The influence of the nucleotide-destroying enzyme apyrase was also tested on p58 budding from semi-intact cells. Different from the same experiment using microsomes (Figure 16), budding of p58 was only slightly impaired by the addition of apyrase to the reaction (from about 10% to 5%, Figure 18, p58, lanes 1 and 4). While inhibition of p58-packaging from both microsomes and semi-intacts was very stringent when cytosol was omitted, in the absence of ATP/GTP, budding from both membrane sources could usually occur to some extent (Figures 15, 16, and 17). Here also, residual p58-budding was found in the vesicle fractions without additional nucleotides (Figure 18, p58, lane 2) whereas no p58 could be detected in the minus
cytosol controls (lanes 3 and 6). This might resemble background budding of p58 with the help of nucleotides present in the supplied cytosol and indicates that no functional cellular COPII proteins, that could promote p58-budding even in the absence of cytosol, are left on SIC and microsomal membranes after preparation. When apyrase was added to the minus ATP/GTP reaction, cytosolic nucleotides were no longer sufficient to generate p58-containing vesicles (Figure 18, p58, lane 5). The calnexin signals detectable in the vesicle fractions were dependent on the presence of extra nucleotides but independent of cytosol addition and therefore the presence of COPII components (Figure 18, calnexin, 1-6). Their appearance did not correlate with p58 budding at all. Since microsomal membranes tend to fragment in vitro (Figure 12), this may sometimes occur to intracellular membranes of semi-intact cells, too. Thus, the calnexin bands were most probably the result of slight fragmentation during the budding incubation rather than being a result of weak but specific calnexin-packaging.

<table>
<thead>
<tr>
<th>budding reactions</th>
<th>ATP</th>
<th>GTP</th>
<th>cytosol</th>
<th>apyrase</th>
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<td></td>
<td>M</td>
<td>-</td>
<td>100% total</td>
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Figure 18. The phosphohydrolase apyrase destroys nucleotides and diminishes p58-packaging from SIC in vitro. Semi-intact CHO cells were subjected to in vitro budding in the absence (lanes 1-3) or presence of apyrase (lanes 4-6), which destroys nucleotides. Addition of this enzyme to the in vitro reactions has an inhibitory effect on p58-budding from semi-intact cells as it has on protein packaging from microsomes. Calnexin (clx) shows slight background signals, which most likely result from fragmentation processes. Budding reactions (lanes 1, 4), negative control reactions without nucleotides (lanes 2, 5), without cytosol (lanes 3, 6), molecular weight marker (lane 7), empty lane (lane 8), 100% total SIC (lane 9), molecular weight marker sizes are indicated.
3.3.3.3 VESICLES GENERATED FROM SEMI-INTACT CELLS ARE MAINLY OF COPII ORIGIN

Using cytosol for *in vitro* vesicle budding, the possibility of generating various kinds of vesicles apart from COPII vesicles only cannot be excluded. This problem principally does not only apply when using semi-intact cells but also when using microsomes. EndoH treatments on SIC and microsomal lysates have shown that the ratios of EndoH-resistant and EndoH-sensitive MHC class I molecules are similar in both membrane sources (data not shown). Since EndoH-resistant glycoproteins are present in microsomal preparations, enriched ER membranes must be contaminated with other types of cellular membranes (e.g. trans Golgi, plasma membrane) so that the likely generation of different vesicle populations cannot be excluded by simply using microsomes for *in vitro* budding.

The usage of purified COPII proteins instead of cytosol as in the case of yeast *in vitro* budding (see chapter 3.2) would eliminate the problem since only vesicles of COPII origin were made. However, contrary to the literature [Espenshade et al. 2002] we could not get the assay to work with purified yeast COPII components and mammalian membranes (*Figure 14*). We wanted to circumvent the problem by showing indirectly that among others also COPII vesicles are generated by the *in vitro* system. Mammalian Sar1AT39N (a threonine at amino acid position 39 in the Sar1A protein is substituted by an asparagine) is a soluble GDP-restricted mutant unable to bind to ER membranes [Kuge et al. 1994]. Interestingly, this mutant has been shown to inhibit protein budding in a dominant fashion when added to *in vitro* vesicle reactions [Espenshade et al. 2002, Kim at al. 2005].

To verify the presence of ER-derived COPII vesicles generated from semi-intact cells, the influence of wildtype Sar1A (Sar1Awt) or mutant Sar1AT39N on p58-budding was investigated. *In vitro* budding reactions using human T1 semi-intact cells (for cell line, see *Table 3*) were either treated as usual or mixed with either mutant or wildtype Sar1A (*Figure 19*, lanes 1, 5, and 6, respectively). The addition of Sar1AT39N decreased packaging of ERGIC-53 (human p58) 2fold (ERGIC-53, lanes 1 and 5). The difference between both signals corresponds to the amount of ERGIC-53-containing COPII vesicles, the generation of which was abolished by 500 nM of mutant Sar1A. In other words, at least 50% of vesicular ERGIC-53 in a normal budding reaction (lane 1) were of COPII origin. Actually, the portion of *in vitro* generated COPII vesicles might have been even higher if the concentration of
supplied Sar1AT39N was not sufficient to inhibit COPII vesicle generation entirely. In contrast to the literature [Espenshade et al. 2002, Kim at al. 2005], 500 nM wildtype Sar1A was able to enhance ERGIC-53-budding into COPII vesicles 3fold (Figure 19, ERGIC-53, lanes 1 and 6). It is not clear whether upon addition of wildtype Sar1A more COPII vesicles are generated or whether more ERGIC-53 is packaged into the same number of COPII vesicles. Anyway, the effect of wildtype and mutant Sar1A is very dependent on the cytosol used. As there is always a competition between cytosolic and purified Sar1A, the effect of purified Sar1A decreases the more functional endogenous Sar1 the supplied cytosol contains. Thus, the effective concentration of purified Sar1A has to be tested individually for each cytosol batch.

| ATP  | + | - | + | + | + | + | + |
| GTP  | + | - | + | + | + | + | + |
| cytosol | + | + | - | + | + | + | + |

**Figure 19.** The marker protein ERGIC-53 is packaged into COPII vesicles generated from T1 semi-intact cells *in vitro*. Dominant-negative Sar1T39N specifically inhibits COPII budding from the ER and hence abolishes p58-packaging while addition of wildtype Sar1 shows the opposite effect. Incubation of the reactions at 4°C non-specifically inhibits vesicle generation and protein packaging. Addition of Triton prior to high-speed spinning solubilizes vesicular membranes and thus prevents membrane proteins from pelleting. Normal budding reaction (lane 1), negative control reactions without nucleotides (lanes 2) and without cytosol (lanes 3), budding reaction incubated at 4°C (lane 4), budding reactions with Sar1AT39N (lane 5) and with wildtype Sar1A (lane 6), addition of Triton prior to high-speed spinning (lane 7), total SIC are shown in a titration range between 30% and 5% (lanes 8-10), molecular weight markers are indicated, calnexin (clx).

Weak budding of ERGIC-53 was detected without additional ATP/GTP whereas there was no detectable packaging if cytosol was omitted (*Figure 19*, ERGIC-53, lanes 2 and 3, respectively). Since vesicle generation needs a certain temperature, budding was strongly inhibited when reactions were incubated at 4°C instead of 25°C.
RESULTS

(lane 4). In order to confirm that the ERGIC-53 signal really represents a membrane-bound but no soluble protein, 1% of Triton X-100 was added to the medium-speed supernatant prior to the high-speed spin (lane 7). Addition of the detergent lead to vesicle lysis and solubilisation of ERGIC-53. As a result, the protein could no longer be pelleted by a 100,000× g spin, and the signal is missing. The specificity of the assay was confirmed by the calnexin control, since no calnexin signals could be detected in the vesicle fractions (Figure 19, calnexin, lanes 1-7).

3.4 IN VITRO BUDDING OF OVEREXPRESSIONED H-2D<sup>b</sup> MOLECULES IN FIBROBLASTS

The establishment of a mammalian in vitro budding assay using calnexin and p58/ERGIC-53 as negative and positive model proteins, respectively, enabled us to investigate MHC class I transport. Due to antibody availability, we decided to first concentrate on transfected class I, namely on the mouse allele H-2D<sup>b</sup>. This molecule was stably expressed in CHO (CHO/D<sup>b</sup>) and TAP2-deficient CHO cells (CHO/D<sup>b</sup>/TAP2<sup>def.</sup>). Lacking TAP2, assembly of a functional heterodimeric TAP complex fails in CHO/D<sup>b</sup>/TAP2<sup>def.</sup> cells such that peptide translocation from the cytosol in the ER lumen cannot take place (see chapter 1.2). In a peptide-depleted environment, MHC class I fails to bind high-affinity peptides, does not fold properly, and potentially gets degraded [Lankat-Buttgereit and Tampe 1999].

EndoH treatment showed that the main portion of H-2D<sup>b</sup> in both TAP2-proficient and -deficient CHO cells remained EndoH-sensitive suggesting intracellular retention. However, H-2D<sup>b</sup> efficiently budded into vesicles in vitro regardless of the TAP2-status and therefore the peptide supply suggesting cycling ability of potentially peptide-receptive H-2D<sup>b</sup>.

3.4.1 VERIFICATION OF THE TAP2 STATUS IN CHO/D<sup>b</sup> AND CHO/D<sup>b</sup>/TAP2<sup>def.</sup> CELLS BY ENDO-H TREATMENT

TAP2-deficient CHO cells were derived from normal CHO-K1 cells after treatment with the point mutation inducing reagent ICR191 [Nilabh Shastri, personal conversation and PhD thesis Thomas Serwold, unpublished data]. Northern blot and immunoblot analysis did not reveal detectable TAP2 protein expression [PhD thesis...
RESULTS

Thomas Serwold, unpublished data]. Unfortunately, the mouse monoclonal anti-TAP2-antibody (clone 435.3) did not react with the hamster protein (data not shown) so that we took advantage of EndoH treatment on total lysates to indirectly verify the TAP2-deficient status of the cell line. TAP2-proficient and -deficient CHO cells were stably transfected with and expressing the mouse MHC class I molecule H-2D\textsuperscript{b} (CHO/D\textsuperscript{b} and CHO/D\textsuperscript{b}/TAP2\textsubscript{def.}, respectively). Immunoblot detection of H-2D\textsuperscript{b} was performed with the T18 antibody (Table 2) that does not crossreact with endogenous hamster class I (data not shown).

Figure 2. EndoH-treatment on H-2D\textsuperscript{b} in CHO and CHO/TAP2\textsubscript{def.} cells. Total cell lysates were subjected to EndoH-treatment and immunoblotted with anti-H-2D\textsuperscript{b} T18-antibody. In TAP2-proficient CHO/D\textsuperscript{b} cells, only a small portion of mouse H-2D\textsuperscript{b} acquires EndoH resistance, while in CHO/D\textsuperscript{b}/TAP2\textsubscript{def.} H-2D\textsuperscript{b} remains entirely EndoH-sensitive. Fully glycosylated, EndoH-resistant H-2D\textsuperscript{b}, 50 kDa (Endo\textsubscript{H}r), core glycosylated, EndoH-sensitive H-2D\textsuperscript{b} (Endo\textsubscript{H}s), core glycosylated, untreated H-2D\textsuperscript{b}, 45 kDa (cg), CHO/D\textsuperscript{b} cells (lanes 1 and 2), CHO/D\textsuperscript{b}/TAP2\textsubscript{def.} cells (lanes 3 and 4), untreated lysates (lanes 2 and 4), EndoH-treated lysates (lanes 1 and 3), molecular weight markers are indicated.

Without EndoH application, H-2D\textsuperscript{b} appears as one 45 kDa band in CHO/D\textsuperscript{b}/TAP2\textsubscript{def.} cells at steady-state (Figure 20, lane 4). In TAP2-proficient CHO/D\textsuperscript{b} cells, a small portion of H-2D\textsuperscript{b} also runs at approximately 50 kDa and is most likely highly glycosylated (lanes 1 and 2). However, the vast majority of H-2D\textsuperscript{b} in CHO/D\textsuperscript{b} cells belonged to the 45 kDa fraction also found in CHO/D\textsuperscript{b}/TAP2\textsubscript{def.}. Without functional TAP protein, H-2D\textsuperscript{b} molecules cannot reach the median Golgi where EndoH resistance is acquired. So, the entire 45 kDa H-2D\textsuperscript{b} population remained EndoH-sensitive in CHO/D\textsuperscript{b}/TAP2\textsubscript{def.} cells (Figure 20, lane 3). In CHO/D\textsuperscript{b} cells, the 45 kDa band shifted upon EndoH treatment whereas the upper 50 kDa signal
resembled EndoH-resistant H-2D\textsuperscript{b} (lane 1). Different from other MHC class I alleles, core and fully glycosylated mouse H-2D\textsuperscript{b} forms tend to run differently on a gel. This is also true for endogenous H-2D\textsuperscript{b} in lymphoid TAP2-positive RMA cells even though with a much higher portion of EndoH-resistant class I at steady-state (about 50%, data not shown).

It is interesting that 90% of H-2D\textsuperscript{b} in TAP2-proficient CHO/D\textsuperscript{b} cells stayed EndoH-sensitive despite full TAP function. Only less than about 10% of total H-2D\textsuperscript{b} became EndoH-resistant in the median Golgi and probably even reached the cell surface. Not only at steady-state but also in pulse-chase experiments on freshly synthesized H-2D\textsuperscript{b}, no EndoH resistance could be acquired in neither CHO/D\textsuperscript{b}/TAP2\textsubscript{def.} nor CHO/D\textsuperscript{b} cells [Garstka, Borchert et al., to be submitted]. Instead, EndoH-sensitive H-2D\textsuperscript{b} molecules became degraded after two hours of chase. The biochemical observations are consistent with immunofluorescence microscopy data on H-2D\textsuperscript{b} in both cell lines where almost no cell surface expression could be detected in CHO/D\textsuperscript{b} cells [Garstka, Borchert et al., to be submitted].

3.4.2 IN VITRO BUDDING OF H-2D\textsuperscript{b} FROM CHO/D\textsuperscript{b} AND CHO/D\textsuperscript{b}/TAP2\textsubscript{def.} SEMI-INTACT CELLS

The vast majority of H-2D\textsuperscript{b} molecules in CHO/D\textsuperscript{b} and all detectable H-2D\textsuperscript{b} molecules in CHO/D\textsuperscript{b}/TAP2\textsubscript{def.} cells cannot reach the median Golgi and subsequently the cell surface (Figure 20). However, it is unclear whether strict ER-retention or forward transport to the ERGIC and cis Golgi followed by recycling can take place.

To address this question, in vitro budding was performed on semi-intact CHO/D\textsuperscript{b} and CHO/D\textsuperscript{b}/TAP2\textsubscript{def.} cells. Detection of H-2D\textsuperscript{b} was performed by immunoblotting with the T18 antibody (Table 2). Figure 21 shows that H-2D\textsuperscript{b} was packaged to similar extents from both TAP2-proficient and TAP2-deficient membranes (lanes 1 and 5). Packaging was severely reduced when either nucleotides or cytosol were omitted (Figure 21, lanes 2 and 3, respectively). In vitro budding of p58 and H-2D\textsuperscript{b} was correlating, i.e. H-2D\textsuperscript{b} and p58 signals were present or absent in the same samples. Both proteins may bud into different or even the same individual vesicles. As p58 was suggested to have cargo receptor function [Appenzeller et al. 1999], a direct or indirect involvement in transport of class I as a potential cargo protein is not unlikely.
RESULTS

<table>
<thead>
<tr>
<th>CHO/Db</th>
<th>budding reactions</th>
<th>total</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
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<tr>
<td>cytosol</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>CHO/Db/TAP2def</th>
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<th>total</th>
</tr>
</thead>
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<td>cytosol</td>
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Figure 21. In vitro packaging of mouse H-2D\textsuperscript{b} from CHO/Db and CHO/Db/TAP2\textsubscript{def}. Semi-intact cells. Budding reactions were carried out as usual with subsequent immunoblotting against the marker proteins calnexin (clx) and p58 as well as mouse H-2D\textsuperscript{b} molecules (T18 antibody). From both CHO/Db (upper panel) and CHO/Db/TAP2\textsubscript{def} SIC (lower panel), H-2D\textsuperscript{b} is packaged with similar efficiencies which are below those of p58. Fully glycosylated H-2D\textsuperscript{b}, 50 kDa (fg), core glycosylated H-2D\textsuperscript{b}, 45 kDa (cg), budding reactions (lanes 1 and 5), negative control reactions without nucleotides (lane 2), without cytosol (lane 3), empty lanes (lanes 4 and 6), 5% and 30% total SIC (lanes 7 and 8), molecular weight markers are indicated.

Despite similar band intensities, p58 and H-2D\textsuperscript{b} budding efficiencies were very different. While approximately 30% of total p58 was packaged, around 10% of the mouse class I could be found in the vesicles (Figure 21, p58 and H-2D\textsuperscript{b} in lanes 1 and 5). In CHO/Db cells, where a small portion of H-2D\textsuperscript{b} becomes fully glycosylated and
EndoH-resistant (*Figure 20*), only the core glycosylated band was specifically packaged (*Figure 21*, upper panel, lanes 1, 5, and 8). A certain amount of *in vitro* generated vesicles carrying p58 and H-2D<sup>b</sup> must be of COPII origin (*Figure 19*) while the remaining portion of the isolated vesicle population may be COPI-derived. Thus, H-2D<sup>b</sup> molecules are able to leave the ER to the ERGIC and the cis Golgi in both CHO/D<sup>b</sup> and CHO/D<sup>b</sup>/TAP2<sub>def</sub> membranes without gaining EndoH resistance and cell surface access (*Figure 20*). So, the absence of H-2D<sup>b</sup> from the cell surface is not due to static ER-retention mechanisms but instead must result from cycling processes through ER, ERGIC, and probably cis Golgi.

It is unclear why the main portion of H-2D<sup>b</sup> in TAP2-proficient CHO/D<sup>b</sup> cells is kept away from the cell surface since it should be peptide-bound. Unfortunately, we cannot distinguish between peptide-loaded and peptide-receptive (i.e. peptide-free) H-2D<sup>b</sup> by immunoblot, as the T18 antibody may recognize both forms of the mouse allele. On the other hand, H-2D<sup>b</sup> can be found in ER-derived vesicles from CHO/D<sup>b</sup>/TAP2<sub>def</sub> semi-intact cells, where virtually no peptide is available. This suggests that the majority of H-2D<sup>b</sup> detected in the vesicular fractions indeed is peptide-receptive or even devoid of any peptide.

Surprisingly, incubation with the calnexin antibody showed a double-signal in both CHO/D<sup>b</sup> and CHO/D<sup>b</sup>/TAP2<sub>def</sub> cells, which could not be seen in normal CHO-K1 with the same antibody (e.g. *Figure 19*). Whether there exist two forms of calnexin or whether one band reflects an unspecific crossreaction, remains unclear.

### 3.5 IN VITRO BUDDING OF ENDOGENOUS HLA MOLECULES IN LYMPHOCYTES

H-2D<sup>b</sup> overexpressed in TAP2-negative and -positive fibroblasts shows efficient *in vitro* packaging (*Figure 21*) suggesting that peptide-receptive class I molecules can leave the ER and recycle back. It still remains an open question if this is also true for endogenous MHC class I.

To exclude cycling of peptide-receptive class I being an artefact of overexpression, *in vitro* budding assays using T1 and T2 semi-intact cells were performed. Both cell lines are of human lymphoid origin and endogenously express HLA-A2 and -B5 alleles (*Table 3*). T2 cells, which are derived from the TAP-proficient T1 cell line,
carry a large deletion in the class II region encompassing the TAP1 and TAP2 genes [Salter and Cresswell 1986, Powis et al. 1991]. For investigation of endogenous class I budding, we decided to focus on lymphocytes rather than fibroblasts, since immune cells were expected to have higher MHC expression and more efficient transport. The status of TAP2 was confirmed in both T1 and T2 cells by anti-TAP2 immunoblot and HLA EndoH treatment. In vitro budding studies revealed no differences in endogenous HLA packaging from both TAP2-proficient T1 and TAP2-deficient T2 semi-intact cells. Though much lower budding efficiencies were observed for endogenous HLA than overexpressed H-2D\textsuperscript{b}, these data suggest that overexpression may not be the cause for cycling of peptide-receptive class I molecules in TAP-deficient cells.

### 3.5.1 VERIFICATION OF THE TAP2 STATUS IN T1 AND T2 CELLS

To verify the identity of T1 and T2 cells, immunoblotting was performed on total lysates from both cell lines using the monoclonal anti-TAP2 antibody 435.3. This antibody was originally selected against human TAP2 but also reacted against the mouse (RMA and RMA-S) but not the hamster (CHO) protein in immunoblots (data not shown). Human TAP2 is unglycosylated and has an apparent size of 75 kDa. In immunoblots on T1 lysates, two protein bands at around 70-75 kDa appeared upon incubation with 435.3 (Figure 22B, lane 1). The lower signal was absent in T2 cells (Figure 22B, lane 2), most likely corresponding to the TAP2 protein. An HLA-immunoblot served as loading control.

The influence of a functional TAP-complex on trafficking of endogenous HLA in T1 and T2 cells, was further monitored by EndoH treatment on total lysates and subsequent immunoblot using a commercial pan-HLA antiserum (Table 2). This antibody may react against both the HLA-A2 and -B5 alleles to similar extents. Thus, it is not possible to state which allele to what extent is reflected by the bands. Approximately 50% of endogenous HLA in T1 gained EndoH resistance at steady-state while in T2 cells the entire HLA population remained EndoH-sensitive (Figure 22A, lanes 1 and 3, respectively). This observation is consistent with pulse-chase data in which endogenous HLA in T1 cells became EndoH-resistant but not in T2 [Garstka, Borchert et al., to be submitted]. In T1 cells, immunofluorescence microscopy revealed that the majority of HLA is at the cell surface whereas HLA in T2 showed ER staining [Garstka, Borchert et al., to be submitted]. Therefore, the
EndoH-resistant HLA band (Figure 22A, lane 1) most probably corresponds to cell surface class I. This suggests that HLA loading and transport is fully functional in T1 cells. Due to the failure of peptide supply to the ER, HLA in T2 cells might be in an empty or peptide-receptive state prohibiting access to the cell surface. However, no distinction between peptide-loaded and peptide-receptive HLA can be made by immunoblot. Like the H-2D^b antibody T18 (see chapter 3.4), the pan-HLA antiserum might detect every form of both HLA-A2 and -B5.

In contrast to H-2D^b molecules in CHO/D^b cells, fully glycosylated HLA molecules cannot be distinguished from core glycosylated HLA without further EndoH-treatment (Figure 20 and Figure 22A, respectively). Glycosylation intermediates of mouse H-2D^b molecules may be more easily separated from each other due to additional glycosylation sites. While human alleles are glycosylated at position 86, mouse H-2D^b molecules carry two more glycosylation sites [Bjorkman et al. 1987].

**Figure 22. Verification of the TAP2-status in T1 and T2 cells by EndoH-treatment and anti-TAP2 immunoblot.**

**A.** EndoH-treatment on T1 and T2 lysates. About half of the HLA population is EndoH-resistant (EndoH_r) in T1 cells while in T2 cells, all HLA molecules remain EndoH-sensitive (EndoH_s). T1 cells (lanes 1 and 2), T2 cells (lanes 3 and 4), EndoH-treated (lanes 1 and 3), untreated (lanes 2 and 4).

**B.** Anti-TAP2 immunoblot with monoclonal 435.3. In T1 cells, two bands light up, one of which is absent in T2 lysates and likely corresponds to human TAP2. The other band is an unknown protein. HLA serves as loading control. T1 (lane 1) and T2 cells (lane 2). Molecular weight markers are indicated.
3.5.2 *IN VITRO* BUDDING OF HLA FROM T1 AND T2 SEMI-INTACT CELLS

In contrast to the H-2D\textsuperscript{b} situation in CHO fibroblasts (Figure 20), HLA localization is very different between T1 and T2 cells (Figure 22A). While the majority of endogenous HLA in T1 cells has access to the cell surface, it does not reach the *median* Golgi in T2 cells. In order to elucidate whether this selection takes place at the level of ER exit or at a later state by recycling, HLA *in vitro* budding from T1 and T2 semi-intact cells was investigated. This was the first time that an *in vitro* budding assay was performed with membranes not derived from adherent fibroblasts but instead from suspension cells.

Endogenous HLA molecules were packaged into the generated vesicles with similar efficiencies from both T1 and T2 membranes (Figure 23, *HLA*, lane 1) indicating that the intracellular localisation is not dependent on ER exit. Even without TAP function and therefore peptide availability in T2 cells, HLA is able to leave the ER, suggesting the influence of recycling mechanisms for peptide-receptive HLA molecules. Thus, packaging of MHC class I from TAP2-deficient membranes is not simply a result of overexpression (H-2D\textsuperscript{b} in CHO/D\textsuperscript{b}/TAP2\textsubscript{def.} cells, Figure 21) but also holds true for endogenous class I molecules (HLA alleles in T2 cells, Figure 23). However, the pan-HLA antiserum as well as the H-2D\textsuperscript{b} antiserum T18 may react against both peptide-loaded and peptide-receptive forms of class I molecules. We therefore do not know whether peptide-receptive or even truly empty class I molecules have been packaged in our assay.

*In vitro* budding of HLA correlated with that of ERGIC-53 (human p58) meaning that both proteins were present or absent in the same samples. For example, packaging of both proteins was reduced when nucleotides or cytosol were omitted (Figure 23, lanes 2 and 3). Similarly, packaging of both ERGIC-53 and HLA was impaired when samples were incubated at 4°C for budding (lane 4). Addition of GDP-restricted Sar1AT39N diminished *in vitro* budding of both proteins (lanes 5) confirming their presence in ER-derived COPII vesicles. However, packaging was more efficient for ERGIC-53 than HLA (approximately 20% versus 1%, respectively). The crossreactive band right above the ERGIC-53 signal belonged to a slightly larger protein. As human ERGIC-53 and rodent p58 were named according to their molecular weights (53 and 58 kDa, respectively), the crossreactive signal most likely resembles hamster p58 coming from the supplied cytosol.
Figure 23. *In vitro* packaging of endogenous HLA from T1 and T2 cells. Budding reactions were carried out as usual with subsequent immunoblotting against calnexin (clx) and ERGIC-53 as well as human HLA. Endogenous HLA molecules are packaged with similar efficiencies from both T1 (upper panel) and T2 cells (lower panel). HLA budding efficiencies are much below those of endogenous ERGIC-53. *In vitro* packaging of ERGIC-53 and HLA is inhibited by low temperature incubation and addition of Sar1AT39N indicating that both proteins are packaged into COPII vesicles. Budding reactions (lane 1), negative control reactions without nucleotides (lanes 2), without cytosol (lanes 3), budding reaction incubated at 4°C (lane 4), budding reactions with Sar1AT39N (lanes 5), 5% and 30% total SIC (lanes 6 and 7), molecular weight markers are indicated.
Budding efficiencies were much lower for endogenous HLA than for overexpressed H-2D\(^b\) (approximately 1% versus 10%, respectively). This might result from H-2D\(^b\) overexpression though *in vitro* packaging efficiencies may vary between different class I alleles. Furthermore, *in vitro* budding assays on radiolabelled T1 and T2 cells followed by immunoprecipitation showed higher transport efficiencies for freshly synthesized HLA molecules [Garstka, Borchert et al., to be submitted]. *In vitro* budding efficiencies of endogenous HLA molecules from T1 lymphocyte membranes were comparable to those from HeLa fibroblasts (data not shown) indicating that at steady-state class I transport is not enhanced in immune cells.

In the budding samples on the anti-calnexin immunoblot, it is difficult to state which one of the two bands corresponds to calnexin as they run very closely together (*Figure 23*, calnexin, lanes 1-6). However, appearance and intensity of both signals seems to be random and independent of ATP/GTP, temperature and addition of purified Sar1AT3\(\Delta\)N but dependent on cytosol. This suggested contamination of the supplied cytosol with calnexin-containing membrane fragments which were responsible for the generation of background signals rather than fragmentation of the donor membranes or specific packaging of that protein.

### 3.5.3 Budding and Endo-H Treatment on Endogenous HLA in T1 Cells

In T1 cells, more than half of the HLA population was EndoH-resistant (*Figure 22A*) and therefore beyond the median Golgi. However, it was unclear whether the HLA portion detected in the isolated vesicles was EndoH-resistant or -sensitive. Addition of purified Sar1AT3\(\Delta\)N verified the presence of HLA in COPII vesicles (*Figure 23*) but could not exclude the generation of vesicles from different origin, e.g. clathrin vesicles generated at the plasma membrane. As fully glycosylated and core glycosylated HLA molecules cannot be distinguished in T1 cells, *in vitro* budding samples from T1 semi-intact cells were EndoH-treated to test for EndoH sensitivity of vesicular HLA.

Approximately 50% of total HLA was EndoH-sensitive (*Figure 24*, lane 5). The situation was the same for HLA detected in the vesicles (lane 1). Thus, vesicular HLA consisted of an EndoH-sensitive but also of an EndoH-resistant population. However, only the EndoH-sensitive form was specifically packaged *in vitro* in an
nucleotide- and cytosol-dependent manner while EndoH-resistant HLA was also present with varying intensities in both negative control reaction (Figure 24, lanes 3 and 4). This speaks against specific packaging of HLA molecules from post-Golgi compartments (e.g. transport of HLA molecules from the trans Golgi to the plasma membrane in clathrin-coated vesicles), that should also depend on the presence of cytosol and nucleotides. EndoH-resistant HLA may rather result from contamination of the budding reactions with post-Golgi fragments of the donor membranes. Addition of wildtype Sar1A slightly increased in vitro budding of ERGIC-53 and EndoH-sensitive HLA molecules confirming their packaging into COPII vesicles (Figure 24, lane 2). However, the generation of COPI vesicles in this assay is not unlikely as both, ERGIC-53 and EndoH-sensitive HLA, could also be present in this vesicle type. Without addition of wildtype Sar1A, packaging efficiencies were extremely low for both proteins (even for ERGIC-53 below 5%) indicating that COPII components (especially Sar1) were limiting in the supplied cytosol. Calnexin and ERGIC-53 are unglycosylated proteins (lanes 5 and 6).

<table>
<thead>
<tr>
<th>T1</th>
<th>budding reactions</th>
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<td>ATP</td>
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<tr>
<td>cytosol</td>
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<tr>
<td>EndoH</td>
<td>+</td>
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<tr>
<td>-</td>
<td>Sar1A wt</td>
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5%

Figure 24. EndoH-treatment on budding reactions from T1 semi-intact cells. In vitro budding reactions were carried out as usual. EndoH-treatment was performed following high-speed spinning. Proteins were detected by immunoblot. Calnexin (clx) and ERGIC-53 are not glycosylated. EndoH-sensitive HLA molecules are specifically packaged in vitro while the presence of EndoH-resistant HLA in the vesicle fractions may rather result from contamination with donor membranes. Budding reaction (lane 1), budding reaction with wildtype Sar1A (lane 2), negative control reactions without nucleotides (lane 3), without cytosol (lane 4), 5% of total SIC, EndoH-treated (lane 5), and untreated (lane 6), molecular weight markers are indicated.
4 DISCUSSION

Peptide-receptive MHC class I molecules are usually retained intracellularly. In this study, an in vitro vesicle generation assay was used to identify whether they are still able to leave the ER in COPII vesicles or whether they are strictly retained in this compartment. The rather simple yeast in vitro COPII budding system was established prior to a more sophisticated mammalian one. The yeast pheromone precursor ppαF was in vitro translated and post-translationally translocated into yeast semi-intact cells. Translocation and simultaneous N-linked glycosylation of gpαF was verified by protease protection, EndoH treatment, and ConA precipitation. Using purified yeast COPII components and gpαF-containing yeast semi-intact cells, packaging of both gpαF and the SNARE protein Sec22p into COPII vesicles could be reconstituted in vitro. The yeast in vitro budding assay was used as a model to establish a similar protocol for mammalian cells using enriched ER membranes as well as semi-intact cells and liver cytosol as COPII source. Endogenous ERGIC-53 (p58), a mammalian cargo receptor was efficiently packaged into the generated vesicles while the ER-resident lectin chaperone calnexin was excluded. These proteins served as positive and negative budding controls, respectively. In vitro packaging of transfected H-2D\(^b\) and endogenous HLA-A2 and -B5 was investigated in TAP-proficient and TAP-deficient CHO as well as in T1 and T2 cells, respectively. Both, H-2D\(^b\) and HLA molecules were found to enter the generated vesicles in vitro to same extents in TAP-proficient and TAP-deficient cells indicating retrieval of peptide-receptive class I forms from post-ER compartments.

4.1 DEVELOPMENT OF AN IN VITRO VESICLE GENERATION ASSAY IN YEAST AND MAMMALS

Both, yeast and mammalian in vitro budding systems have been adopted from published protocols [Shimoni and Schekman 2001, Kim et al. 2005]. Several factors and conditions had to be optimized including identification of reliable control proteins, suitable donor membranes, the COPII source, the nucleotide system, as well as incubation temperature and time. Some of them were similar for the yeast and the mammalian system, while others turned out to be very different.
4.1.1 CONTROL PROTEINS FOR IN VITRO BUDDING

The first important step was to identify reliable control proteins in both in vitro budding systems. We chose well studied model proteins, which had already been successfully used by other groups. These proteins were necessary to develop the assay and to evaluate each sample.

For the yeast studies, ppaF, the precursor of the yeast mating pheromone α-factor, served as a cargo protein and therefore as marker for the generation of COPII vesicles. This protein is not only very efficiently transported in vivo and in vitro [Julius et al. 1984, Baker et al. 1988], it can also be translated and radiolabelled in vitro prior to ER translocation [Rothblatt and Meyer 1986] making its detection independent of antibodies. Instead, the protein can directly be visualized in cell lysates without further immunoprecipitation or immunoblotting procedures. Glycosylation can be used as a convenient diagnostic of protein translocation into the ER since occurring simultaneously. In vitro synthesized ppaF was post-translationally translocated into yeast semi-intact cells prior to budding experiments. As translocated and signal sequence-cleaved gpαF exhibits three N-linked glycosylation sites [Wuestehube and Schekman 1992], we could easily differentiate between untranslocated 35S-ppαF and translocated, glycosylated 35S-gpαF by their different gel mobilities. Consistent with published data [Rothblatt and Meyer 1986], post-translational translocation of 35S-ppαF into yeast semi-intact cells in vitro was strongly dependent on ATP. Protection from external proteases, EndoH treatment, and ConA precipitation confirmed membrane translocation and glycosylation of 35S-gpαF.

Yeast semi-intact cells containing 35S-gpαF were then used for in vitro budding experiments. After separation from the SIC donor membranes by a medium-speed spin, only translocated 35S-gpαF could be found in the isolated vesicle fractions (medium-speed supernatant, high-speed pellet) while untranslocated 35S-ppαF was absent. This confirmed specific packaging of 35S-gpαF. Thus, 35S-gpαF was not only synthesized and post-translationally translocated into the ER of yeast semi-intact cells, it was also able to be packaged in vitro. As yeast 35S-gpαF is a soluble cargo protein, we also wanted to check for in vitro budding of an endogenous yeast membrane protein. Sec22p as a SNARE is involved in vesicle trafficking and fusion and therefore continuously cycles between ER and Golgi [Jahn and Scheller 2006]. Like 35S-gpαF, Sec22p is a control protein frequently used for yeast in vitro budding.
studies [Campbell and Schekman 1997]. Here, Sec22p and $^{35}$S-gpαF were detected in the same vesicle fractions confirming that both proteins were simultaneously packaged \textit{in vitro}. It is unclear whether they were even incorporated into the same individual vesicles or into different vesicles of the same population. Consistent with published data [Bednarek et al. 1995, Kuehn et al. 1998, Belden and Barlowe 2001], the membrane protein Sec61p belonging to the translocon channel could not be found in the generated vesicles and seems to be strictly retained in the ER of yeast cells. Finding reliable control proteins for a mammalian \textit{in vitro} budding system was more complicated. So far, post-translational translocation of mammalian proteins into mammalian ER membranes could not be realized. It is possible to translocate and radiolabel co-translationally but \textit{in vitro} packaging of such proteins has not yet been shown. As no system comparable to yeast α-factor \textit{in vitro} translocation and budding is available in mammalian cells, we had to search for antibodies against endogenous proteins that could serve as budding controls. Two proteins, one efficiently exported from the ER and the other one strictly retained inside the ER were identified to control for the mammalian \textit{in vitro} vesicle generation assay. The lectin human ERGIC-53 (its rodent homologue is called p58) is a membrane-bound receptor for soluble cargo proteins continuously cycling between ER, ERGIC, and cis Golgi [Kappeler et al. 1997, Appenzeller et al. 1999, Appenzeller-Herzog et al. 2005]. It has been used extensively for mammalian \textit{in vitro} budding studies [Rowe et al. 1996, Kim et al. 2005]. Here too, in all tested cell lines ERGIC-53 (p58) turned out to be efficiently packaged \textit{in vitro} into vesicles that could be pelleted by a high-speed spin. The lipid character of the vesicular carrier was further demonstrated by addition of the detergent Triton X-100 prior to high-speed spinning. This resulted in lysis of the vesicle membrane and solubilisation of the original transmembrane protein p58. The transmembrane protein calnexin is an ER-resident lectin chaperone [Ellgaard and Helenius 2003] and was already been shown not to be packaged \textit{in vitro} [Rowe et al. 1996]. This is in accordance with our data, since we never observed specific incorporation of calnexin in the p58-containing vesicle fractions. In this assay, p58 and calnexin were found to be reliable positive and negative control proteins for evaluation of mammalian \textit{in vitro} budding.
4.1.2 DONOR MEMBRANES SUITABLE FOR VESICLE GENERATION FROM THE ER

In order to reconstitute an \textit{in vitro} vesicle generation assay, we had to find suitable donor membranes. Microsomes or semi-intact cells can serve as source of ER membranes. Microsomes are enriched in ER membranes but contamination with Golgi and other cellular membranes cannot be avoided. Semi-intact cells have a perforated plasma membrane while intracellular membranes are largely unaffected (hence “semi-intact”). The cell wall has to be destroyed additionally prior to microsome or SIC preparations from yeast cells (spheroplasting). We used semi-intact cells for yeast \textit{in vitro} budding studies since they are faster and more convenient to prepare than microsomes.

In mammals, similar \textit{in vitro} vesicle generation and isolation has so far been described from microsomal membranes only [Nohturfft et al. 2000, Kim et al. 2005] therefore we started from this point. Following the protocols described by Nohturfft et al. (2000), we could not observe specific p58-packaging from mammalian microsomes. Instead, p58 as well as calnexin both appeared in the vesicle fractions. This was also the case when either nucleotides or the source of coat proteins (i.e. cytosol) were omitted, both indispensable for vesicle generation \textit{in vitro} (see below). We therefore concluded that incomplete separation of donor membranes from the generated vesicles was taking place. Mammalian microsome preparation by the needle technique as described here is a harsh process that can result in fragmentation of intracellular membrane systems including the ER. If these fragments are in the size of the generated vesicles (~ 60 nm) and become included in the reaction, they cannot be separated from them by a medium-speed spin, resulting in contamination of the budding fractions with ER membranes. To avoid this problem, CHO microsomes were cleared from smaller membrane fragments by a low-speed spin as suggested by Kim et al. (2005) prior to incubation. This centrifugation step only allowed bigger membranes to sediment while smaller fragments remained in the supernatant and were discarded. Furthermore, fragmentation did not happen at all when CHO microsomes were incubated on ice since biochemical reactions take place at nearly physiological temperatures. This is also true for the process of vesicle budding, which was shown to be inhibited when reactions were kept on ice. In the yeast system however, fragmentation of the donor membranes did not render \textit{in vitro} budding studies.
Isolation of microsomes always comes along with the loss of a certain portion of the original ER membranes in the cell. This can result in detection problems for proteins expected to have a low packaging efficiency like MHC class I molecules. Thus, we thought of a way to prepare mammalian semi-intact cells competent for vesicle generation in vitro. In 1987, Beckers et al. published a protocol for reconstitution of protein transport from the ER to Golgi apparatus in semi-intact fibroblasts [Beckers et al. 1987]. However, they measured protein arrival at the Golgi instead of isolating the transport vesicles to further investigate their cargo. A procedure to permeabilize the plasma membrane for immunofluorescence techniques served as model to prepare mammalian semi-intact cells by freeze-thaw [Mardones and Gonzalez 2003]. From these cells, we could generate and deliberate p58-containing vesicles in vitro confirming access of coat proteins into and exit of generated vesicles out of the permeabilized plasma membrane. Furthermore, mammalian semi-intact cells were shown to contain approximately ten times more protein than the microsome preparations.

Efficiency of p58-packaging from both SIC and microsomes varied from experiment to experiment. Vesicle generation and incorporation of cargo proteins is clearly dependent on the quality of membranes, of the coat protein source (cytosol, purified proteins), and of nucleotides, on the concentration of the various components, as well as pH, incubation temperature and time. Furthermore, the intensity of a budding signal can appear increased or decreased because more or less vesicles with the same portion of cargo proteins (e.g. p58, gpαF) have been generated. On the other hand, the number of generated vesicles can be equal whereas more or less cargo molecules have been packaged into the same number of vesicles. Both situations would positively or negatively influence the in vitro budding efficiency of a protein but we are not able to distinguish between them.

4.1.3 THE COAT PROTEIN SOURCE TO DRIVE COPII VESICLE GENERATION

The identity of generated vesicles from both the yeast and the mammalian system, is determined by the source of coat proteins present in the reaction. Neither microsomes nor SIC consist of ER membranes solely but are contaminated with different cellular membranes instead. To promote vesicle generation from the ER, the donor
membranes have to be combined with COPII components. Therefore, the use of purified COPII proteins guarantees the generation of COPII vesicles only. In yeast, they have been shown to successfully promote in vitro vesicle generation from microsomal and SIC membranes as well as from synthetic liposomes [Campbell and Schekman 1997, Matsuoka 1998]. Cytosol, the fraction of cytoplasm minus membrane-bound organelles, can be used as COPII source instead. However, cytosol preparations still contain all different protein coat complexes (e.g. COPII, COPI, clathrin) and are therefore capable to induce the generation of all kinds of vesicles. This renders the identification of the vesicles generated in presence of cytosol.

Yeast in vitro budding could be easily reconstituted with yeast semi-intact cells and purified yeast COPII proteins. Sec22p has been shown to directly bind to the COPII coat [Springer and Schekman 1998] while soluble gpaF can interact indirectly via the cargo receptor Erv29p [Belden and Barlowe 2001]. When COPII components were not included in the reaction, neither $^{35}$S-gpaF nor Sec22p were found to be packaged in vitro confirming that both proteins specifically left the donor ER in COPII-derived vesicles. It was even possible to follow up the fate of in vitro synthesized $^{35}$S-gpaF from entry into and leave from the ER once more emphasizing the strength of this system.

Generation of COPII vesicles from mammalian microsomes with the help of purified yeast COPII proteins has been described once [Espenshade et al. 2002]. However, we could not reproduce these results. Interestingly, even purified mammalian COPII components were not able to induce vesicle budding in vitro without the presence of additional cytosolic compounds [Kim et al. 2005]. Thus, we had to use cytosol instead of purified proteins for COPII vesicle generation from mammalian membranes. Because of the much higher yield, cytosol was prepared from tissue rather than cultured cells. Hamster or rat liver cytosols were shown to promote efficient vesicle budding in both CHO- and lymphoid T1/T2-derived membranes while pig brain cytosol failed in this respect (data not shown). A certain disadvantage of using cytosol in COPII-budding reactions is the generation of background signals when detecting proteins by Immunoblot. Even removal of the supernatant after high-speed spinning the vesicle fractions, can still result in contamination with cytosolic proteins that could unspecifically react with antibodies. The unspecific cytosol signals could be eliminated by washing the vesicle pellets before gel loading. Packaging of p58 was absolutely cytosol-dependent excluding unspecific fragmentation of ER
membranes and indicating vesicle generation due to cytosolic coat proteins. As no p58-budding could be observed at all in the absence of cytosol, we concluded that donor membranes were free of endogenous coat proteins sticking to them and unspecifically promoting vesicle generation. Human ERGIC-53 contains sequence motifs that have been shown to interact with the COPII coat [Kappeler et al. 1997]. However, the identity of the vesicles generated from mammalian membranes in the presence of cytosol, still remained elusive. To test for the presence of COPII vesicles, we included purified hamster Sar1A forms in the reactions. Here, wildtype Sar1A was able to stimulate p58-packaging while mutant Sar1AT39N had an inhibitory effect on vesicle generation. Sar1AT39N is a GDP-restricted mutant of this GTPase known to suppress COPII budding from the ER in vitro [Kuge et al. 1994]. This mutant is unable to bind GTP and hence to insert to the ER membrane. Recruitment of Sec23/24 and Sec13/31 cannot take place and COPII vesicle generation is inhibited. It is still unclear how Sar1AT39N is able to inhibit cytosolic Sar1 in the reaction since a competition for membrane binding may not take place. Addition of wildtype Sar1A does not always have a stimulatory effect on packaging [Kuge et al. 1994, Espenshade et al. 2002, Kim et al. 2005] as shown here. The effect of wildtype Sar1A is most likely dependent on the concentration of Sar1 in the applied cytosol. When Sar1 is the limiting factor in the cytosol, addition of Sar1Awt could stimulate COPII vesicle budding while no effect would be observed under saturating Sar1 levels. These results showed that the p58-containing vesicles generated in vitro are indeed mainly COPII-derived. Inhibition of p58-budding by addition of Sar1AT39N was incomplete indicating insufficient concentration of the mutant protein or presence of p58 in other vesicles than COPII (e.g. COPI).

4.1.4 IMPORTANCE OF NUCLEOTIDES FOR VESICLE GENERATION

The presence of nucleotides (ATP, GTP or a non-hydrolyzable analogue like GMP-PNP) is indispensable for vesicle generation from both yeast and mammalian membranes in vitro [Baker et al. 1988, Bednarek et al. 1995, Todorow et al. 2000, Espenshade et al. 2002, Kim et al. 2005]. GTP (or GMP-PNP) is necessary for initiation of vesicle budding by activation and membrane insertion of Sar1. If Sar1 fails to bind to the ER membrane, assembly of the remaining coat protein complexes
is abolished, and vesicle generation cannot occur. Though ATP is required for ER to Golgi transport in vivo and in vitro [Novick et al. 1981, Beckers et al. 1987], its exact role in this process is still unclear. It may act as phosphate reservoir for GTP regeneration.

Packaging of the cargo proteins $^{35}\text{S-gp}\alpha\text{F}$ and Sec22p from yeast semi-intact cells was totally abolished in the absence of nucleotides while residual p58-budding from mammalian microsomes and SIC could still be observed in the respective samples. This difference is most likely due to usage of cytosol for mammalian in vitro packaging studies, which does not only contain proteins like the COPII components but also non-proteinaceous factors including different forms of nucleotides. Thus, even in the absence of additional ATP/GTP, residual cytosolic nucleotides may still initiate vesicle generation and protein packaging from mammalian donor membranes.

Addition of apyrase to mammalian in vitro budding reactions reduced packaging of p58 even in the presence of additional ATP/GTP. Apyrase is a phosphohydrolase catalyzing the removal of gamma and beta phosphates from nucleoside tri- and diphosphates (e.g. ATP, GTP, UTP, ADP, but not AMP). When present in the budding reaction, nucleotides become depleted and vesicle generation is inhibited. The inhibitory effect is dependent on nucleotide and enzyme concentrations.

Non-hydrolyzable guanosine nucleotides like GMP-PNP can substitute for GTP in Sar1 activation and vesicle generation. In both the yeast and the mammalian systems, GMP-PNP was sufficient to promote vesicle generation and packaging of control cargo proteins ($^{35}\text{S-gp}\alpha\text{F}$, Sec22p, p58). Interestingly, the $^{35}\text{S-gp}\alpha\text{F}$ signal in the budding fractions was stronger when GMP-PNP was used instead of GTP. However, GTP hydrolysis is necessary for uncoating. Vesicles generated in the presence of GMP-PNP still contain their COPII protein coat and are unable to fuse with a donor compartment [Barlowe et al. 1994]. As the coat proteins are still attached, these vesicles are more dense and therefore may settle more easily during the high-speed spin than those generated in the presence of GTP. This rather than more efficient $^{35}\text{S-gp}\alpha\text{F}$-packaging might explain the stronger $^{35}\text{S-gp}\alpha\text{F}$ signal.

Mammalian in vitro budding experiments were performed in the presence of GTP. GMP-PNP was sufficient to promote vesicle generation and p58-packaging. However, using cytosol for in vitro budding, the concentrations of COPII proteins supplied are unknown and most likely the limiting factor in the reaction. Addition of non-hydrolyzable GTP analogues could eventually deplete the cytosol from free
COP II components by inhibiting their recycling following GTP hydrolysis and coat disassembly. This problem does not exist if purified COP II proteins are used since they were added in excess and apparently did not become limiting after several rounds of coat assembly and vesicle generation.

4.2 \textit{IN VITRO} BUDDING OF MHC CLASS I MOLECULES

4.2.1 \textit{IN VITRO} PACKAGING OF CLASS I MOLECULES IN TAP-PROFICIENT LYMPHOCYTES AND FIBROBLASTS

\textit{In vitro} budding studies of endogenous HLA molecules in T1 cells turned out to be a challenging task with packaging efficiencies at the limit of detection. Finding an HLA-antibody still reactive against the lowest protein concentrations in immunoblots was therefore the first requirement for successful experiments. However, it is unknown if the commercially available anti-human class I antibody used in this study preferentially recognizes certain HLA-alleles. HLA-C molecules usually have a lower cell surface expression than HLA-A and -B gene products [Neefjes and Ploegh 1988, Neisig et al. 1998]. By immunoblot, we could not distinguish between different alleles expressed by T1 cells.

HLA budding could reliably be shown from semi-intact cells only while from microsomes the amount of protein that had been incorporated into vesicles was often below the detection limit (data not shown). Despite extreme low budding efficiencies with approximately 0.5-2\%, HLA-packaging was nucleotide-, cytosol-, and temperature-dependent. It could be partially inhibited by the addition of Sar1AT39N confirming the presence of HLA molecules in COP II vesicles. It is unknown whether MHC I can bind to the COP II coat directly or indirectly with the help of cargo receptors recognizing specific ER exit signals exposed by class I molecules as suggested by Spiliotis et al. (2000). Direct interaction with the COP II components had been shown for HLA-F, a class I-like protein, which binds to the coat with its cytosolic tail [Boyle et al. 2006]. The same could be true for classical HLA-A, -B, and -C alleles.

\textit{In vitro} budding of HLA furthermore correlated with that of p58 meaning both proteins where either present or absent in the same vesicle fractions and similar fluctuations in packaging efficiencies (i.e. if p58 showed more or less efficient
budding this was also true for HLA). It was unknown whether the proteins were incorporated into the same individual vesicles but they were clearly present in the same vesicle population. However, budding efficiencies of HLA molecules were very much lower than those of p58. This was not surprising at all since the cargo receptor has been shown to be the most successfully packaged endogenous protein used for mammalian in vitro budding so far.

Immunofluorescence microscopy showed that HLA molecules in T1 cells were localized to the cell surface at steady-state [Garstka, Borchert et al., to be submitted]. Furthermore, EndoH treatment on total T1 lysates confirmed that more than 50% of endogenous HLA was EndoH-resistant and had passed the median Golgi to become sialylated. The TAP2 transporter subunit could also be detected by immunoblot. Addition of exogenous HLA-A2-specific peptides to the in vitro budding reactions did not increase HLA-packaging (data not shown). Assuming the presence of a still functional peptide transporter in the semi-intact cells, these results indicated that the extremely low budding efficiencies observed for HLA molecules in T1 cells were not due to lack of endogenous peptides or inefficient peptide loading. We concluded instead they might rather reflect the transport situation of an endogenous cell surface protein. Unfortunately, we had no comparison since in vitro budding of another endogenous cell surface protein had not been shown so far. The vesicular stomatitis virus glycoprotein (VSV-G) is transported to the plasma membrane of infected cells. Packaging of VSV-G in vitro is quite efficient [Espenshade et al. 2002] but it does not reflect the situation of an endogenous cell surface protein and is therefore not a suitable control. Immunoblot detection of a plasma membrane ATPase and the CD45 cell surface marker by monoclonal antibodies failed (data not shown). In yeast, the glycosyl phosphatidylinositol (GPI)-anchored protein Gas1p was packaged in vitro with an efficiency of around 5% [Doering and Schekman 1996]. Presenilin, a protein linked to early-onset Alzheimer’s disease, is localized in the ER and the Golgi complex. In vitro budding studies of the endogenous protein showed a packaging efficiency of around 2% [Kim et al. 2005], which is comparable to that observed for endogenous HLA molecules in T1 cells.

However, percentage of HLA-packaging referred to the total amount of HLA expressed including freshly synthesized class I molecules inside the ER, those that had already made their way to the Golgi or further to the cell surface, and even that population of old proteins inside endosomes destined for degradation while p58 is
distributed in ER, ERGIC-, and the Golgi complex. EndoH treatment of budding reactions and total membranes showed that both EndoH-sensitive and EndoH-resistant HLA molecules were present in the vesicle fractions. The EndoH-sensitive population was specifically packaged into the generated vesicles in a cytosol- and nucleotide-dependent manner while the EndoH-resistant molecules also appeared in the samples, in which cytosol and nucleotides were omitted. This indicated a contamination with fragmented donor membranes containing EndoH-resistant HLA forms (e.g. remnants of the plasma membrane, endosomes).

*In vitro* budding of mouse H-2D<sup>b</sup> from CHO semi-intacts was nucleotide- and cytosol-dependent and could be partially inhibited by addition of Sar1AT39N (the latter not shown). Interestingly, it was packaged with an efficiency of approximately 10-20%, which was still below that one of endogenous p58 (~30%) but much more than that one generally observed for endogenous HLA molecules in T1 cells (0.5-2%). The antibody used for detection of H-2D<sup>b</sup> in immunblots did not react against endogenous hamster class I molecules (data not shown). Thus, the signals solely reflect *in vitro* budding of transfected H-2D<sup>b</sup> molecules so that efficient packaging most likely resulted from overexpression.

Different from HLA in T1 cells, only a very small population of H-2D<sup>b</sup> acquired EndoH resistance in CHO cells, and no cell surface expression could be detected by immunofluorescence microscopy [Garstka, Borchert et al., to be submitted]. Even though H-2D<sup>b</sup> molecules could leave the ER of CHO cells quite efficiently *in vitro*, they were retained intracellularly at steady-state indicating a retention mechanism by cycling between the ER and the early Golgi complex. Since TAP function should be normal in these cells, the reason for intracellular retention is unclear. Fibroblasts might need external stimulation by cytokines to optimize peptide generation, translocation, and loading to achieve class I cell surface expression. On the other hand, it cannot be excluded that mouse H-2D<sup>b</sup> is unable to interact with the hamster loading complex, resulting in suboptimal peptide loading and the generation of peptide-receptive H-2D<sup>b</sup> molecules. An overload of the ER quality control system due to overexpression could be the reason for the high *in vitro* packaging efficiencies of H-2D<sup>b</sup> molecules from CHO semi-intact cells.
4.2.2 IN VITRO PACKAGING OF PEPTIDE-RECEPTIVE CLASS I MOLECULES IN FIBROBLASTS AND TAP-DEFICIENT LYMPHOCYTES

Surprisingly, in both TAP-deficient CHO and T2 cells, in vitro packaging of H-2D$^b$ and HLA, respectively, was as efficient as in their wildtype counterparts. Despite higher budding efficiencies of overexpressed H-2D$^b$, also endogenous HLA molecules were packaged from TAP-deficient cells excluding the possibility that overexpression was responsible for the ER exit of peptide-receptive class I molecules. TAP deficiency was confirmed by EndoH-treatment on total lysates showing that in both cell lines MHC class I populations were entirely EndoH-sensitive and not localized to the cell surface at steady-state as verified by immunofluorescence microscopy [Garstka, Borchert et al., to be submitted].

In TAP-deficient and -proficient CHO cells, the H-2D$^b$ localisation did not differ much since the majority of H-2D$^b$ molecules remained EndoH-sensitive and were kept away from the cell surface in the wildtype cell line, too. Even in the TAP-proficient CHO cells, peptide supply might be the limiting factor for cell surface expression. This may be generally true for fibroblasts, as addition of exogenous specific peptides resulted in redistribution of H-2D$^b$-GFP from the ER to the Golgi apparatus and finally the cell surface in Vero cells [Garstka, Borchert et al., to be submitted]. However, in T2 cells, HLA localisation was very different from that in T1 cells. Here, the lack of peptide-supply due to TAP-deficiency was clearly the reason for intracellular retention of HLA molecules. However, partial inhibition of HLA in vitro packaging by addition of hamster Sar1AT39N confirmed that HLA molecules could exit the ER in TAP-deficient T2 cells to the same extent as they did in TAP-proficient T1 cells. Taken together, these data indicated that peptide-receptive MHC class I molecules are capable of leaving the ER to the ERGIC and even to the cis Golgi before being retrieved. This is in agreement with previous studies that have detected peptide-receptive class I molecules in the Golgi apparatus or in the ERGIC of TAP-deficient cells [Hsu et al. 1991, Paulsson et al. 2002].

Ljunggren et al. (1990) found out that in a TAP-incompetent mouse lymphoma cell line endogenous H-2 molecules showed a high level of cell surface expression when cells were incubated at reduced temperatures (19-33°C). They were stabilized at the cell surface at physiological temperatures by addition of specific peptides to the medium. Our in vitro budding assays had to be run at 25°C, since higher temperatures increased fragmentation of donor membranes. It cannot be excluded the reduced
temperature was responsible for in vitro packaging of class I molecules in TAP-deficient T2 cells. However, this effect could not be observed for HLA molecules in human T2 cells [Baas et al. 1992] and might therefore depend on the alleles examined. Furthermore, we could not detect increased ER exit of HLA molecules in the TAP-proficient cell line compared to the TAP-deficient counterpart. If the low budding temperature were responsible for in vitro packaging of peptide-receptive class I molecules, this should also happen in TAP-competent cells. Here, peptide-receptive and peptide-occupied (i.e. loaded with optimal, high-affinity peptides) molecules should be exported resulting in more efficient packaging in the wildtype cell line. Thus, ER exit of peptide-receptive HLA molecules in TAP-deficient T2 cells (and of H-2D\textsuperscript{b} in both CHO cell lines) might not be an artefact caused by low incubation temperatures but might instead reflect physiological processes.

Peptide-free class I molecules devoid of any peptide and those loaded with suboptimal peptides were both defined “peptide-receptive”, since both forms are unstable and usually not cell surface expressed. However, this term simply serves as construct to differentiate from fully assembled class I molecules bound to optimal, high-affinity peptides, which are stably expressed at the cell surface. Actually, differentiation of peptide-free from suboptimal class I/peptide complexes is very difficult and the antibodies used in this study (anti-H-2D\textsuperscript{b}, anti-HLA) most likely could not distinguish between them. Therefore, we do not know whether “empty” class I molecules can exit the ER or only those that are associated with any peptide.

When specific peptides were added to the post-lysis vesicle fractions, more HLA molecules could be immunoprecipitated in T1 and T2 cells using the monoclonal W6/32 antibody, which recognizes HC/\beta_2\text{m} complexes [Garstka, Borchert et al., to be submitted]. As empty dimers are unstable in the absence of peptide, the subunits tend to dissociate from each other very quickly. Addition of exogenous peptides can help to stabilize the interaction of the heavy chain and \beta_2\text{m}. Increased immunoprecipitation signals with W6/32 following supply of specific peptides indicated the presence of “empty”, peptide-free dimers inside the vesicles capable to bind peptide. However, it is unclear whether originally “empty” class I molecules were packaged or those loaded with suboptimal peptides, which could have been lost during incubation and exchanged by the high-affinity peptides added.

In TAP-deficient cells, such low-affinity peptides cannot be of cytosolic origin. Indeed, data exist that suggest efficient assembly of class I molecules with TAP-
independent peptides. Certain alleles, particularly HLA-A2 also expressed by T1 and T2 cells, can bind signal sequence-derived peptides in the absence of TAP [Henderson et al. 1992, Wei and Cresswell 1992]. It is therefore possible that in TAP-deficient T2 cells, this allele is preferentially packaged. Premature termination of proteins undergoing Sec61-mediated translocation into the ER could be another source, as well as ER degradation of membrane proteins [Snyder et al. 1997]. Many HLA-B molecules do not even interact with TAP and can bind peptides outside of the peptide loading complex [Neisig et al. 1996]. However, this is not true for HLA-B5, which strongly associates with TAP [Neisig et al. 1996]. This allele might be packaged efficiently in T1 but not in T2 cells. Thus, similar class I budding efficiencies observed in both lymphoid cell lines might not indicate packaging of the same alleles. In anti-HLA immunoblots we might instead observe ER exit of different molecules depending on the cell line.

4.3 FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

We have shown for the first time in vitro budding of a cell surface protein from the ER of mammalian semi-intact cells. Despite intracellular retention, peptide-receptive MHC class I molecules were able to leave the ER in COPII vesicles in vitro as efficiently as fully assembled ones (Figure 25, step 1). While the latter are subsequently transported through the later stages of the secretory pathway to reach the cell surface, peptide-receptive class I molecules would be retrieved back to the ER from the ERGIC or even the cis Golgi (Figure 25, step 2 and 3, respectively). Our data thus support that continous cycling between the early stages of the secretory pathway may contribute to intracellular retention of peptide-free and/or suboptimally loaded class I molecules. Further immunoprecipitation experiments with conformation-specific antibodies (HC10 for free heavy chains, W6/32 for β2m-associated class I) after in vitro budding could reveal whether class I molecules devoid of any peptide would be able to leave the ER and cycle back. In this study, peptide-receptive class I molecules were investigated in TAP-deficient cells. In vitro packaging experiments could be performed following inhibition of the proteasome or using β2m-negative cells to further check for cycling of peptide-receptive HC/β2m dimers and heavy chain monomers, respectively.
Figure 25. Proposed cycling of peptide-receptive class I molecules through the early stages of the secretory pathway. Fully assembled as well as peptide-receptive (represented by a peptide-free dimer) class I molecules leave the ER in COPII vesicles (1). Both forms could reach post-ER compartments where peptide-receptive molecules would be recognized by unknown control mechanisms. While fully assembled class I is allowed to pass forward to the cell surface (2), peptide-receptive forms may subsequently be retrieved back to the ER from the ERGIC and/or the cis Golgi. This retrieval could be mediated in COPI vesicles (3).
In combination with retrieval processes, active ER retention mechanisms (e.g. by association with ER-resident chaperones like calnexin) may still play a role in intracellular retention of peptide-receptive class I molecules. However, it remains uncertain where and how peptide-receptive molecules are recognized for retrieval back to the ER. They could exhibit a non-native conformation that is identified by chaperones capable of cycling between the compartments of the early secretory pathway. General chaperones that interact with MHC class I molecules (e.g. calnexin and calreticulin) could take over this task. In this study, we did not detect specific packaging of calnexin. Leakage of a small calnexin population below the limit of detection could still have taken place. Calreticulin has been shown to be localized to the ER by retention as well as retrieval processes operating independently [Sönnichsen et al. 1994] and could thus be responsible for retrieval of non-native proteins from post-ER compartments. MHC class I-specific chaperones like tapasin may also cycle together with peptide-receptive molecules. In this respect, it would be interesting to know whether members of the PLC (e.g. tapasin, TAP) can be packaged in vitro. This is not unlikely as both TAP and tapasin have also been found in post-ER compartments [Kleijmeer et al. 1992, Paulsson et al. 2002]. Consequently, peptide loading might not be restricted to the ER but could even proceed in COPII vesicles, the ERGIC, or the cis Golgi.

Recycling of a class I-like protein, HLA-G, could be halted by addition of high-affinity peptides [Park et al. 2001], hence suggesting that the quality of bound peptide determines if a molecule is retained by retrieval and transported forward to the cell surface. This suggests a post-ER quality control process that is able to sense the peptide occupancy of trafficking MHC class I molecules. The ERGIC has been shown to contribute to general protein quality control [Pröpstring et al. 2005]. If a general post-ER quality control exists, ER exit and in vitro packaging should also be observed for other immature proteins. Suitable candidates for such experiments would be the viral VSV-G protein or the ABC-transporter cystic fibrosis conductance regulator (CFTR). Apparently misfolded variants of both proteins (VSV-G ts045, ΔF508 CFTR) were found to partially exit the ER and be retrieved from post-ER compartments [Hammond and Helenius 1994, Gilbert et al. 1998]. Retrieval was also shown for a subunit of the mammalian translocon channel Sec61 [Greenfield and High 1999], an ER-resident protein, while the yeast homologue seems to be strictly retained inside the ER [Belden and Barlowe 2001]. This indicates that ER quality
control and protein sorting mechanisms may not be the same in different eukaryotic species. Despite similar pathways and homologeous proteins involved, *in vitro* budding assays turned out to have different requirements in yeast and mammals. However, identification of an independent control protein comparable to yeast $\alpha$-factor would facilitate mammalian *in vitro* packaging experiments.

Immunological and cell biological aspects were combined in this project. Investigation of the mechanisms involved in intracellular transport of MHC class I molecules could give further insight in the regulation of the adaptive immune response. On the other hand, the conformation-dependent localisation of an exemplary membrane protein in the mammalian secretory pathway should contribute to better understand the development of trafficking-based diseases like cystic fibrosis or Alzheimer’s disease.
5 REFERENCES


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