Exploiting the Yeast L-A Viral Capsid for the In Vivo Assembly of Chimeric VLPs as Platform in Vaccine Development and Foreign Protein Expression

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INTRODUCTION
Viral expression systems can be classified into three types based on the regulatory and/or structural viral component that drives protein expression: (i) plasmid-based vectors containing promoter elements from either pro- or eukaryotic viruses; (ii) infectious viral vectors in which the gene of interest is integrated into the viral genome and expressed from a viral promoter in an appropriate host; (iii) virus-like particles (VLPs), also called pseudovirions, representing subunit structures composed of multiple copies of a viral capsid and/or envelope protein capable to self-assemble into VLPs of defined spherical symmetry in vivo [1–3]. Currently, VLPs composed of a structural protein are often used as particulate antigen in the design of prototype vaccines as they possess several advantages over conventional monomeric protein immunogens [4]. Firstly, most VLPs can be produced in large quantity in a heterologous host. Secondly, due to their particle structure and high molecular weight, VLPs can be easily purified in a preparative scale. Thirdly, a number of particle forming proteins tolerate insertion of foreign amino acid sequences without affecting in vivo self-assembly competence. Such chimeric or hybrid VLPs, exploited as platform for the display of antigenic determinants in a polyvalent manner, have already been shown to be promising candidates in the development of various subunit vaccines [5].

Here, a novel expression system based on the non-infectious yeast (Saccharomyces cerevisiae) dsRNA virus L-A was designed. This mycovirus represents an autonomously replicating, encapsidated dsRNA element that stably persists in the cytoplasm of an infected yeast cell without conferring a recognizable phenotype upon its host [6]. As member of the Totiviridae family, L-A contains a linear non-segmented dsRNA genome (4.6 kb) comprising two overlapping ORFs, gag and pol. While gag encodes the major capsid protein Gag (76 kDa), pol specifies a multifunctional RDRP which is in vivo expressed as a 171 kDa Gag/Pol fusion protein by a −1 ribosomal frame-shift event [6,7]. As Gag has been shown to be sufficient to drive in vivo self-assembly into VLPs, Pol is dispensable for viral coat assembly [8]. However, N-acetylation of Gag (catalyzed by Mak3p of the host cell) is an essential prerequisite for VLP formation in vivo [9]. The 40 nm L-A capsid has a 120-subunit structure composed of 118 Gag proteins and two copies of Gag/Pol configured as an icosahedron of triangulation class T = 1 [7,10–12]. In the present study, we used Gag - and specifically designed variants thereof - for the in vivo assembly of VLP chimeras suitable for heterologous protein production and display of vaccine-relevant immunogens.

RESULTS
Chimeric Gag assembles into yeast VLPs
Since in the natural L-A virus, Pol (as C-terminal part of Gag/Pol) extends into the interior of the capsid to ensure replication and transcription of the viral genome [11], we replaced Pol by a truncated version of the immunodominant phosphoprotein pp65 from human cytomegalovirus (HCMV) to modify the inner surface of the capsid. The truncated protein (ppp65) comprised the C-terminal amino acids 358-561 of pp65 flanked by the CD8α-subunit, and a particle-associated and fully recyclable biotechnologically relevant enzyme (esterase A). Thus, yeast viral Gag represents a unique platform for the in vivo assembly of chimeric VLPs, equally attractive and useful in vaccine development and recombinant protein production.

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as well as non-modified (naked) Δpp65 (24.9 kDa) were separately expressed in yeast and analyzed for expression level and protein stability. In the Gag/Δpp65 protein fusion, Δpp65 is fused in the [0]-frame to the 3′-end of gag resulting in a protein fusion that is ought to self-assemble (via its Gag domain) into VLPs encapsulating Δpp65 as C-terminal cargo (Figure 1A). Western analysis of cell extracts from yeast expressing either naked Δpp65 or Gag/Δpp65 revealed only a weak signal for non-fused Δpp65 in contrast to an intense signal seen in cells expressing Gag/Δpp65 (Figure 1B). The observed instability of the naturally short-lived Δpp65 protein in the multiple protease-deficient mutant strain s086c could not even be prevented in mutant hosts defective in behaviour as natural L-A virions (Figure 2A and 2B).

DST (Figure 1B). The competence of Gag/Δpp65 expressed in a particulate manner as C-terminal protein fusion and effectively protected from proteolytic degradation when expressed in a particulate manner as C-terminal protein fusion to Gag (Figure 1B). The competence of Gag/Δpp65 for in vivo self-assembly into hybrid VLPs was demonstrated by analyzing its sedimentation profile during sucrose gradient centrifugation and by electron microscopy of gradient-purified VLPs: Gag/Δpp65 formed isometric particles which showed a similar sedimentation behaviour as natural L-A virions (Figure 2A and 2B).

**Gag/Δpp65 activates HCMV-specific CD8⁺ memory T-cells ex vivo**

Antigenicity of recombinant Gag/Δpp65 particles was determined in an ex vivo stimulation assay allowing quantification of HCMV-specific memory T cell responses in human whole blood by FACS analysis [14,15]. For such an assay, both VLPs (Gag and Gag/Δpp65) were expressed and assembled in yeast and partially purified by centrifugation through a sucrose cushion (Figure 3A). T cell stimulation assays performed on whole blood of a HCMV seropositive donor indicated that pp65-specific CD4⁺ and CD8⁺ T-lymphocytes were strongly activated to maximal frequencies of 2.64% and 0.22% by HCMV positive control antigens, while no immune response was seen in the negative control. Interestingly, cushion-purified Gag/Δpp65 as well as non-modified Gag only poorly activated CD4⁺ cells (<0.1%, Figure 3B), while chimeric Gag/Δpp65 particles induced a pronounced CD8⁺ T-cell response in a dose-dependent manner that was even higher than in the positive control (0.35% versus 0.22%; Figure 3C). In contrast to Gag/Δpp65, unmodified Gag did not significantly activate HCMV-specific CD8⁺ cells, not even at the highest concentration tested (Figure 3C). To demonstrate that the observed CD8⁺ T cell response was caused by the Δpp65 moiety of the chimeric particles, recombinant VLPs were isolated from yeast, purified by sucrose gradient centrifugation and subsequently analyzed by SDS-PAGE and Coomassie-Blue staining. As shown in Figure 4A, gradient-purified VLPs only contained two protein species representing Gag and Gag/Δpp65, thereby demonstrating that both preparations were of high purity (>95%). For T cell stimulation, whole blood of HCMV-seropositive donors was supplemented with 5 μg gradient-purified VLPs; a lysate from HCMV-infected fibroblasts served as positive control, a HCMV-seronegative blood sample as negative control to demonstrate antigen specificity of the immune response. As expected, no T cell response was detectable in the negative control, while a significant CD4⁺ T lymphocyte response was seen in seropositive samples against the positive control antigen (Figure 4B and 4C). In contrast to unmodified Gag, chimeric Gag/Δpp65-VLPs caused a significant activation of CD4⁺ T cells only in donor 2 (Figure 4B). As shown before for cushion-purified particles, gradient-purified Gag/Δpp65 showed significantly elevated frequencies of activated CD8⁺ T cells that were up to 25-fold increased over unmodified Gag (Figure 4C). Qualitatively the same result was obtained by analyzing chimeric VLPs in which the antigenic Δpp65 moiety was expressed on the outer VLP surface by in-frame insertion into surface-exposed loops of Gag immediately upstream of amino acid position S182 and flanked by flexible spacers (Powileit and Schmitt, unpublished). These data demonstrate that Gag/Δpp65 expressed and assembled into yeast VLPs exposing Δpp65 either inside the particle or at the outer VLP surface, both possess antigenic properties (in particular to activate CD8⁺ memory T cells) that are due to their HCMV-specific Δpp65 moiety. To further investigate the potential of Gag/Δpp65 particles as unique yeast vaccine, we are currently analyzing native Gag/Δpp65 particles in a murine HCMV model of HLA transgenic mice for the induction of a protecting in vivo immune response. That antigens exposed inside chimeric yeast VLPs are indeed efficiently processed by immune cells in vivo resulting in a humoral immune response was demonstrated by using native Gag/K28Δx VLPs (assembled in and isolated from a GTXx expressing yeast strain) which induced K28Δx-specific antibodies in rabbit (Figure 5A and

**Figure 1. Expression of a Gag/Δpp65 fusion protein in yeast.** (A) Schematic outline of Gag/Δpp65 before and after in vivo assembly into chimeric yeast VLPs. (B) SDS-PAGE and anti-pp65 immunoblot of crude extracts from yeast expressing either Δpp65 (lane 1), Gag (lane 2), or Gag/Δpp65 (lane 3). To ensure in vivo translation initiation of N-terminally truncated Δpp65 (24.9 kDa), a methionine residue was added to the N-terminus of Δpp65 [M, prestained PAGE Ruler, Fermentas].

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5B). In addition, since the in vivo expression of the α-subunit of the viral α/β toxin K28 is known to be toxic (in particular when expressed in the ER lumen [16]), successful expression of Gag/K28 particles demonstrates that chimeric Gag-VLPs are also suitable for the expression of a per se lethal protein.

Chimeric VLPs as platform for protein expression and purification

In contrast to monomeric protein fusions, hybrid VLPs are of high molecular weight and can be easily prepared from crude cell extracts by ultracentrifugation [4]. In this context, we exploited Gag as particle-forming carrier to express and purify the green fluorescent protein GFP as model polypeptide. For this purpose, a Gag variant was constructed encoding a 105 kDa protein fusion containing Gag at its N-terminus (to ensure in vivo VLP assembly and GFP encapsulation), followed by an 11 amino acid T7 epitope tag (for immunological detection) and a factor Xa cleavage site to release mature GFP from Gag (Figure 6A). Yeast transformants expressing such a construct (GTXG) were used for VLP preparation, and western analysis of sucrose gradient fractions revealed that GTXG (105 kDa) assembled into VLPs that showed...
Figure 3. Gag/Δpp65 expressed in yeast assembles into VLP chimeras strongly activating CD8+ memory T cells in human whole blood. (A) Western blot of Gag/Δpp65 (lane 1) and Gag (lane 2) expressed in yeast and partially purified as sucrose cushion pellet after ultracentrifugation. Aliquots (20 μl each) of the indicated VLP preparation were subjected to SDS-PAGE followed by Coomassie-Blue staining and western analysis probed with anti-Gag and/or anti-pp65 [M, full range rainbow marker, Amersham]. (B) Frequencies of antigen-specific CD4 and (C) CD8 T cell activation after stimulation by sucrose cushion-purified yeast Gag and Gag/Δpp65 particles. Activated T cells were identified as CD69/IFN-γ double-positive lymphocytes by flow cytometry. Antigen samples were added to whole blood from HCMV seropositive donor 1. A VLP-free sample containing PBSE buffer was included as negative control (NC), a lysate from HCMV-infected fibroblasts served as positive control (PC). The threshold of significant T cell responses (0.05% of counted lymphocytes [15]) is indicated as dashed line.

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Figure 4. Purified Gag/Δpp65 chimeras induce an extensive human CD8 T cell response. (A) SDS-PAGE and anti-pp65 immunoblot of sucrose gradient purified Gag and Gag/Δpp65 particles expressed and assembled in yeast [Coomassie-Blue staining and BSA (2.7 μg) were used for semi-quantitative signal detection; M, full range rainbow marker, Amersham]. (B and C) Whole blood cells from three HCMV seropositive donors were stimulated by the addition of either Gag or Gag/Δpp65 (5 μg each), and specifically activated CD4 (B) and CD8 (C) T cells were quantified as CD69/IFN-γ-double-positive lymphocytes by flow cytometry. A lysate from HCMV-infected fibroblasts served as positive control (PC), whereas a lysate from noninfected fibroblasts, a VLP-free buffer sample as well as blood cells from HCMV seronegative donor 5 were used as negative controls (NC). The threshold of significant T cell responses (0.05% of counted lymphocytes [15]) is indicated as dashed line.

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a sedimentation profile portraying that of natural yeast VLPs (Figure 6B). To check whether the protein fusion is accessible to factor Xa cleavage and subsequent release of its GFP moiety, GTXG-VLPs (288 μg protein/ml) were treated with Triton X-100, thereafter incubated with factor Xa and subsequently subjected to SDS-PAGE and western analysis. As shown in Figure 6C, detergent-treated GTXG-VLPs were efficiently processed by factor Xa, liberating two protein moieties from the GTXG precursor (both absent in negative controls) whose calculated molecular weights are consistent with the presence of monomeric (26.8 kDa) and dimeric (53.6 kDa) GFP. In direct support, dimeric GFP was only seen in non-reducing SDS-PAGE and completely disappeared under reducing conditions in the presence of β-mercaptoethanol (data not shown). To investigate the efficiency of preparative GFP purification, detergent-treated GTXG particles (274 μg) were incubated in the presence of factor Xa, VLP debris was removed by high-spin centrifugation (100,000 x g) and the resulting supernatant was treated with Xarrest agarose to eliminate residual endopeptidase. Soluble GFP released from the VLPs was precipitated by the addition of ammonium sulfate, and the resulting pellet and supernatant fraction was subsequently analyzed by SDS-PAGE probed with anti-GFP and anti-T7. By this procedure, monomeric GFP (26.8 kDa) and the larger GTX cleavage fragment (77.8 kDa) were successfully released from the 105 kDa GTXG precursor (Figure 6D). Furthermore, GFP could be purified in a single step by hydrophobic interaction chromatography (HIC) as judged by SDS-PAGE and western analysis (Figure 6D). GFP containing fractions from the HIC column were 100-fold concentrated by ultrafiltration, and the pooled HIC fractions as well as the retentate after ultrafiltration were analyzed by SDS-PAGE and Coomassie-Blue staining. As expected, GFP concentration in the retentate was higher than in the pooled HIC fractions or in the filtrate (Figure 6E). Moreover, the preparation was highly pure, only showing two GFP-specific signals on non-reducing SDS gels, a major 26.8 kDa protein representing monomeric GFP and a minor 33.6 kDa species representing dimeric GFP (Figure 6E). Based on the signal intensity after Coomassie-Blue staining, the overall yield of GTXG-derived GFP after sucrose gradient centrifugation and HIC purification was in the range of 0.2 mg purified protein from 1 liter yeast culture (and a density of 5 x 10^10 cells/ml).

VLP chimeras as recyclable biocatalyst
To demonstrate the flexibility of the viral carrier for the expression of a biotechnologically relevant enzyme, the GFP moiety in GTXG was replaced by the carboxylesterase EstA from Burkholderia gladioli [17], the resulting Gag/EstA protein fusion was expressed in yeast and electron microscopy of sucrose gradient-purified Gag/EstA particles confirmed in vivo assembly into recombinant VLPs (Figure 7A). To demonstrate esterase activity in the VLP chimeras, gradient-purified Gag/EstA particles were analyzed in an enzyme activity assay using 4-nitrophenylacetate as substrate. Since EstA is located inside the particle, the substrate must pass the capsid pores to be converted into acetate and 4-nitrophenol. Under the assay conditions used, the release of 4-nitrophenol was monitored through its absorption at 405 nm that was shown to be linearly correlated to a concentration of up to 1 mM (regression coefficient = 0.9974; Figure 7B). Based on these parameters, gradient-purified Gag/EstA (70 ng and 280 ng) and non-modified Gag (560 ng) were subsequently analyzed for esterase activity; a VLP-free sample served as negative control to detect autohydrolysis and unspecific breakdown of the ester substrate. In contrast to unmodified VLPs, Gag/EstA chimeras catalyzed the release of 4-nitrophenol and under steady state conditions (reaching reaction equilibrium within 48 min), 67.9% of the initial substrate were enzymatically converted into 4-nitrophenol (Figure 7C).

Gag/EstA particles allow multiple substrate conversions
To investigate whether particle-associated esterase can be recycled and reused in multiple rounds of substrate conversion, gradient-purified Gag/EstA particles (22.5 μg) were used in an enzyme reaction (5 ml), isolated by ultracentrifugation, subsequently subjected to SDS-PAGE and western analysis, and compared to the same VLP charge prior to substrate conversion. Based on the esterase signal intensity obtained after SDS-PAGE and Coomassie-Blue staining, approximately one-third of the original particle preparation had been recovered in the pellet fraction after a single ultracentrifugation step (Figure 7D). Since Gag/EstA particles were significantly diluted prior to ultracentrifugation, esterase protein remaining in the final supernatant was not detectable in immunoblots. Most interestingly however, catalytic activity of Gag/EstA-VLPs after recovery was not negatively affected and rather resembled EstA activity in the original non-recycled VLPs. Using an equal volume of Gag/EstA particles before and after recycling, absolute esterase activity was three-fold lower in recycled VLPs; however, given that one-third of the initial VLP amount (750 ng) had been recycled, specific esterase activity in the Gag/EstA chimeras before and after recycling was almost identical (Figure 7E), demonstrating that VLP-associated EstA can be recycled and repeatedly used in multiple rounds of enzyme-catalyzed substrate conversion. Most interestingly however, the remarkable efficacy of the VLP-associated enzyme becomes evident when specific esterase activity of Gag/EstA particles (20.8 U mg⁻¹ protein) is compared to that after EstA cell surface expression in either yeast (S. cerevisiae) or bacteria (E. coli): in both cases, esterase activity was significantly lower and ranged from 1.3 to 2.7 U mg⁻¹ protein in yeast [18] and 0.001 to 0.023 U mg⁻¹ protein in bacteria [19].

DISCUSSION
Viral expression systems are not only useful in gene transfer experiments, but also in heterologous protein production. In most cases, structural or regulatory elements of animal and human viruses represent the key elements in these systems, restricting their
Figure 6. GFP expression and purification via recombinant yeast VLPs. (A) schematic outline of a Gag/GFP fusion (GTXG) for *in vivo* VLP assembly and purification of the model protein GFP. The particular function of each domain within the protein fusion is indicated. (B) SDS-PAGE and anti-GFP western analysis of recombinant GTXG particles assembled in yeast and purified by sucrose gradient centrifugation. (C) Release of GFP from GTXG particles by factor Xa cleavage [rGFP, recombinant GFP; M, full range rainbow marker, Amersham]. (D) Single-step purification of GFP obtained after factor Xa treatment and ammonium sulfate (AS) precipitation (S, supernatant; P, pellet) by hydrophobic interaction chromatography on a HIC column. Samples were separated by SDS-PAGE and probed with anti-GFP and/or anti-T7. (E) Coomassie-Blue staining of pooled GFP-containing HIC fractions after Amicon ultrafiltration through a 10 kDa cut-off membrane [rGFP, recombinant GFP; M, full range rainbow marker, Amersham].

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Figure 7. Chimeric yeast VLPs expressing bacterial esterase (EstA) function as recyclable bioreactor and show efficient substrate conversion. (A) Electron micrograph of recombinant Gag/EstA particles prepared from yeast were purified by sucrose gradient centrifugation, negatively stained with uranyl acetate/methyl cellulose and subsequently used for electron microscopy (magnification 340,000). (B) Linear correlation between the 4-nitrophenol concentration of up to 1 mM and its absorption at 405 nm. (C) Kinetics of Gag/EstA-driven hydrolysis of 4-nitrophenylacetate (280 μM) to 4-nitrophenol and acetate at 25°C in PBS50 buffer (pH 7.0). (D) Coomassie-Blue staining and western analysis of Gag/EstA particles before and after catalysis and recycling by ultracentrifugation. BSA (1 and 3 μg) was used as loading control [M, full range rainbow marker, Amersham]. (E) Specific activity of chimeric Gag/EstA particles before and after recycling.

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application to higher eukaryotic cells as host. In the present study we engineered the yeast toivirus L-A and demonstrated its feasibility for being used as unique expression system in a lower eukaryotic host. The potential of its capsid as platform for the presentation of immunogens was demonstrated by using the HCMV tegument protein pp65 as model antigen. This structural protein represents the major target of cellular immune response during HCMV infection [20,21], and also in vitro HCMV-infected cells are recognized by 70–90% of cytotoxic T lymphocytes (CTLs) [22]. Besides inducing a strong CD8+ T cell response, pp65 can also activate CD4+ T cells [23] making it an ideal candidate in developing an HCMV vaccine ensuring both, humoral and cellular immunity.

To analyze recombinant yeast VLPs for their potential as non-replicating particle vaccine, we fused an N-terminally truncated pp65 variant (App65) of HCMV containing immunodominant T cell epitopes to the C-terminus of Gag and showed that it self-assembled into VLP chimeras when expressed in the yeast cell cytosol. Electron microscopy revealed a spherical symmetry of the recombinant particles (Gag/App65) in which the App65 moiety was buried inside the capsid, analogous to Pol in the natural L-A virus [11]. The “in vivo” localization of App65 either inside or outside the particle was judged in two ways: (i) by immunogold labelling and electron microscopy, and (ii) by analyzing cosedimentation profiles of Gag/App65 and monoclonal anti-pp65 in a sucrose density gradient (Powilleit and Schmitt, unpublished). In contrast to non-modified (naked) App65 which was only weakly expressed and subject to proteolytic degradation in vivo, particle-associated App65 was highly stable and effectively protected against the action of host cell proteases. In an ex vivo stimulation assay in which memory T cell stimulation can be quantified and characterized in human whole blood [14], purified Gag/App65 particles - in contrast to non-modified Gag - resulted in a significant activation of CD8+ T lymphocytes, while frequencies of activated CD4+ helper T lymphocytes (HTLs) were generally low. The same holds true for chimeric VLPs in which the App65 moiety was exposed at the outer VLP surface by insertion into surface-exposed loops of Gag immediately N-terminal to position S182 and flanked by flexible spacer elements (Powilleit and Schmitt, unpublished). In all cases the observed bias in activation of CD8+ T lymphocytes by exogenous antigen points to an alternative presentation pathway favouring association of pp65 epitopes with MHC I. In the classical pathway of antigen presentation, peptides derived from exogenous proteins or particles are exposed in complex with MHC II molecules on the cell surface where interaction with complementary T cell receptors leads to an activation of CD4+ HTLs. This mechanism is apparently true for the positive control antigen used in this study, a lysate of HCMV-infected fibroblasts containing both soluble as well as virion-associated pp65. In one out of four blood samples, Gag/App65 also induced a CD4+ T cell response, indicating that App65 peptides can also be presented in complex with MHC II. Due to the N-terminal truncation in App65, HTL epitopes in pp65 such as peptides 11, 71 and/or 72 are lacking [23], probably attenuating its ability to induce a more frequent CD4+ T cell response.

In contrast to MHC II, MHC I-associated presentation of peptides is considered to be restricted to endogenously synthesized proteins, initiating with proteasomal processing in the cytoplasm. Upon targeting on the cell surface, the MHC I/peptide complex can activate CD8+ T cells through interaction with the corresponding T cell receptor [24]. More recent studies have indicated that proteins taken up by phagocytosis can also be presented by MHC I molecules, thereby promoting CD8+ CTL proliferation [25]. Such alternative antigen presentation (also known as cross-presentation) has been observed in phagocytic cells upon engulfment of bacterial cells or viral particles [26–30]. Since recombinant Gag/App65-VLPs share the particulate nature with these antigens, a cross-presentation pathway might also exists for them as well as for pp65 associated to intact HCMV virions (present in the positive control).

Current strategies in HCMV vaccine development imply the application of live, attenuated virus strains, DNA vectors coding for immunodominant HCMV proteins and/or genetically modified carrier viruses [31]. Although these approaches might be well-tolerated and immunogenic, they bear the risk of reverting to original virulence, inducing anti-DNA antibodies or recombining with the host cell genome [32,33]. In addition, the production of most of these vaccines in human cell lines is costly, time-consuming and hardly suitable to an industrial scale-up [34]. In contrast, yeast is regarded as safe due to its GRAS status and widely accepted as a profitable host to produce biotechnologically and pharmaceutically relevant proteins [35]. As proof of principle for a yeast vaccine based on recombinant VLP chimeras, we intend to use Gag/pp65 particles and HLA transgenic mice in a murine HCMV model to analyze in vivo immune responses and to evaluate vaccine potential of chimeric yeast VLPs.

Besides being attractive in vaccine development, the yeast viral expression system described here is also interesting in foreign protein production. This was demonstrated for a gene fusion in which the 3’-end of gag was sequentially extended by a T7 epitope, a factor Xc cleavage site, and the coding sequence of GFP as model protein. After in vivo expression, the protein fusion self-assembled into hybrid yeast VLPs from which GFP could be entirely released from its Gag carrier by factor Xc cleavage. Single-step purification via hydrophobic-interaction chromatography and subsequent ultrafiltration resulted in a highly pure GFP preparation with an overall yield of 0.2 mg purified and biologically active GFP from 1 liter yeast culture (and a density of 5×10^6 cells/ml). The overall yield in heterologous GFP production via chimeric yeast VLP expression falls within the broad-range levels of GFP fusion protein production which has been shown to range over 3 orders of magnitude, from 4 µg/liter to 4 mg/liter yeast cell culture [36,37]. Furthermore and in contrast to expression systems based on yeast Ty retrotransposons exposing foreign proteins at the outer VLP surface [38,39], recombinant Gag particles described here contain their cargo within the inner capsid, thereby effectively preventing proteolytic degradation. Especially for the production of short-lived and unstable proteins (such as pp65 from HCMV), the L-A-derived expression system might be superior as it efficiently protects its cargo from proteolytic degradation in the host cell cytosol. Furthermore, by using the α-subunit of K29 toxin - which is cytotoxic when expressed in yeast [16] - we could demonstrate that recombinant Gag-VLPs are also suitable for the in vivo expression of a protein which is per se toxic. In addition, Gag was also shown to be effective in the expression of a particle-associated and recyclable biotechnical enzyme, carboxylesterase A from B. globuli [18]. Gag/EstA protein fusions expressed in yeast assembled into VLP chimeras that were catalytically active and effectively converted 4-nitrophenylacetate into 4-nitrophenol and acetate. A hallmark of this VLP-based “bioreactor” is its reusability in multiple substrate conversions without loss in enzyme activity and its overall yield in particle-associated specific esterase activity, being significantly higher than esterase activity after cell surface display in E. coli or S. cerevisiae [18,19]. In sum, these data demonstrate the efficiency of the yeast L-A viral expression system in the production and purification of recombinant proteins/ enzymes in a particle-associated manner, providing substantial yields of a functional protein in sufficient quality without the need of time-consuming
purification procedures. In addition, the ease of fermentation in low-cost media makes S. cerevisiae and its chimeric Gag-VLPs attractive for foreign protein production.

**MATERIALS AND METHODS**

**Strains, oligonucleotides and plasmids**

*E. coli* strain TOP10 [F 'merA Ap'par-kad RMS-mecB C F080lacZAM15 
\[58x242]\] 42,769 galU galK gluR (Su') endA nagG (Invitrogen) used for plasmid propagation was grown at 37°C in Luria Broth supplemented with 100 μg/ml ampicillin. All plasmids and oligonucleotide primers used in this study are listed in supplementary Table S1 and Table S2, respectively. Target gene amplification was performed using High-Fidelity Tag polymerase (Roche) according to the manufacturer’s instructions. PCR products were subcloned into pCR®II-TOPO (Invitrogen) and checked by DNA sequencing using primers M13for and/or M13rev (5'-labeled with infra-red dye 800; MWG). For *App65* amplification, template JW4303 and primers 5’pp65epi+3’CMVepi were used. To obtain plasmid pPGK-App65, the *App65* fragment was inserted into pPGK via EcoRI/BamHI. The gag-ORF, amplified using pTIL05 [40] as template and primers 5’-L-A ORF1+3’-L-A ORF1, was cloned as *HindIII*/BamHI fragment into vector pPGK to give pG. To obtain expression plasmids pGAG/App65 and pGTXG, PCR reactions were carried out using the template/prime combinations JW4303/ 
\[58x285] 3’CMVepi and pUG36/5’T7Xa-GFP+3’GFP, respectively. Upon subcloning, both fragments were inserted as *SalI*/ 
\[58x285] BamHI fragment into pG. Plasmid yGTXG was constructed by introducing the *HindIII*/BamHI GTXG fragment from pGTXG into YEp352. The Xz gene (encoding the z-subunit of killer toxin K28) was ampliﬁed using vector pM28-SL [16] as template and primers 5’SpeXal//3’albaBgl. Subsequently, the 5’-terminal *SpeI* 
\[58x285] BamHI fragment was integrated into yGTXG (*SpeI*/BamHI) to give yGTXZa. GTXZa [Hin dIII/BamHI] was then inserted into pPGK, and the GTXz fusion was completed by inserting the 3’-terminal *BglII*/BamHI fragment of Xz into the BamHI digested vector pGTXZa. For amplification of the Ce fragment, template JW4303 and primers 5’pp65epi+3’CMVepi were used. To obtain plasmid pCe, the Ce fragment was inserted into pPGK via EcoRI/ 
\[58x285] BamHI. The multiple protease-deﬁcient S. cerevisiae strain S86c [MATa ura3-2 his3 can1-102 leu2-3,112 met15 0.6% 2-mercaptoethanol] and heated at 100°C was applied onto 7.5% SDS-polyacrylamide gels and run in Tris/Tricine buffer [43]. Upon separation, proteins were either stained with Coomassie Brilliant Blue R250 (Roti) or blotted onto polyvinyl diﬂuoride membranes [46]. Blots were probed with monoclonal anti-pp65 (Novocastra), anti-17 (Novagen), anti-GF (Roche) or polyclonal antibodies raised in rabbit against native Gag-VLPs (anti-Gag) or chimeric Gag/K292z particles (anti-Gag/K292z) followed by treatment with an alkaline phosphatase-coupled secondary anti-mouse immunoglobulin (Sigma). For colorimetric signal detection blots were covered with NBT/BCIP solution (Roche) according to the instructions of the manufacturer. Protein concentration was determined by using a bicinchoninic acid assay kit (Sigma). Alternatively, deﬁned amounts of bovine serum albumin (BSA; Sigma) served as standard for semi-quantitative determination of protein concentration after SDS-PAGE and Coomassie Blue staining.

**Rapid extraction (S80 method) and detection of intracellular proteins**

Yeast cells from a 1 ml overnight culture were collected by 15 min centrifugation at 500 xg (4°C) and washed in cold PBS/E. Thereafter, cells were resuspended in 10 ml PBS/E (150 mM NaCl, 10 mM Na2HPO4 pH 7.4, 10 mM EDTA) and disrupted by vortexing seven times for 1 min (with 1 min breaks in between to cool samples on ice) in the presence of 12 g glass beads (0.45–0.55 μm in diameter). The resulting raw extracts were supplemented with 10 ml PBS/E and centrifuged at 10,000 xg for 1 h (4°C) to sediment glass beads and cell debris. The supernatant was adjusted with PBS/E to 23 ml and then layered onto a cushion of 15 ml 45% sucrose. During ultracentrifugation at 69,260 xg overnight (4°C; Beckman SW28 rotor) only structures of high molecular weight pass the cushion and form a pellet. Subsequently, the cushion pellet was resuspended in 1 ml PBS/E and layered onto a linear density gradient (38 ml) of 20–70% sucrose. Upon further ultracentrifugation at 76,740 xg overnight (4°C) the gradient was fractionated into 18–20 fractions (each 2 ml) while the gradient pellet was resuspended in 2 ml PBS/E. Aliquots of each fraction were subjected to SDS-PAGE followed by western analysis or Coomassie blue staining. For reisolation of recombinant VLPs, a maximum of 12 fusion protein containing gradient fractions was pooled, supplemented with PBS/E to 38 ml, and again ultracentrifuged at 76,740 xg overnight (4°C). Finally, the VLP pellet was resuspended in 100–500 μl PBS/E. The procedure described above was also used to prepare natural L-A particles from yeast strain BY4741 starting from a 200 mlYPD culture grown to a density of 5 x 10^6 cells/ml.

**VLP preparation**

Transformants of the indicated yeast strain were incubated in 400 ml Ura-d/o at 220 rpm (30°C, in a 1 L-Erdemeyer flask) to a density of 5 x 10^8–5 x 10^9 cells/ml, harvested by 15 min centrifugation at 5,000 xg (4°C), washed in prechilled H2O, thereafter in 1 M sorbitol, and finally resuspended in 50 ml cold PBS/E (150 mM NaCl, 10 mM Na2HPO4 pH 7.4, 10 mM EDTA, 1 M sorbitol). Subsequently, 2-mercaptoethanol (1:2,000) and 2.5 mg zymolase 20T (Seikagaku, Japan) were added. Upon 1.5 h incubation at 120 rpm (30°C), spheroplasts were collected by 15 min centrifugation at 500 xg (4°C) and washed in cold PBS/E. Thereafter, cells were resuspended in 10 ml PBS/E (150 mM NaCl, 10 mM Na2HPO4 pH 7.4, 10 mM EDTA) and disrupted by vortexing seven times for 1 min (with 1 min breaks in between to cool samples on ice) in the presence of 12 g glass beads (0.45–0.55 μm in diameter). The resulting raw extracts were supplemented with 10 ml PBS/E and centrifuged at 10,000 xg for 1 h (4°C) to sediment glass beads and cell debris. The supernatant was adjusted with PBS/E to 23 ml and then layered onto a cushion of 15 ml 45% sucrose. During ultracentrifugation at 69,260 xg overnight (4°C; Beckman SW28 rotor) only structures of high molecular weight pass the cushion and form a pellet. Subsequently, the cushion pellet was resuspended in 1 ml PBS/E and layered onto a linear density gradient (38 ml) of 20–70% sucrose. Upon further ultracentrifugation at 76,740 xg overnight (4°C) the gradient was fractionated into 18–20 fractions (each 2 ml) while the gradient pellet was resuspended in 2 ml PBS/E. Aliquots of each fraction were subjected to SDS-PAGE followed by western analysis or Coomassie blue staining. For reisolation of recombinant VLPs, a maximum of 12 fusion protein containing gradient fractions was pooled, supplemented with PBS/E to 38 ml, and again ultracentrifuged at 76,740 xg overnight (4°C). Finally, the VLP pellet was resuspended in 100–500 μl PBS/E. The procedure described above was also used to prepare natural L-A particles from yeast strain BY4741 starting from a 200 mlYPD culture grown to a density of 5 x 10^6 cells/ml.

**Transmission electron microscopy**

An aliquot of gradient-puriﬁed VLPs was layered onto a copper grid (mesh 300–400; coated with poly-L-lysine) and allowed to
bind for 5 min at room temperature. Upon washing three times with 30 μl TBS (150 mM NaCl, 100 mM Tris/HCl pH 7.5) the grid was incubated in uranyl acetate/methyl cellulose (1.8%/0.2%) for 5 min at room temperature (negative staining) before it was slowly dried. For analysis and documentation of VLP samples, a transmission electron microscope type TECNAI G2 (FEI) equipped with a MegaView III camera (Olympus) was used.

Protein processing using factor Xa and hydrophobic interaction chromatography (HIC)

To release the GFP moiety from chimeric particles, GTXG-VLPs were prepared from gradient fractions and resuspended in 0.9 ml H2O. Upon addition of Triton X-100 (1% final concentration) the sample was rotated overnight at 20°C. Digestion was carried out using 55 μl factor Xa/μg GTXG protein in a total volume of 4.5 ml at 20°C under rotation for 4 h. To remove residual intact capsid and factor Xa, the sample was ultracentrifuged at 102,000 × g (4°C) for 1 h, and the supernatant was treated with Xarrest agarose (Novagen) according to the manufacturer’s instructions. The resulting sample (10 ml) was carefully supplemented with 830 mM ammonium sulfate and subsequently centrifuged at 12,400 × g (4°C) for 1 h. The pellet was resuspended in 10 ml H2O while 2.5 ml aliquots of the supernatant were applied onto a column of Phenyl Superose HR5/5 (2 ml; Amersham Pharmacia) equilibrated in HIC buffer (pH 7.4) containing 830 mM ammonium sulfate and 100 mM KH2PO4. The column was washed with the same buffer and bound proteins were eluted in a linear gradient (15 ml) from 850 to 51 mM ammonium sulfate/100 mM KH2PO4 (pH 7.4). The column was run at 0.5 ml/min, and 1 ml fractions were collected and analyzed by SDS-PAGE.

Microtiter activity assay

Catalytic activity in esterase-coupled VLPs was determined in microtiter plates (96 flat-bottom wells; Nunc) using an MF reader V2.9-0 (EMS). A stock solution of 80 mM 4-nitrophenylacetate (dissolved in dimethyl sulfoxide) was diluted to the indicated concentrations. Aliquots of the supernatant were applied onto a column of Phenyl Superose HR5/5 (2 ml; Amersham Pharmacia) equilibrated in PBS50 buffer (150 mM NaCl, 50 mM Na2HPO4, pH 7.0), finally completed with 5 mM NaCl and 50 mM Na2HPO4, pH 7.0, and bound proteins were eluted in a linear gradient (15 ml) from 850 to 51 mM ammonium sulfate/100 mM KH2PO4 (pH 7.4). The column was run at 0.5 ml/min, and 1 ml fractions were collected and analyzed by SDS-PAGE.

REFERENCES

1. Valenzuela P, Medina A, Rutter WJ, Ammerer G, Hall BD (1982) Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature 298: 347–350.
2. Kornhauser R, Booy F, Cheng N, Lowey DR, Schiller JT (1992) Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc Natl Acad Sci USA 89: 12180–12184.
3. Kost TA, Goudreay JP (1999) Recombinant baculoviruses as expression vectors for insect and mammalian cells. Curr Opin Biotechnol 10: 428–433.
4. Kruger DH, Ulrich R, Gerlich WH (1999) Chimeric virus-like particles as vaccines. Biol Chem 380: 275–276.
5. Ulmer JB, Valley U, Rappuoli R (2006) Vaccine manufacturing: challenges and solutions. Nat Biotechnol 24: 1377–1383.
6. Wickner RB (1996) Double-stranded RNA viruses of eukaryotic cells. Curr Opin Biotechnol 10: 428–433.
7. Dinman JD, Icho T, Wickner RB (1991) A mechanism of RNA virus replication is studied using the Cellquest software. DOC
8. Fujimura T, Ribas JC, Makhot AM, Wickner RB (1992) Pol of gag-pol fusion protein required for encapsidation of viral RNA of yeast L-A virus. Nature 359: 746–749.
9. Tercero JC, Wickner RB (1992) MAK3 encodes an N-acetyltransferase whose modification of the L-A gag NH2 terminus is necessary for virus particle assembly. J Biol Chem 267: 2027–2033.
10. Cheng RH, Caston JR, Wang GJ, Gu F, et al. (1994) Fungal virus capsids, cytoplasmic compartments for the replication of double-stranded RNA, formed as isosahedral shells of asymmetric Gag dimers. J Mol Biol 244: 253–258.
11. Naitow H, Tang J, Canady M, Wickner RB, Johnson JE (2002) L-A virus at 3.4 A resolution reveals particle architecture and RNA decapping mechanism. Nat Struct Biol 9: 725–728.
12. Reinisch KM (2002) The dsRNA viridae and their catalytic capsids. Nat Struct Biol 9: 714–716.
13. Solomon AI, Morgan CL, Dodi AI, Morte C, Scott I, et al. (1999) Identification of three HLA-A*0201-restricted cytotoxic T cell epitopes in the cytomegalovirus protein pp65 that are conserved between eight strains of the virus. J Immunol 163: 5512–5518.
14. Breining F, Heintel T, Schumacher A, Meyerhans A, Schmitt MJ (2003) Specific activation of GMV-primed human T lymphocytes by cytomegalovirus pp65 expressed in fusion yeast. FEMS Immunol Med Microbiol 38: 231–239.
15. Breinig F, Sester M, Sester U, Meyerhans A (2006) Antigen-specific T cell responses: determination of their frequencies, homing properties, and effector functions in human whole blood. Methods 38: 77–83.

16. Heiligenstein S, Eifeld K, Sendzik T, Jimenez-Becker N, Breinig F, Schmitt MJ (2006) Retrotranslocation of a viral A/B toxin from the yeast endoplasmic reticulum is independent of ubiquitination and ERAD. EMBO J 25: 4717–4727.

17. Schlacher A, Stanzer T, Osprian I, Mischitz M, Klingsbichel E, et al. (1998) Detection of a new enzyme for stereoselective hydrolysis of linalyl acetate using simple plate assays for the characterization of cloned esterases from Botrytis cinerea. J Biotechnol 62: 47–54.

18. Breinig F, Diehl B, Rau S, Zimmer C, Schwab H, Schmitt MJ (2006) Cell surface expression of bacterial esterase A by Saccharomyces cerevisiae and its enhancement by constitutive activation of the cellular unfolded protein response. Appl Environ Microbiol 72: 7140–7147.

19. Schultheiss E, Paar C, Schwab H, Jose J (2002) Functional esterase surface display by the autoexporter pathway in Escherichia coli. Mol Catal B Enzym 18: 89–97.

20. McLaughlin-Taylor E, Pande H, Forman SJ, Tanamachi B, Li CR, et al. (1994) Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. J Med Virol 43: 103–110.

21. Gyulas Z, Endrezs V, Burian K, Pincus S, Toldy J, et al. (2000) Cytotoxic T lymphocyte (CTL) responses to human cytomegalovirus pp65, IE1-Exon1, gB, pp150, and pp28 in healthy individuals: reevaluation of prevalence of IE1-specific CTLs. J Infect Dis 181: 1537–1546.

22. Wills MR, Carmichael AJ, Mynard K, Jin X, Weeks MP, et al. (1996) The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. J Virol 70: 7569–7579.

23. Kern F, Bunde T, Faulhaber N, Kiecker F, Khatamzam E, et al. (2002) Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. J Infect Dis 185: 1709–1716.

24. Van Kaer L (2002) Major histocompatibility complex class I-restricted antigen processing and presentation. Tissue Antigens 60: 1–9.

25. Ackermann AL, Crosswell P (2004) Cellular mechanisms governing cross-presentation of exogenous antigens. Nat Immunol 5: 678–684.

26. Pfleger JD, Wick MJ, Roberts RL, Findlay K, Normark SJ, Harding CV (1993) Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. Nature 361: 359–362.

27. Schirrmbeck R, Melber K, Reimann J (1995) Hepatitis B virus small surface antigen particles are processed in a novel endosomal pathway for major histocompatibility complex class I-restricted epitope presentation. Eur J Immunol 25: 1063–1070.

28. Kovacovic-Bankowski M, Rock KL (1995) A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science 267: 243–246.

29. Moron VG, Rueda P, Sedlik C, Leclerc C (2003) In vivo, dendritic cells can cross-present virus-like particles using an endosome-to-cytosol pathway. J Immunol 171: 2242–2250.

30. Tsunetsugu-Yokota Y, Morikawa Y, Isopai M, Kawana-Tachikawa A, Odawara T, et al. (2003) Yeast-derived human immunodeficiency virus type 1 p55(gag) virus-like particles activate dendritic cells (DCs) and induce perforin expression in Gag-specific CD8+ T cells by cross-presentation of DCs. J Virol 77: 10250–10259.

31. Schleis M (2005) Progress in cytomegalovirus vaccine development. Herpes 12: 66–75.

32. Rabońowicz NR, McMunes P, Klein DL, Hall BF (1994) Vaccine technologies: view to the future. Science 265: 1491–1494.

33. Hansson M, Nygren PA, Stahl S (2000) Design and production of recombinant subunit vaccines. Biotechnol Appl Biochem 32: 95–107.

34. Kari M, Paff H (2001) Expression systems for production of heterologous proteins. Curr Sci 80: 1121–1128.

35. Hensing MC, Rouwenhorst RJ, Heijnen JJ, van Dijken JP, Pronk JT (1995) Physiological and technological aspects of large-scale heterologous-protein production with yeasts. Antonie Van Leeuwenhoek 67: 261–279.

36. Chang HC, Kaiser CM, Hartl FU, Barral JM (2005) De novo folding of GFP fusion proteins: high efficiency in eukaryotes but not in bacteria. J Mol Biol 353: 397–409.

37. Huang D, Shusta EV (2006) Yeast platform for the production of single-chain antibody-green fluorescent protein fusions. Appl Environ Microbiol 72: 7748–7759.

38. Gilmore JE, Senior JM, Burns NR, Ensouf MP, Gull K, et al. (1989) A novel method for the purification of HIV-1 p24 protein from hybrid Ty virus-like particles (Ty-VLPs). AIDS 3: 717–723.

39. Burns NR, Gilmore JE, Kingsman SM, Kingsman AJ, Adams SE (1994) Production and purification of hybrid Ty-VLPs. Mol Biotechnol 1: 137–145.

40. Echo T, Wickner RB (1989) The double-stranded RNA genome of virus-L-A encodes its own putative RNA polymerase by fusing two open reading frames. J Biol Chem 264: 6716–6723.

41. Cooper A, Busey H (1989) Characterization of the yeast KEX1 gene product: a carboxypeptidase involved in processing secreted precursor proteins. Mol Cell Biol 9: 2706–2714.

42. Schiestl RH, Gietz RD (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr Genet 16: 339–346.

43. Widner WR, Wickner RB (1993) Evidence that the SKI antiviral system of Saccharomyces cerevisiae acts by blocking expression of viral mRNA. Mol Cell Biol 13: 4331–4341.

44. Brown JT, Bai X, Johnson AW (2000) The yeast antiviral proteins Sklp2p, Sklp3p, and Sklp exist as a complex in vivo. RNA 6: 449–457.

45. Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166: 368–379.

46. Ausubel FM (2005) Current protocols in molecular biology John Wiley & Sons, Inc.

47. Hill JE, Myers AM, Koerner TJ, Tzagoloff A (1986) Yeast/E. coli shuttle vectors with multiple unique restriction sites. Yeast 2: 163–167.

48. Kang YS, Kane J, Kurjan J, Stadel JM, Tipper DJ (1990) Effects of expression of a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. J Med Virol 31: 487–499.

49. Ausubel FM (2005) Current protocols in molecular biology John Wiley & Sons, Inc.