HIV-1 p55Gag Encoded in the Lysosome-associated Membrane Protein-1 as a DNA Plasmid Vaccine Chimera Is Highly Expressed, Traffics to the Major Histocompatibility Class II Compartment, and Elicits Enhanced Immune Responses*

Ernesto T. A. Marques, Jr., Priya Chikhlikar, Luciana Barros de Arruda‡, Ihid C. Leao§, Yang Lu, Justin Wong, Juei-Suei Chen, Barry Byrne&&, and J. Thomas August††

From the Department of Pharmacology and Molecular Sciences, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205

Several genetic vaccines encoding antigen chimeras containing the lysosome-associated membrane protein (LAMP) translocon, transmembrane, and cytoplasmic domain sequences have elicited strong mouse antigen-specific immune responses. The increased immune response is attributed to trafficking of the antigen chimera to the major histocompatibility class II (MHC II) compartment where LAMP is colocalized with MHC II. In this report, we describe a new form of an HIV-1 p55gag DNA vaccine, with the gag sequence incorporated into the complete LAMP cDNA sequence. Gag encoded with the translocon, transmembrane and cytoplasmic lysosomal membrane targeting sequences of LAMP, without the luminal domain, was poorly expressed, did not traffic to lysosomes or MHC II compartments of transfected cells, and elicited a limited immune response from DNA immunized mice. In contrast, addition of the LAMP luminal domain sequence to the construct resulted in a high level of expression of the LAMP/Gag protein chimera in transfected cells that was further increased by including the inverted terminal repeat sequences of the adeno-associated virus to the plasmid vector. This LAMP/Gag chimera with the complete LAMP protein colocalized with endogenous MHC II of transfected cells and elicited strong cellular and humoral immune responses of immunized mice as compared with the response to DNA-encoding native Gag, with a 10-fold increase in CD4+ responses, a 4- to 5-fold increase in CD8+ T-cell responses, and antibody titers of >100,000. These results reveal novel roles of the LAMP luminal domain as a determinant of Gag protein expression, lysosomal trafficking, and possibly of the immune response to Gag.

MHC II-directed antigen activation of CD4+ T-cells is vital to the function of genetic vaccines as demonstrated in studies

* This work was supported in part by NIAID, National Institutes of Health Grants R37-AI41908 and R21-AI44317 (to J. T. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.
§ Supported by World Health Organization Grant TDR 960158.
&& Present address: University of Florida, 1600 S. West Archer Rd., Gainesville, FL 32610.
†† To whom correspondence should be addressed: Dept. of Pharmacology and Molecular Sciences, The Johns Hopkins School of Medicine, 725 N. Wolfe St., Biophysics Rm. 309, Baltimore, MD 21205, Tel.: 410-955-8484; Fax: 410-955-1894; E-mail: taugust@jhmi.edu.
1 The abbreviations used are: MHC, major histocompatibility complex; MIIIC, MHC class II-enriched compartment; LAMP, lysosome-associated membrane protein; APC, antigen-presenting cell; RRE, Rev-responsive elements; HPRT, hypoxanthine-guanine phosphoribosyltransferase; HIV-1, human immunodeficiency virus, type 1; CTL, cytotoxic T lymphocyte; INS, inhibitory sequence; AAV-ITR, adeno-associated virus inverted terminal repeat; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; IL-1, interleukin-1; ELISA, enzyme-linked immunosorbent assay; IFN, interferon.
plasmids were constructed using nucleotides 1–1503 of the HIV-1 HXB2 p55gag gene (GenBank™ ac-
9eXho
I and
38
9
9
l
5
MHC II

n
plasmid 5
9
LAMP/gag

yellow site of the viral Rev protein to Rev-responsive elements (RRE) for

LAMP chimera proteins as described above and then with anti-

1503

9

5

LAMP/gag

s

3

1

9

2

1

HIV-1 gag DNA vaccine, because it is relatively conserved among diverse HIV strains and subtypes, and broad cross-clade anti-Gag cytotoxic T lymphocytes (CTLs) responses have been demonstrated in HIV-infected patients (43–46). Moreover, several studies relate both Gag-specific CD4+ and CD8+ responses to the control of viremia following infection (47–55). Application of native gag as a DNA vaccine is limited by a strong dependence on binding of the viral Rev protein to Rev-responsive elements (RRE) for the nuclear export and stability of Gag mRNA (56, 57). These inhibitory sequences (INS) have been identified, and their removal by silent-site mutations or humanization of codon usage has resulted in increased expression of Gag protein by DNA vectors in the absence of Rev (58–63). DNA vaccines encoding these modified Rev/RRE-independent forms of Gag have been reported to elicit Gag-specific immune responses of mice, both antibody and CTL (59, 61, 64).

In our development of a HIV-1 gag DNA vaccine, we have analyzed two forms of LAMP/gag chimeras as DNA vaccines. The initial construct corresponded to the LAMP/antigen chimera containing the LAMP translocon, transmembrane, and cytoplasmic domains, but lacking the luminal domain (Gag/lamp), that was previously successfully used with several DNA-encoded antigens. It was found, however, that the majority of the Gag/lamp chimera did not traffic to the lysosome or MHC II compartments of transfected cells, despite the presence of the LAMP cytoplasmic YQTI targeting sequence. Also, the Gag/lamp chimera did not elicit an appropriately enhanced immune response when injected into mice. These problems were overcome by including the LAMP luminal domain in the construct, thus placing Gag within the lumen of the complete LAMP molecule, proximal to the transmembrane domain (LAMP/Gag). In this case, the LAMP/Gag chimera protein trafficked to the MHC II compartment and application of this construct as a DNA vaccine resulted in enhanced immune responses by immunized mice, as shown herein, and in ongoing studies with non-human primates (data not shown). Additionally, there was a novel finding that this DNA elicited a high level expression of unmodified Gag protein by transfected cells.
HIV-1 LAMPgag Chimera DNA Vaccine

MHC II, by incubating the cells for 1 h with FITC-labeled goat anti-mouse I-A$^E$ (14-4-8) (BD Pharmingen) at a 1:75 dilution. The cells were then washed three times with PBS, and the coverslips were mounted onto glass slides using ProLong Antifade reagent (Molecular Probes, Eugene, OR). Confocal microscopy was performed using a Wallac confocal laser scanning microscope. Colocalization of the proteins with MHC II was thus determined by merging fluorophore images individually captured and digitally colored by use of Photoshop 5.0 (Adobe).

Antibody Responses of Vaccinated Mice—BALB/c mice (Charles Rivers, Wilmington, MA), 6–8 weeks old, were immunized intramuscularly with 50$\mu$I of the specified plasmid. The mice were immunized four times at 3-week intervals with the same protocol. Blood was collected by tail bleeding 9 days after the second immunization and at day 94 after fourth immunization. Blood was centrifuged with the serum, and the serum was collected and stored at 4 °C for immediate use. HIV gag lysate (ABD, Rockville, MD) was diluted in 0.1$\times$I sodium carbonate-bicarbonate buffer, pH 9.4 (Pierce) at a concentration of 5$\mu$I/ml, and 50$\mu$I of the solution was added to each well of a 96-well plate (Nunc, Roskilde, Denmark). After overnight incubation at 4 °C, the solution was removed and the plates were washed six times with PBS containing 0.05% Tween 20 wash buffer. The plates were incubated for 2 h at 37 °C with 200$\mu$I of blocking buffer (PBS with 0.05% Tween 20 and 5% fetal bovine serum) and then washed three times. Serum samples were prepared in eight (3-fold) serial dilutions in blocking buffer starting at 1:100. 10$\mu$I of each dilution was added to the blocked plate, and the plate was incubated overnight at 4 °C. For IgG detection, a monoclonal antibody was developed using goat anti-mouse anti-IgG horseradish peroxidase (Cappel, Durham, NC) at a 1:5000 dilution. For IgM and IgG$\alpha$, biotinylated anti-mouse anti-IgM and IgG$\alpha$, antibody (BD Pharmingen) diluted 1:500 was conjugated with avidin-horseradish peroxidase (BD Pharmingen) diluted 1000-fold, and 100$\mu$I of each antibody was added to each well for 2 h at 37 °C. Tetrameric antibody (BD Pharmingen) diluted 1:200 was added to each well, and the plates were washed once and blocked with anti-mouse CD16/CD32, Fc receptor antibody FITC-conjugated rat antibody (BD Pharmingen). The cells were washed three times, resuspended in culture medium, and diluted to 2 × 10$^5$ cells/ml. The target cells (1 × 10$^5$) were seeded onto 96-well plates with equal volumes of 2% Triton (maximum release), media alone (minimum release), or a suspension of splenocytes from mice immunized for Gag-specific CD8$^+$-mediated responses (effector cells) at different effector:target ratios, starting at 100:1, all in triplicates. After 4-h incubation at 37 °C, 50$\mu$I of supernatant was transferred to a LumaPlate (Packard, Meriden, CT), dried, and counted in a TOPCOUNT NXT scintillation counter (Packard). The percentage of specific lysis (percent killing) was calculated as: 100 × [(cpm released with effector) − (minimum release)]/(maximum release) − (minimum release). 

Tetramer Staining—Splenocytes from mice immunized for Gag-specific CD8$^+$-mediated responses were washed in FACS buffer at 4 °C and blocked with anti-mouse CD16/CD32, Fe$\gamma$II/III receptor antibody (BD Pharmingen) diluted 1:200. 100$\mu$I of blocking buffer (PBS with 0.05% Tween 20 and 5% fetal bovine serum) was added to each well for 10 min at 4 °C. The splenocytes (1 × 10$^6$ cells/well) were stained in duplicate with phycoerythrin-conjugated tetramer (H-2K$^d$/AMQMLKETI, NIAID, National Institutes of Health, Atlanta, GA) and the immunoglobulin isotype control rat IgG1 antibody (BD Pharmingen) at a dilution of 1:100 for 1 h at 4 °C. The cells were washed twice with FACS buffer and incubated with the secondary antibody FITC-conjugated rat anti-mouse CD8 (BD Pharmingen) at a dilution of 1:100 for 30 min at 4 °C. Finally, the cells were washed twice with FACS buffer. Analysis was done on a BD Biosciences FACS can with CellQuest software. A minimum of 200,000 events was analyzed, and results were expressed as a percent of total CD8$^+$ cells.

Intracellular IFN-γ Staining—Intracellular IFN-γ staining for Gag-specific CD8$^+$-mediated responses was performed using the Cytofix/Cytoperm Plus (with GolgiStop$^{TM}$) kit (BD Pharmingen). Briefly, GolgiStop$^{TM}$ containing monensin was added to the splenocytes harvested from the immunized mice for 2 h at 37 °C. After incubation, cells were washed twice with FACS buffer and non-specific binding was blocked by incubating cells with anti-FcRγ antibody (BD Pharmingen) at a concentration of 10$\mu$I/ml for 10 min at 4 °C. The splenocytes (1 × 10$^6$ cells/well) were stained in duplicate with FITC-conjugated rat anti-mouse CD8 (BD Pharmingen) at a dilution of 1:100 for 30 min at 4 °C. The cells were washed twice with FACS buffer and resuspended in 200$\mu$I of Cytofix/Cytoperm solution at 4 °C. The cells were then washed twice with Perm/Wash solution and stained with phycoerythrin-conjugated rat anti-mouse IFN-γ antibody or the immunoglobulin isotype control rat IgG1 antibody (BD Pharmingen) diluted 1:100. Analysis was done on a BD Biosciences FACS can with CellQuest software. A minimum of 200,000 events was analyzed, and the results were expressed as a percent of IFN-γ$^+$ cells on total CD8$^+$ cells.

Statistical Analyses—Unpaired t test analyses and all graphs were made using StatView 5.0 (SAS Institute Inc., Cary, NC).

RESULTS

LAMP Luminal Domain Enhances Expression of Native HIV-1 p55 Gag as a LAMP/Gag$_{p55}$ Protein Chimera—Several DNA plasmids encoding HIV-1 p55Gag or as LAMP protein chimeras were synthesized in both pcDNAs3.1 (Invitrogen) and AAV-ITR-modified (pITR) (65) vector backbones for studies of Gag protein expression and cellular trafficking in transfected cells.
cells (Fig. 1A). In a typical experiment to examine protein expression (Fig. 1B), COS cells transfected with the pCDNA3.1 or pITR plasmid constructs were analyzed by Western blotting and immunostaining with Gag-specific monoclonal antibodies. Because of cis-acting inhibitory sequences (56, 57), less Gag protein was present in the absence of HIV-1 Rev in cells transfected with the pCDNA3.1 plasmid expressing the native Gag (GagN) or native Gag modified to contain the LAMP translocon, transmembrane, and cytoplasmic sequences (SS/GagN/lamp) (lanes 1 and 2). Gag expression of transfected cells was increased with the mutated Gag lacking inhibitory sequences (GagNINS) (lane 3), as previously reported (61), and with GagNINS containing LAMP translocon, transmembrane, and cytoplasmic domain sequences (SS/GagNINS/lamp) (lane 4). The SS/GagNINS/lamp chimera migrated at the expected deducted molecular mass of ~60 kDa, indicating that the transmembrane and cytoplasmic domains were translated and remained attached to the p55Gag protein (lanes 4 and 7). A novel finding of these experiments was that cells transfected with the plasmid containing native Gag inserted into the luminal domain of the complete LAMP sequence (LAMP/GagN) (lane 5) expressed an increased amount of the ~200-kDa LAMP/GagN chimera protein, even with the unmodified native p55Gag DNA sequence. Additionally, LAMP/GagN protein expression was further enhanced in cells transfected with the pITR LAMP/GagN construct (lane 8) as compared with the pCDNA3.1 construct (lane 5), the pITR plasmid expression of native Gag (lane 6), or SS/GagNINS/lamp (lane 7).

The presence of the LAMP/GagN chimera in protein bands of >220 kDa (lane 8) is attributed to multimerization of Gag at high concentration, and the Gag-specific protein bands below 100 kDa are attributed to proteolysis. A major Gag-specific protein fragment, with a molecular mass approximate to that expected for Gag joined to the LAMP transmembrane and cytoplasmic domains, suggests specific proteolytic cleavage of the LAMP luminal domain from the LAMP/GagN chimera. As a control, there was no staining with anti-Gag monoclonal antibodies of extracts of cells transfected with the control vector encoding LAMP without the Gag insert. Ongoing studies of Gag protein expression by transfected cells, including 293 and 3T3 cells, and the use of both mouse and human LAMP constructs and other plasmid vectors, have consistently shown that LAMP mediated enhanced expression of native Gag and that the 5′ sequences of the LAMP luminal domain are critical to the expression of HIV-1 Gag protein (data not shown). Because of greater protein yield, the constructs in the pITR vector were selected for further studies of trafficking to the MHC II compartment and of the immune response of mice injected with the DNA.

**LAMP Luminal Domain Is Required for Gag Endosomal/Lysosomal Trafficking**—The cellular localizations of the LAMP/GagN chimera expressed in transfected DCEK-I-CAM.Hi7, a mouse fibroblast cell line engineered to express MHC class II I-Ek, ICAM-1, and B7 molecules (67), was examined by immunofluorescence microscopy, comparing the localization of GagN, endogenous LAMP-1 and LAMP-2, and MHC II labeled with monoclonal antibodies. In these cells, the endogenous LAMP-1 (Fig. 2A, panels a–c) and LAMP-2 (Fig. 2A, panels d–f) were extensively colocalized with MHC II. In general, the majority of vesicles containing LAMP-1 and LAMP-2 also contained MHC II; in contrast, a significant number of MHC II-containing vesicles lacked the LAMP molecules. Cells transfected with pITR plasmids encoding native Gag (GagN) without any specific trafficking signal showed Gag present throughout the cell in a diffuse distribution typical for cytoplasmic proteins in fibroblast cells (71) (Fig. 2B, panel a). A critical finding was that the majority of the Gag/lamp chimera molecules containing the translocon, transmembrane, and cytoplasmic sequences (SS/GagNINS/lamp), due to the presence of the YQTI lysosomal membrane targeting sequence at the carboxyl terminus of the cytoplasmic domain, were not colocalized with endogenous LAMP or MHC II of DCEK cells but were instead found as a vesicular/tubular component distributed in a manner similar to that seen in labeling of Golgi stacks (71) (Fig. 2B, panel b). Trafficking of Gag as a LAMP/GagN chimera to lysosomes was found to require the presence of the LAMP luminal domain in the construct (Fig. 2B, panel c). Confocal immunofluorescence microscopy confirmed these findings of colocalization of Gag with MHC II of the DCEK cells as a LAMP/GagN chimera (Fig. 2C, panels d–f), but not of the SS/GagNINS/lamp (Fig. 2C, panels a–c). Additional analysis of DCEK cells transfected with the LAMP/GagN chimera encoding the complete LAMP, with 10 serial sections of the confocal Z-plane, showed that Gag as the LAMP/GagN chimera (Fig. 2D, panels a1 and a2) and MHC II...
FIG. 2. LAMP/Gag$_{\text{N}}$ colocalize with MHC II in mouse antigen presenting cells. A, colocalization of LAMP-1 and LAMP-2 with MHC II molecules in mouse DCEK cells. Proteins and antibodies are indicated in each panel: a, immunofluorescence microscopy of mouse DCEK cells stained with anti-LAMP 1 monoclonal antibody in red; b, MHC II molecules stained with anti-MHC II in green; c, merged images of a and b; d,
**FIG. 3.** The LAMP/GagN chimera enhances antigen-specific CD4-mediated cytokine responses. Mice, in groups of five animals for each plasmid construct, were injected twice with 50 μg of DNA on days 1 and 21 with pITR plasmids encoding the control LAMP without Gag sequences (LAMP), native p55Gag control (GagN), Gag/lamp chimera lacking the LAMP luminal domain (SS/Gag<sub>lamp</sub>/lamp), and the LAMP/Gag<sub>N</sub> chimera in the complete LAMP sequence (LAMP/Gag<sub>N</sub>). Animals were sacrificed on day 31, and splenocytes were prepared for assay of CD4<sup>+</sup> T-cell responses as described under “Experimental Procedures.” Results from the medium control and Gag-specific assays of a representative experiment are shown as mean ± S.D. A, Gag-specific induction of IFN-γ production. B, Gag-specific induction of CD4<sup>+</sup> IFN-γ production by splenocytes incubated with anti-CD4 and anti-CD8 blocking antibodies and Gag as indicated. C, statistical analyses of the CD4<sup>+</sup> mediated IFN-γ production. The results of immune assays obtained from several different experiments performed in a similar manner were combined in this graph and grouped according to each vaccine plasmid. The “n” is the number of experiments performed with a particular plasmid, and each experiment represents the average response of two or three mice. No results were excluded from the analyses. The error bars indicate ± S.E. D, Gag-specific up-regulation of IL-2 mRNA expression. E, Gag-specific up-regulation of IL-4 mRNA expression.

(1g, 2D, panels b1 and b2) were colocalized on most of the vesicles that strongly stained for the two proteins (Fig. 2D, panels c1 and c2). A significant amount of MHC II was also visualized in compartments not containing LAMP/Gag. These confocal images are representative of the majority of the cell staining.

CD4<sup>+</sup>-mediated Responses of Immunized Mice—DNA vaccines encoding LAMP chimeras of HIV gp160 and human papilloma virus E7 have been shown to provide robust antigen-specific CD4<sup>+</sup>-mediated responses as measured by assays of T cell proliferation (34, 35, 38). In the present study, we have measured CD4<sup>+</sup>-mediated responses by assays of mouse spleen cells for Gag-specific IL-2 and IL-4 mRNA expression and IFN-γ protein production (Fig. 3). Mice, in groups of five animals for each plasmid construct, were injected twice with 50 μg of DNA on days 1 and 21 with pITR plasmids encoding: 1) the control LAMP without gag sequences, 2) gag<sub>N</sub>, 3) SS/gag<sub>lamp</sub>/lamp chimera lacking the LAMP luminal domain, and 4) LAMP/gag<sub>N</sub>, with Gag incorporated into the complete LAMP-1 immediately proximal to the LAMP transmembrane domain.

Animals were sacrificed on day 31, and splenocytes were prepared for assay of CD4<sup>+</sup> T-cell responses as described under “Experimental Procedures.”

The mutated SS/Gag<sub>lamp</sub>/lamp, with the LAMP targeting signals and increased protein expression, elicited a greater response than did the native Gag, but in repeated experiments it was severalfold less effective than LAMP/Gag<sub>N</sub> (Fig. 3A). Experiments that included CD4<sup>+</sup> and CD8<sup>+</sup> T-cell blocking antibodies showed that IFN-γ protein production was specific to CD4<sup>+</sup> cells, being completely blocked by prior incubation of splenocytes with an anti-CD4<sup>+</sup> but not an anti-CD8<sup>+</sup> monoclonal antibody (Fig. 3B). Statistical analysis of the CD4<sup>+</sup> IFN-γ protein production following immunization with these vaccine constructs was analyzed with pooled data taken from all experiments performed with comparable protocols in the schedule, doses, and immunization routes (Fig. 3C). Each experiment involved spleens from two or three mice, with “n” equal to the number of experiments performed with the indicated plasmid construct. The greater number of LAMP/Gag<sub>N</sub>
experiments is derived from this vaccine construct used as a positive control in a large number of experiments testing other hypotheses or systems not included in this report. In all experiments there was an ~10-fold greater CD4⁺ IFN-γ response to the complete LAMP/Gag₅ chimera antigen as compared with the wild-type Gag (p < 0.01). In addition, there were greater IL-2 and IL-4 cytokine mRNA responses of Gag-stimulated spleen cells from mice immunization with the LAMP/Gag₅ as compared with the response of cells from mice injected with pLTR-DNA encoding the native p55Gag or SS/Gag₅/lamp (Fig. 3, D and E).

CD₈⁺-mediated Responses of Immunized Mice—DNA vaccines are able to generate CD₈⁺ T-cell responses through MHC class I presentation of antigenic epitopes by transfected cells, but secondary expansion and memory in CD₈⁺ cells requires stimulus from CD₄⁺ T-helper cells (3). Gag-specific CD₈⁺-mediated responses were measured after a single immunization with 50 μg of the plasmid DNA as described above for CD₄⁺-mediated responses, followed in 3 weeks by in vivo expansion of Gag-activated T cells through inoculation with recombinant vaccinia-gag-pol (rVV-gag-pol). Five days later, the mice were injected with an immunodominant H-2Kb-restricted Gag peptide epitope; after 2 h they were sacrificed and spleen cells were prepared. Gag-specific CTL was assayed for CD₈⁺-specific tetramer staining and intracellular IFN-γ staining and by chromium release from P815 cells pulsed with the Gag peptide, all performed ex vivo (Fig. 4).

In general, the CD₈⁺ responses of mice injected with the unmodified gag were stronger than the corresponding responses of CD₄⁺ cells, as is commonly found with DNA vaccines. Nevertheless mice immunized with the complete LAMP/gag₅ DNA uniformly showed greater CD₈⁺ responses in all three assays (Gag tetramer binding, intracellular IFN-γ staining, and Gag-specific cell killing) than did those injected with the wild-type gag or SS/gag₅/lamp DNA preparations (Fig. 4, A–C). Pooled data from all experiments (Fig. 4, D–F) confirmed these results, with the average epitope-specific CD₈⁺ tetramer binding and IFN-γ responses several-fold higher than the responses to native Gag or SS/Gag₅/lamp (p < 0.02). Under this experimental protocol of a single immunization with the DNA vaccine followed by in vivo expansion, 5–10% of the total CD₈⁺ cells became Gag antigen-specific. Removal of CD₈⁺ cells from the effector population abolished the Gag-specific cell killing, but removal of CD₄⁺ did not (not shown).

Combined Immunization with DNA Plasmids Encoding Native Gag and LAMP/Gag₅—The response of mice to immunization with a mixture of the two plasmids encoding native Gag and the LAMP/Gag₅ chimera was studied to investigate the possibility that trafficking of the LAMP/Gag₅ chimera to lysosomes could result in a deficiency in the amount of protein available to the MHC I processing and presentation pathway of transfected cells. Experiments with cultured cells have shown that the great majority of endogenous LAMP, >95%, has a steady-state localization in the lysosomal membrane (15–19). Because all Gag in the transfected cells of mice injected with the DNA is expressed as a LAMP/Gag₅ chimera targeted to

**Fig. 4.** The LAMP-targeted LAMP/Gag₅ chimera induces more efficient CD₈ priming. Groups of mice (two to three/group) were immunized with 50 μg of the indicated pLTR plasmids. Three weeks after immunization, they were boosted intraperitoneally with 10⁶ plaque-forming units of rVV-Gag-Pol. Five days later, 10 μg of the H-2Kb-binding HIV-1 Gag peptide was injected intravenously, and splenocytes were harvested after 2 h and analyzed for ex vivo activity. A, flow cytomtery quantification of tetramer binding in the CD₈⁺ splenocyte population. B, flow cytomtery quantification of Gag-specific CD₈⁺ T-lymphocytes producing IFN-γ. C, cytolytic activity in a 4-h ⁵¹Cr release assay using P815 target cells pulsed with the H-2Kb-binding HIV-1 Gag peptide. The effector cells from the different immunized mice were as indicated. Nonspecific lysis (using unpulsed P815 target cells) was <5% for all groups (not shown). D–F, statistical analyses of the CD₈⁺ response. The results of immune assays obtained from several different experiments performed in a similar manner were combined and grouped according to each vaccine plasmid. The “n” is the number of experiments performed with a particular plasmid, and each experiment represents the average response of two or three mice. No results were excluded from the analyses. The error bars indicate ± one S.E. D, H-2Kb tetramer-binding CD₈⁺ T-lymphocytes. E, Gag-specific IFN-γ-producing CD₈⁺ T-lymphocytes. F, cytolytic activity in a 4-h ⁵¹Cr release assay at the indicated effector:target ratios.
lysosomes, it was thought that the amount of protein available for MHC I presentation to CD8\(^+\) T-cells might be limiting and that this could be corrected by communication with DNA encoding native Gag. Mice were injected with equal amounts of pITR plasmids encoding Gag\(_N\) and LAMP/Gag\(_N\). Animals were sacrificed on day 31, and splenocytes were prepared for assay of CD4\(^+\) T-cell responses as described under “Experimental Procedures.”

**Fig. 5.** The immune response to LAMP/Gag\(_N\) is not increased by combined immunization with DNA plasmids encoding native Gag (Gag\(_N\)) and LAMP/Gag\(_N\). A and B, mice, in groups of five animals for each plasmid construct, were injected on days 1 and 21 with 50 \(\mu\)g of the pITR plasmid DNA constructs encoding the control LAMP without Gag sequences (LAMP), the native p55Gag (Gag\(_N\)), the LAMP/Gag\(_N\) chimera, and with a mixture of DNA plasmids, 25 \(\mu\)g each, encoding Gag\(_N\) and LAMP/Gag\(_N\). Animals were sacrificed on day 31, and splenocytes were prepared for assay of CD4\(^+\) T-cell responses as described under “Experimental Procedures.” Results from the medium control and Gag-specific assays of a representative experiment are shown as mean ± S.D. A, Gag-specific induction of IFN-\(\gamma\) production. B, Gag-specific up-regulation of IL-2 mRNA expression. C–E, groups of mice (two to three/group) were immunized with 50 \(\mu\)g of the indicated pITR plasmids (25 \(\mu\)g each of the Gag\(_N\) and LAMP/Gag\(_N\) combination). Three weeks after immunization they were boosted intraperitoneally with 10\(^7\) plaque-forming units of rVV-Gag-Pol. Five days later, 10 \(\mu\)g of the H-2K\(d\)-binding HIV-1 Gag peptide was injected intravenously, and splenocytes were harvested after 2 h and analyzed ex vivo. C, flow cytometry quantification of tetramer binding in the CD8\(^+\) splenic population. D, flow cytometry quantification of Gag-specific CD8\(^+\) T-lymphocytes. E, cytolytic activity in a 4-h \(^{51}\)Cr release assay at the indicated effector:target ratios.

**DISCUSSION**

These studies document a novel HIV-1 p55gag DNA vaccine constructed as a chimera with the Gag sequence inserted into the luminal domain of the LAMP lysosomal membrane protein. This construct was developed as a means to provide additional necessary protein elements required for Gag trafficking to the vesicular lysosomal sites of antigen-presenting cells that contain MHC II and act in the presentation of antigen peptide epitopes to CD4\(^+\) T helper cells. Gag encoded with the LAMP/gag chimera increased the antibody titers for both immune responses only after four immunizations, with equivalent IgG1 and IgG2a titers of 8,100. Thus the LAMP/Gag\(_N\) protein chimera increased the antibody titers for both immunogoldin isotypes, but more strongly for IgG1. These results are consistent with an increase in IFN-\(\gamma\) production and IL-4 mRNA up-regulation, which was not detected in mice immunized with DNA encoding the native Gag.

**Dose Response to Immunization with pITR/LAMP/gag**—This new p55gag DNA construct, with the high Gag protein expression, was maximally active in mice at the 10- to 50-\(\mu\)g range of DNA for both antibody and cellular responses (Fig. 7, A–D). Mice injected twice intramuscularly with 0.1–50 \(\mu\)g of LAMP/gag\(_N\) DNA showed maximum CD8\(^+\) and CD4\(^+\) IFN-\(\gamma\) responses to 10 \(\mu\)g of DNA, whereas the antibody response was dependent on a greater dose of DNA.
taining the YXXØ lysosomal membrane targeting signal, without the LAMP luminal domain, did not colocalize with endogenous LAMP or MHC II molecules of transfected cells. This was in contrast to similar LAMP chimeras of membrane proteins, the CD44 hyaluronate receptor (21), HIV gp160 envelope (34), and the premembrane-envelope of dengue 2 virus, which do traffic to lysosomes as chimeras containing only the lamp transmembrane and cytoplasmic domains (39, 40). Unlike these membrane proteins that normally enter the cellular vesicular system, Gag is a cytoplasmic protein, and we speculate that the lack of lysosomal membrane trafficking resulted from the absence of sequences recognized by chaperones or other molecules (72), or required vesicular trafficking elements such as appropriate protein folding or glycosylation. Our rationale for synthesizing Gag as a chimera with the complete LAMP molecule was that the LAMP luminal domain might provide additional necessary structural and perhaps functional elements that facilitate the vesicular trafficking of the associated Gag protein. This proved to be the case, as demonstrated by confocal immunofluorescence microscopy of transfected DCE-K.ICAM.Hi7 cells, which express MHC II in a vesicular compartment colocalized with the endogenous LAMP-1 and LAMP-2. The extent of the LAMP/Gag chimera localization with MHC II of these cells was striking, with Gag present in most of large MHC II-containing vesicles as found by serial confocal sections of individual cells. It should be noted that these results with the Gag protein may not apply to all cytoplasmic proteins; for example, a chimera of the human papilloma virus E7 lacking the LAMP luminal domain was shown to elicit an enhanced immune response of mice (35, 36, 38).

The stimulation of immune responses of mice injected with DNA encoding the LAMP/Gag chimera, including antibody, CD4+ T-cell IL-2 and IL-4 mRNA, and IFN-γ protein production, and CD8+ T-cell tetramer binding, intracellular IFN-γ production by splenocytes harvested 2 weeks after the second immunization, as analyzed by ELISA after in vitro stimulation of splenocyte with the p55 Gag protein. Results are means ± S.D.
staining, and Gag-specific chromium release from target cells, adds to the now substantial evidence that trafficking of DNA-encoded antigens to the LAMP endosomal/lysosomal vesicular compartments can be an important factor in the function of genetic vaccines. Additionally, in other ongoing studies, monkeys immunized with a comparable human LAMP/gag chimera construct have shown strong immune responses by each of the five animals after three immunizations (data not shown). A leading hypothesis is that the enhanced immune response results from trafficking of the LAMP/antigen chimera to the MHC II compartment with a resulting increased efficiency of antigen processing and epitope binding to MHC II. However, a variety of alternative mechanisms to explain the LAMP effect may be entertained. For example, the LAMP luminal domain may have some direct role in MHC II antigen processing and presentation independent of the colocalization with MHC II, or in the general activation of lysosomal function during dendritic cell maturation (30). It is also likely that antigen processing and the specific epitopes of the LAMP chimera antigens will differ from those derived from native Gag. LAMP targeting of antigen is not a normal function of APCs, and the products of antigen processing may differ from those associated with conventional endocytic antigen uptake to the MHC II compartment by dendritic cell receptors or other possible trafficking pathways. Trafficking of the LAMP/antigen chimera may provide access to different endosomal/lysosomal antigen-processing compartments where the specificities of the proteases may differ from those present in the natural pathway of the antigen taken into dendritic cells by the normal endocytic route. Moreover, the proteolytic processing might also be conditioned by the structure of the LAMP/antigen chimera or its relationship to other proteins involved in the lysosomal membrane vesicular trafficking. The heavily glycosylated, disulfide cross-linked luminal domain of LAMP is highly protease-resistant (73), as expected for sequences that reside in the lumen of lysosomes, and may partially protect certain Gag sequences from proteolysis. Whatever the mechanism, there is evidence that LAMP chimeras modify the repertoire of epitopes eliciting cellular and humoral immune responses. For example, a CD4+–mediated T-cell response of a minigene peptide epitope was abolished when constructed as a chimera with the lysosomal targeting signal of the cytoplasmic domain from the lysosomal integral membrane protein-II (LIMP-II) (74). It also has been reported that LAMP targeting of antigen increases the number of immunogenic peptide epitopes that activate CD4+ T cells, thus inducing a qualitatively broadened immune response as compared with untargeted antigen (75). In our ongoing studies it is found that both the antibody and T-cell responses to Gag peptides of the LAMP/Gag chimera also have significant differences from the responses to native Gag. A further question is the mechanism of the enhanced CD8+ response. While this can be attributed to the stronger CD4+ responses, the nature of MHC I processing and presentation remain to be elucidated. Presumably, in the case of the LAMP-targeted Gag, the majority, if not all, of the Gag protein is degraded by the hydrolytic enzymes of the endosomal/lysosomal compartments of transfected cells rather than by the cytoplasmic proteasome complex. Nevertheless, immunization with a mixture of DNA plasmids encoding native Gag as well as the LAMP/Gag chimera did not enhance the CD8+ T-cell responses despite the expression of Gag localized to the cytoplasm and available for MHC I processing and presentation. Thus, the strong CTL response to the lysosomal targeting of the LAMP/Gag chimera protein suggests that the LAMP/Gag-processing pathway provided sufficient epitope cross-priming for MHC I presentation, or that the greater CD4+ response compensated for a decrease in MHC I epitope presentation by transfected APCs. There is also the question of how trafficking of the LAMP/Gag chimera enhances antibody responses to Gag. Commonly, DNA vaccines encoding intracellularly expressed proteins do not elicit strong B cell responses (9). Evidently, antibody epitopes of LAMP/antigen chimeras persist and are efficiently presented to B cells. It is known that while LAMP is predominantly localized in lysosomes, a small fraction is present on the cell surface. Our initial identification of LAMP was made by monoclonal antibody binding at the cell surface (68), and cycling of LAMP through the plasma membrane (76) and lysosomal fusion with the plasma membrane (77) have been described. Additionally, some populations of cells transfected in vitro may have lysosomal trafficking patterns that differ from those deduced from previous in vitro studies. For example, mouse peritoneal macrophages are shown to contain a post-translational isoform of LAMP-1 (M150) that is uniquely present on the surface of macrophages and acts as a costimulatory molecule driving the differentiation of naïve CD4+ T cells into the Th1 subset (78). Thus, although we suggest that the principal element in the enhanced immune response is the improved delivery of Gag antigen to the MHC II compartment, there may be additional effects of LAMP trafficking and the LAMP luminal domain on the immune response to the DNA-encoded LAMP/Gag protein chimera.

Another novel finding of this study is the enhanced protein expression of HIV-1 p55 Gag as a LAMP/Gag chimera. Gag expression is minimal in cells transfected with DNA plasmid vectors containing only the native Gag gene. This low expression is attributed to mRNA cis-acting inhibitory or instability region sequence elements, which can be eliminated by gene mutation or humanization of codon usage (56, 57, 59, 60, 62, 63). Additionally, the nuclear export of Gag mRNA has been promoted by the use of DNA chimeras encoding the constitutive transport RNA element of type D retroviruses that binds to a nuclear element and functions as export factors for the constitutive transport RNA element-containing RNAs (79). The effect of the LAMP luminal sequences 5′ to the Gag sequences to increase Gag protein expression has not previously been reported and indicates the presence of additional mechanisms acting on the regulation of HIV-1 Gag mRNA and/or protein expression and stability. Ongoing studies suggest that the amino-terminal sequences of LAMP are critical to the increased protein expression, as indicated by deletions, substitutions, and modifications of the amino-terminal proximal sequence of the LAMP luminal domain (data not shown). It has previously been shown that the enhanced gag gene expression of the codon-optimized gag-pol was not inhibited when native gag sequences were inserted downstream, whereas native gag gene sequences upstream of the codon-optimized gag-pol led to diminished RNA levels (60). Expression of the LAMP/Gag chimera was also increased by the addition of ITR sequences to the plasmid. This effect on transgene protein expression has previously been attributed to the increased stability of the plasmid DNA; the ITR domains can form trans-concatamers or palindromes in a cis configuration and have been shown to induce high level, long term gene expression (65). However, these effects of ITR sequences do not apply to all genes, and we have found no apparent increase in protein expression in several other systems.

Acknowledgments—We thank Dr. Susan Swain, from The Trudeau Institute, Saranac Lake, NY, for the fibroblast cell line DCEK.I-Hi7; Dr. Alan L. Scott, the Johns Hopkins University, School of Public Health, for providing the cytokine standards for quantitative real-time PCR; Dr. Clair Chaungnet, Children’s Hospital Medical Center, Cincinnati, OH, for stimulating discussion and advice on the immune response assays; Dr. Deborah McClellan for editorial assistance; and Betty Earl’s Hart and Dolores Henson for their excellent technical assistance. Several reagents were obtained through the AIDS Research Reagents Program, Division of AIDS, NIAID, National Institutes of...
