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DNA Immunization: Ubiquitination of a Viral Protein Enhances Cytotoxic T-Lymphocyte Induction and Antiviral Protection but Abrogates Antibody Induction†

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DNA immunization can induce cytotoxic T lymphocytes (CTL), antibodies, and protection against microbial challenge. The underlying mechanisms remain obscure and must be understood to permit rational manipulation and optimization of the technique. We set out to enhance the intracellular degradation of a viral antigen, with the intent of improving antigen entry into, and presentation by, the class I major histocompatibility complex pathway. We achieved this goal by cotranslational ubiquitination of a plasmid-encoded viral antigen, lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP). We show that native NP is very stable in cell culture, while the ubiquitinated product is so rapidly degraded that it is barely detectable. This rapid degradation leads to more efficient sensitization of target cells in an in vitro cytotoxicity assay, consistent with enhanced antigen presentation, and both degradation and target cell recognition are blocked by a proteasome inhibitor. We have used the plasmid for in vivo studies and find that, remarkably, ubiquitination leads to a complete abrogation of antibody responses, presumably because the encoded protein is so rapidly and completely degraded that insufficient antigen remains to interact appropriately with B cells. In contrast, in vivo CTL induction is improved by ubiquitination of NP. That CTL are induced at all by this rapidly degraded protein may shed light on the mechanism by which CTL are induced by DNA immunization; it has been suggested that CTL induction following intramuscular DNA injection results not from antigen presentation by cells taking up and expressing the DNA but rather from uptake of soluble protein by specialized antigen-presenting cells (APC). It appears to us unlikely that the ubiquitinated protein could function in this manner, since it is so rapidly degraded in vitro and fails to induce antibodies in vivo. Finally, the ubiquitinated protein confers markedly enhanced protection against LCMV challenge. Mice immunized with a plasmid encoding NP show approximately 100-fold reductions in virus titers compared to controls, while mice immunized with a plasmid encoding the ubiquitinated NP show reductions in virus load of at least 5×10^4- to 5×10^5-fold. This is by far the most effective DNA vaccine that we have yet designed. Ubiquitination therefore may improve DNA immunization, but caution is warranted, since immunity to many microbes depends on induction of good humoral immunity, and we show here that this may be prevented by ubiquitination of the encoded protein.

DNA immunization, the inoculation of plasmid DNA encoding microbial proteins, is a recent addition to the vaccine arsenal. It has many apparent advantages over conventional approaches to vaccination, which we and others have reviewed elsewhere (9, 11, 19, 34). One major benefit is the endogenous synthesis of the encoded protein, allowing presentation of the foreign antigen by major histocompatibility complex (MHC) class I. Furthermore, the release of soluble protein should permit induction of antibody responses; both arms of the immune response may therefore be induced. In this study, we focused on the mechanisms underlying successful DNA immunization and attempted to improve cytotoxic T lymphocyte (CTL) induction and recognition by enhancing the introduction of the encoded protein into the MHC class I pathway. Others have shown that the peptide concentration in the endoplasmic reticulum is often a limiting factor in the maturation of the class I complex (22), suggesting that enhancement of peptide delivery might increase cell surface expression of the mature class I complexes. The importance of the proteasome in peptide generation is broadly accepted, and we therefore sought to determine if induction of CTL by DNA immunization might be enhanced by improving the delivery of the encoded protein to the proteasome; the resulting enhanced proteolysis should produce more peptides for introduction into the MHC class I antigen presentation pathway. Many proteins degraded by the proteasome are targeted to that site by the covalent attachment of the cellular protein ubiquitin. While the importance of ubiquitin in class I presentation was suggested some time ago (20), other workers found little effect (5), and the issue remains controversial (18).

We have carried out our DNA immunization studies using the lymphocytic choriomeningitis virus (LCMV) mouse model system. In this report, we show that intracellular degradation of LCMV nucleoprotein (NP) can be greatly enhanced by a novel approach, in which ubiquitin is attached to NP by a stable covalent bond. The resulting degradation is so efficient that the protein product is barely detectable except in the presence of a proteasome inhibitor; moreover, class I presentation is improved, since transfected tissue culture cells are more sensitive to CTL lysis. We complement the tissue culture studies with in vivo experiments. Mice inoculated with plasmid DNA encoding this rapidly degraded protein are unable to mount an antiviral antibody response. In contrast, there is an enhancement of antiviral CTL induction and of protective antiviral immunity. These results reflect on the mechanisms

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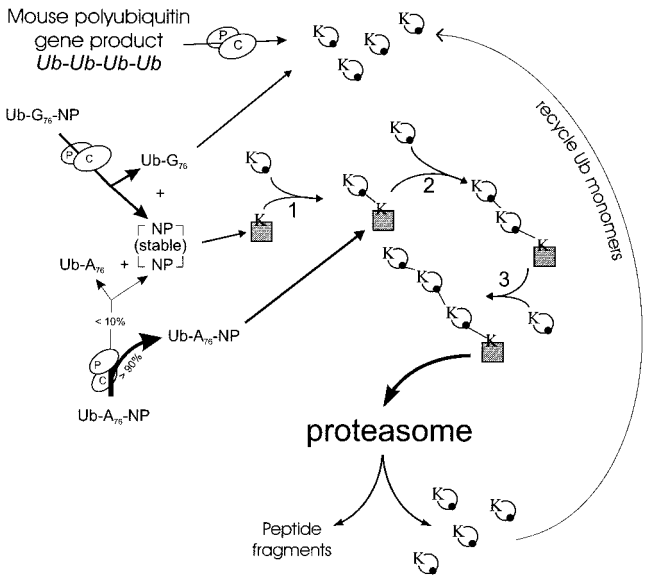

FIG. 1. The ubiquitin pathway and the point of entry for ubiquitin-A_{76}-NP. The ubiquitin pathway is shown on the right. Ubiquitin (Ub) is represented by a circle, with a small dot representing the C-terminal glycine. K = lysine (see text). The target protein is shown as a grey box. On the left is shown the rationale for construction of the cotranslationally ubiquitinated NP (Ub-A_{76}-NP) and its point of entry into the polyubiquitination pathway.

which underlie the immune responses induced by DNA immunization.

MATERIALS AND METHODS

Cell lines and viruses. MC57 (*H2*^{b}) and BALB Cl7 (*H2*^{d}) cell lines were maintained in RPMI (Sigma, St. Louis, Mo.), and Vero 76 cells were maintained in medium 199 (Gibco-BRL, Gaithersburg, Md.) supplemented with 10% fetal calf serum, L-glutamine, and penicillin-streptomycin. Virus used was LCMV (Armstrong strain).

Mouse strains used. Mouse strains (BALB/c [*H2*^{d}] and C57BL/6 [*H2*^{b}]) were obtained from the breeding colony at Scripps Research Institute. Mice were used at 6 to 16 weeks of age.

Virus titration. LCMV titration was performed on Vero 76 cells, which were plated at a density of 6.6×10^5 per six-well plate, 24 h prior to titration. At the time of titration, 10-fold dilutions were made in medium 199 (Gibco-BRL) supplemented with L-glutamine and 10% fetal calf serum and applied to the indicator cells. Following adsorption and infection for 1 h at 37°C under 5% CO_2, the inoculum was withdrawn and replaced with an overlay of 0.5% sterile ME agarose (FMC Biochemicals)-1× complete medium 199. Four days later, the monolayer was fixed with 25% formaldehyde in phosphate-buffered saline (PBS), the agarose plug was removed, and the monolayer was stained with 0.1% crystal violet–20% ethanol in PBS.

Construction of plasmids to enhance proteasomal destruction of LCMV NP. The ubiquitin pathway and the point of entry for ubiquitin-tagged NP are shown in Fig. 1. A small proportion of all intracellular proteins are ubiquitinated, i.e., tagged for proteasomal destruction by the covalent attachment of the 76-amino-acid cellular protein ubiquitin (reviewed in reference 12). Ubiquitin covalently attaches, via its C-terminal glycine residue G_{76}, to a lysine on the target protein (event 1 in the Fig. 1); a second ubiquitin then attaches to a lysine residue on the first (event 2), then a third ubiquitin attaches to the second (event 3), and so on (for clarity, only three cycles are shown). The doomed protein is therefore marked with a polyubiquitin chain, and this complex is taken to the proteasome for processing. Only a small percentage of plasmid-encoded proteins normally become ubiquitinated, and we reasoned that delivery to the proteasome might be enhanced by ensuring ubiquitination of every copy of the plasmid-encoded protein. We have approached this issue by fusing the open reading frame (ORF) of ubiquitin to the ORF of our protein of interest. A coherent description of these gene fusions requires a brief summary of how ubiquitin monomers are made within the normal cell. The mouse ubiquitin gene encodes serial repeats of the 76-amino-acid ubiquitin monomer (8); the primary translation product is, therefore, polyubiquitin (linked tail to head). Were it to remain in that form, this polyprotein would be shuttled to the proteasome; however, a specific cellular protease complex (PC in Fig. 1) recognizes this tail-to-head array and cleaves it after each tail G_{76} residue, releasing free ubiquitin monomers. If we were to make constructs in which an ORF encoding a normal ubiquitin monomer (with the G_{76} residue) lay in frame with the ORF encoding our protein, the resulting protein (for example, ubiquitin-G_{76}-NP [Fig. 1]) would be recognized by the PC and cleaved, releasing free monomeric ubiquitin and free NP. Others have exploited this cleavage to generate proteins with N-terminal amino acids other than methionine. Because of the N-end rule (33), changes in a protein's N-terminal amino acid may result in markedly altered stability (3, 31, 32). With this approach, ubiquitin is attached only transiently to the virus protein, and the PC cleavage is used to manipulate the N-terminal sequence thereof. Our approach is different, since it stably links ubiquitin to the virus protein by a covalent bond which is relatively resistant to cleavage, thus targeting the stably ubiquitinated protein into the polyubiquitination pathway and thence to the proteasome. Others have shown that mutation of ubiquitin G_{76} to A_{76} diminishes the rate of cleavage of a fusion protein by ≥90% in yeast (6) while still permitting the fused ubiquitin to serve as a substrate for addition of a polyubiquitin chain. We wished to evaluate the effect of this approach on antigen presentation, and therefore we made a plasmid construct placing modified ubiquitin (A_{76}) N terminal to, and in frame with, LCMV NP. This is represented diagrammatically in Fig. 1 for our construct ubiquitin-A_{76}-NP; in theory, this fusion protein should be very efficiently introduced into the polyubiquitination pathway, at event 2 in Fig. 1.

Construction of the recombinant plasmids. The gene encoding a monomer of mouse ubiquitin (76 amino acids) was amplified by PCR from BALB/c genomic DNA with two oligonucleotide primers. The 5′ primer supplied an ATG start codon with an appropriate Kozak sequence (16), and the 3′ primer changed the C-terminal amino acid from glycine (G_{76}) to alanine (A_{76}), for reasons explained above. The full-length LCMV NP gene was cloned as a fusion gene with ubiquitin-A_{76} into the *Not*I site of plasmid pCMV (derived by excision of the β-galactosidase gene from pCMV-β [Clontech, Palo Alto, Calif.]) to generate pCMV-U-NP. The control plasmid pCMV-U also was made, with ubiquitin alone cloned into pCMV. These constructs were compared in immunization experiments with our previously evaluated plasmid, pCMV-NP, which encodes a nonubiquitinated protein.

^{35}S metabolic labeling, pulse-chase analysis, and use of a proteasome inhibitor. BALB Cl7 cells were transfected with each plasmid (2 μg of DNA per 10^6 cells), using Lipofectamine and OPTI-MEM (Gibco-BRL) according to the manufacturer's recommendations. At 48 h posttransfection, cells were starved for 30 min in methionine- and cysteine-free medium, after which ^{35}S-labeled amino acids were added (0.3 mCi/ml, final activity). Cells were incubated (pulsed) for 30 min and then either (i) lysed on ice with lysis buffer (0.5% Nonidet P-40, 2 mM EDTA, 0.5 M NaCl, 0.2% sodium dodecyl sulfate, 5 mM β-mercaptoethanol, 50 mM Tris-HCl [pH 8.0]) or (ii) refed with prewarmed medium containing cold methionine and cysteine (chased). Cells were lysed at the indicated times, and proteins were immunoprecipitated for 2 h with 3 μl of anti-LCMV NP monoclonal antibody 1-1-3 (the kind gift of M. J. Buchmeier) or with polyclonal anti-LCMV antibody, along with 30 μl of protein A-agarose beads (Sigma). Beads were washed five times with the lysis buffer without β-mercaptoethanol, and samples were run under reducing conditions on sodium dodecyl sulfate-gradient (8 to 16%) polyacrylamide gels. Gels were then soaked for 30 min in Fluoro-hance (Research Products International Corp., Mount Prospect, Ill.), dried, and exposed to photographic film. Calpain inhibitor I, *N*-acetyl-leucyl-leucyl-norleucinal (ALLN), is a cell-permeable synthetic tripeptide with an aldehyde at its C terminus, which specifically inhibits the activity of cysteine proteases. Where noted, ALLN was added to cells, at a final concentration of 10 μg per ml, 30 min before addition of radiolabel and was maintained at this concentration throughout labeling, harvesting, and immunoprecipitation.

Protocol for DNA immunization. DNA was purified by standard techniques using Nucleobond (The Nest Group, Southborough, Mass.), and all DNA was treated with an endotoxin removal buffer (40% ethanol, 5% acetic acid). BALB/c (*H2*^{d}) and C57BL/6 (*H2*^{b}) mice were immunized intramuscularly three times, at 14-day intervals, with 100 μg of plasmid. DNA was dissolved in 1 N saline at a concentration of 1 mg/ml, and 50 μl (50 μg) was injected into each anterior tibial muscle, using a 28-gauge needle. As positive vaccine control, mice were immunized intraperitoneally (i.p.) with a sublethal dose of LCMV (2×10^5 PFU).

Induction of virus-specific CTL. CTL activity following LCMV infection of previously nonimmune mice peaks at 7 to 9 days postinfection (p.i.) and declines thereafter. CTL activity is difficult to detect in these mice at day 4 p.i. but is readily detectable at this time point in an LCMV-immune animal, in which the presence of memory cells allows an accelerated response to viral challenge. Therefore, mice were inoculated with the various DNAs (or with LCMV as a vaccine positive control) and 6 weeks later received LCMV i.p. Four days later the mice were sacrificed, and their spleens were taken and bisected. Half of each spleen was used in a CTL assay, and the other half was collected for virus titration. A positive CTL response indicates that the prior DNA immunization induced memory CTL.

In vitro cytotoxicity assay. The assays were carried out as previously described (36). Effector cells were either primary splenocytes from mice infected 7 days previously with LCMV (positive control for lysis of target cells) or splenocytes taken 4 days p.i. from mice treated as described above and in the text. Target cells were transfected with the various plasmids (2 μg of DNA per 10^6 cells), coated with epitope peptide, or infected with LCMV; 48 h later, the target cells were labeled for 1 h with ^{51}Cr, washed, and incubated in triplicate for 5 h with effector cells at the indicated effector-to-target ratios. Supernatant was har-

vested, and specific chromium release was calculated by using the following formula: [(sample release − spontaneous release) × 100/(total release − spontaneous release)].

Enzyme-linked immunosorbent assay (ELISA) for detection of anti-LCMV antibodies. Blood was drawn from the tail artery 3 weeks postimmunization, and serum was prepared. Tissue culture plates (Costar 96-well cell culture plate model 3596) were coated with purified LCMV as antigen (100 μl of 2 μg protein/ml in Dulbecco's PBS) by overnight incubation at room temperature in a humid box. Residual liquid was removed, and 200 μl of blocking buffer (3% bovine serum albumin and 0.2% Tween 20 in Dulbecco's PBS) was added to each well. One hour later, the liquid was removed, and the wells were washed with wash buffer (0.2% Tween 20 in Dulbecco's PBS). Serum samples were serially diluted (in twofold steps) in the blocking buffer, and 100 μl of the diluted solution was added to each well and incubated for 1 h at room temperature. Wells were washed, and 100 μl of second antibody (goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase [Sigma A-9917], diluted to a working concentration of 1:20,000 in 3% bovine serum albumin–Dulbecco's PBS) was added per well. Following a 1-h incubation, the liquid was removed, the wells were washed, and 100 μl of *o*-phenylenediamine dihydrochloride substrate (Sigma P-9187) was added. Light was excluded, and the reaction was allowed to proceed for 30 min, when addition of 100 μl 1 N HCl stopped the reaction. The absorbance at 492 nm was measured with a Titertek Multiskan Plus.

In vivo protection studies. Two independent measures were used to evaluate the in vivo protection induced by DNA immunization.

(i) Protection reflected by reduction in LCMV titers following nonlethal viral challenge. Mice were immunized with LCMV (i.p. as a positive vaccine control) or with DNA and 6 weeks later were challenged with LCMV by the i.p. route (2×10^5 PFU). Four days later mice were sacrificed, and their spleens were harvested. Each spleen was bisected, and half was used for analysis of anti-LCMV CTL activity (see above). The remainder was used for analysis of virus titers; low titers (in comparison to nonimmune control mice) indicate prior immunity.

(ii) Protection reflected by resistance to a normally lethal LCMV challenge. When inoculated intracranially (i.c.), LCMV induces a massive lymphocytic response in the choriomeninges, and death supervenes usually 7 to 8 days p.i. The disease is immunopathological, being mediated by $CD8^+$ antiviral CTL; in a naive mouse, by the time a strong CTL response develops, the virus has already disseminated widely, and the resulting antiviral CTL activity is lethal to the mouse. Although the disease is CTL mediated, vaccine-mediated induction of CTL can confer protection (10, 14, 15, 27, 35). For the studies reported here, mice were inoculated with DNA or with LCMV as previously described, and 6 weeks later, the animals were challenged with a potentially lethal amount (20 50% lethal doses [LD_{50}]) of LCMV administered i.c. Mice were observed daily, and all recorded deaths occurred between days 6 and 8 postchallenge.

RESULTS

Ubiquitination markedly enhances intracellular turnover of LCMV NP. To determine the intracellular turnover of the ubiquitinated protein, we transfected BHK cells with pCMV-U-NP and carried out a pulse-chase analysis. Radiolabeled proteins were immunoprecipitated with an NP-specific antibody, and the products were separated on a polyacrylamide gel and visualized by autoradiography. As shown in Fig. 2A, full-length LCMV-NP was readily detected in cells transfected with plasmid pCMV-NP. NP appears relatively stable in these cells, since even after a 90-min chase, a band was still easily visible. In contrast, cells transfected with the ubiquitin-NP construct showed very low levels of detectable protein. The ubiquitin-NP band should be 76 residues longer than the 558-residue NP, but no such band is visible. A faint band of the size of NP is seen (presumably due to some leakiness of the PC cleavage which cleaves the ubiquitin-A_{76}-NP to release free NP as indicated in Fig. 1) along with several lower-molecular-weight bands which may represent degradation products (some of which are abundant in the LCMV track). An alternative interpretation is that plasmid pCMV-U-NP simply fails to make much protein. To distinguish rapid degradation from failure of synthesis, we used the proteasome inhibitor ALLN (26). As shown in Fig. 2B, the quantity of NP in cells transfected with pCMV-NP is barely altered (perhaps slightly increased) in the presence of the inhibitor, consistent with NP being stable in the cell. In contrast, there is a marked effect in cells transfected with pCMV-U-NP; very little material is detected in the absence of inhibitor, but in the presence of ALLN there are readily detectable levels both of NP and of a band whose size is consistent with the cotranslational ubiquitin-NP product. These results demonstrate that plasmid pCMV-U-NP directs protein synthesis but that the protein product is very rapidly degraded by the proteasome. These findings are not cell specific, as similar results were obtained in BALB/c fibroblasts (not shown).

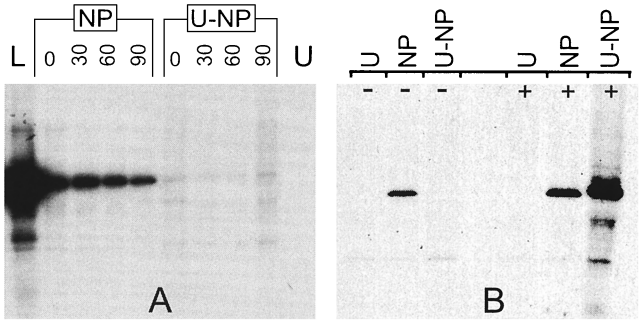

FIG. 2. Cotranslational ubiquitination enhances degradation of NP. (A) BHK cells were transfected with pCMV-NP (NP) or with pCMV-U-NP (U-NP), and 48 h later the proteins were metabolically labeled for 30 min. Some cells were harvested immediately thereafter (tracks 0), while others were chased for 30, 60, or 90 min as shown. Track L, cells infected with LCMV; track U, cells transfected with the control plasmid pCMV-U. An immunoprecipitation was carried out with NP-specific monoclonal antibody 1-1-3, and the products were separated by electrophoresis. (B) Duplicate plates of cells were transfected with pCMV-U (tracks U), pCMV-NP (tracks NP), or pCMV-U-NP (tracks U-NP), and 48 h later the proteins were metabolically labeled. For one set of plates, the proteasome inhibitor ALLN was added at 10 μg/ml 30 min prior to addition of the label and was maintained at this concentration in all further manipulations. An immunoprecipitation was carried out with polyclonal anti-LCMV antibody, and the products were separated by electrophoresis. The absence (−) or presence (+) of ALLN is indicated.

Target cell sensitization is enhanced by covalent attachment of ubiquitin to NP and completely blocked by ALLN. To determine whether this enhanced degradation was reflected in increased presentation of the NP epitope by class I MHC, we carried out an in vitro CTL assay using as effector cells primary anti-LCMV CTL from a BALB/c mouse infected 7 days previously. Target cells were transfected with pCMV-U (a negative control plasmid encoding ubiquitin), pCMV-NP, or pCMV-U-NP. As shown in Fig. 3A, covalent attachment of ubiquitin to NP gave higher levels of chromium release at all effector/target ratios tested, compatible with improved antigen presentation. The enhancement is consistent, occurring on both MHC backgrounds and in several repetitions of the experiment (not shown). Antigen presentation of endogenously synthesized materials required proteasome activity, since lysis of infected or transfected target cells was abolished by inclusion of the proteasome inhibitor, although the inhibitor did not protect against lysis of target cells coated with the appropriate LCMV epitope peptides, as shown in Fig. 3B. Thus, ubiquitination of NP results in improved target cell recognition, which requires proteasome activity.

The foregoing studies were carried out in tissue culture, and we wished to evaluate the effects of ubiquitin in vivo. Below are described the results for induction of antibody, CTL, and antiviral protection.

Ubiquitination of NP completely abrogates the induction of anti LCMV antibodies. Mice infected 3 weeks previously with LCMV served as sources of positive control sera. DNA-immunized mice were inoculated three times at 14-day intervals with either pCMV-U, pCMV-NP, or pCMV-U-NP. There were eight mice in each vaccine group, and both BALB/c and C57BL/6 mice were tested (thus, a total of 16 mice received each vaccine).

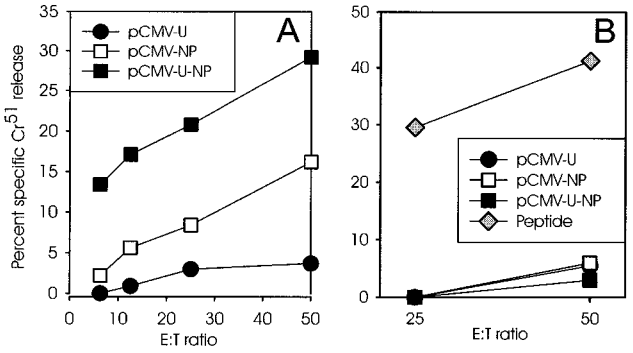

FIG. 3. CTL recognition of NP is enhanced by ubiquitination and is blocked by proteasome inhibitors. (A) BALB Cl7 cells were transfected with pCMV-U, pCMV-NP, or pCMV-U-NP, labeled with ^{51}Cr, and used as targets in an in vitro cytotoxicity assay as described in the text. Effector cells were day 7 anti-LCMV splenocytes, at four effector/target (E:T) ratios as shown. (B) An experiment similar to that in panel A was carried out, but transfected cells were treated with the proteasome inhibitor ALLN (10 μg/ml). To show that the effector cells remained capable of lysis in the presence of ALLN, control target cells, coated with a peptide representing an LCMV CTL epitope and incubated with ALLN (10 μg/ml), were used.

Three weeks later, mice were bled, and anti-LCMV antibody titers were evaluated by ELISA. As shown in Fig. 4, pCMV-NP induced anti-LCMV antibodies, as we have previously demonstrated (39, 40); all 16 mice responded. Remarkably, in both mouse strains, pCMV-U-NP failed to induce anti-LCMV antibodies; the ELISA titers of mice immunized with this plasmid were indistinguishable from those of mice immunized with a plasmid encoding ubiquitin alone. None of the 16 mice responded, and the variation within each group was less than 5%. The failure of the ubiquitin-NP construct to induce antibodies does not reflect an absence of gene expression in vivo, since other immune responses (CTL induction and antiviral protection) were demonstrable, and indeed enhanced, in the same mice, as described below.

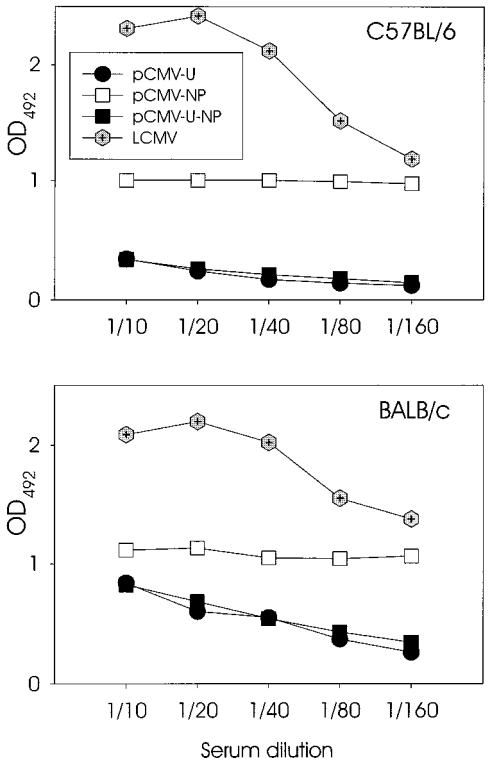

FIG. 4. Ubiquitination of NP abrogates anti-NP antibody induction in two strains of mice. Mice were inoculated with pCMV-U, pCMV-NP, or pCMV-U-NP as described in the text or were infected with a sublethal dose of LCMV (2×10^5 PFU i.p.). Three weeks later, mice were bled, serum was prepared, and an ELISA was carried out. Results are shown for C57BL/6 ($H2^b$) and BALB/c ($H2^d$) mice. OD_{492}, optical density at 492 nm.

Improved CTL induction by ubiquitinated NP. The mice described above were evaluated for CTL activity and for protection against LCMV challenge. Half of the mice (four from each vaccine group, in both mouse strains) were infected with LCMV (2×10^5 PFU i.p.); the remaining mice were challenged with i.c. LCMV (see below). Four days later, the i.p.-challenged mice were sacrificed, and spleens were bisected. Half of each spleen was used in a CTL assay to determine whether primed CTL were present (Fig. 5). If the animals had not been primed by the prior immunization, then CTL activity would be minimal at 4 days p.i. In contrast, if the animals had been effectively immunized, then readily detectable levels of anti-LCMV CTLs would be present at day 4 p.i. Controls shown include a day 7 primary CTL response to LCMV (as a positive control for CTL lysis) (bars labeled LCMV d7), two mice immunized 6 weeks previously with LCMV and then reinfected 4 days prior to assay (as a positive control for the in vivo secondary restimulation) (bars labeled LCMV), and negative control mice inoculated with pCMV-U. Results are shown in Fig. 5. All mice receiving the ubiquitin-NP construct were primed for high levels of anti-LCMV CTL. Mice receiving pCMV-NP (nonubiquitinated protein) also mounted LCMV-specific responses, but levels were lower and in one case was not significantly above control levels; this result is consistent with our previous findings and those of other laboratories using DNA immunization in the LCMV system (23, 41), in which CTL responses are seen in only approximately 50 to 75% of mice following immunization with pCMV-NP.

Ubiquitinated NP confers enhanced protection against LCMV challenge. The biological relevance of the immunity induced was tested in two ways. First, the remaining splenic tissues from the mice described above (four per vaccine group in both mouse strains) were weighed, homogenized, and titrated for LCMV. The results are presented in Fig. 6. Compared to mice immunized either with saline or with pCMV-U alone, mice immunized with pCMV-NP showed significant (2 to 3 logs) reduction in virus titers, as we have previously noted (37, 39, 40). Nevertheless, virus was still detectable in all eight pCMV-NP-immunized mice. In contrast, virus was detectable in none of the mice eight immunized with ubiquitin-NP. This is the most effective protection that we have seen with a DNA vaccine. As a second measurement of protection, the remaining mice (four per vaccine group in both mouse strains) were challenged with a normally lethal dose of LCMV (20 LD_{50}) by the i.c. route. Naive mice succumbed 6 to 8 days p.i. In this experiment, pCMV-NP conferred protection on six of eight mice, while all eight mice immunized with pCMV-U-NP survived (Fig. 7).

In summary, therefore, stable covalent attachment of ubiquitin to NP enhances degradation and antigen presentation in tissue culture, abrogates antibody induction in vivo, and enhances CTL induction and antiviral protection in vivo. These studies strongly support the contention that ubiquitination of protein leads to enhanced class I MHC presentation in vivo and may be of utility in enhancing DNA immunization.

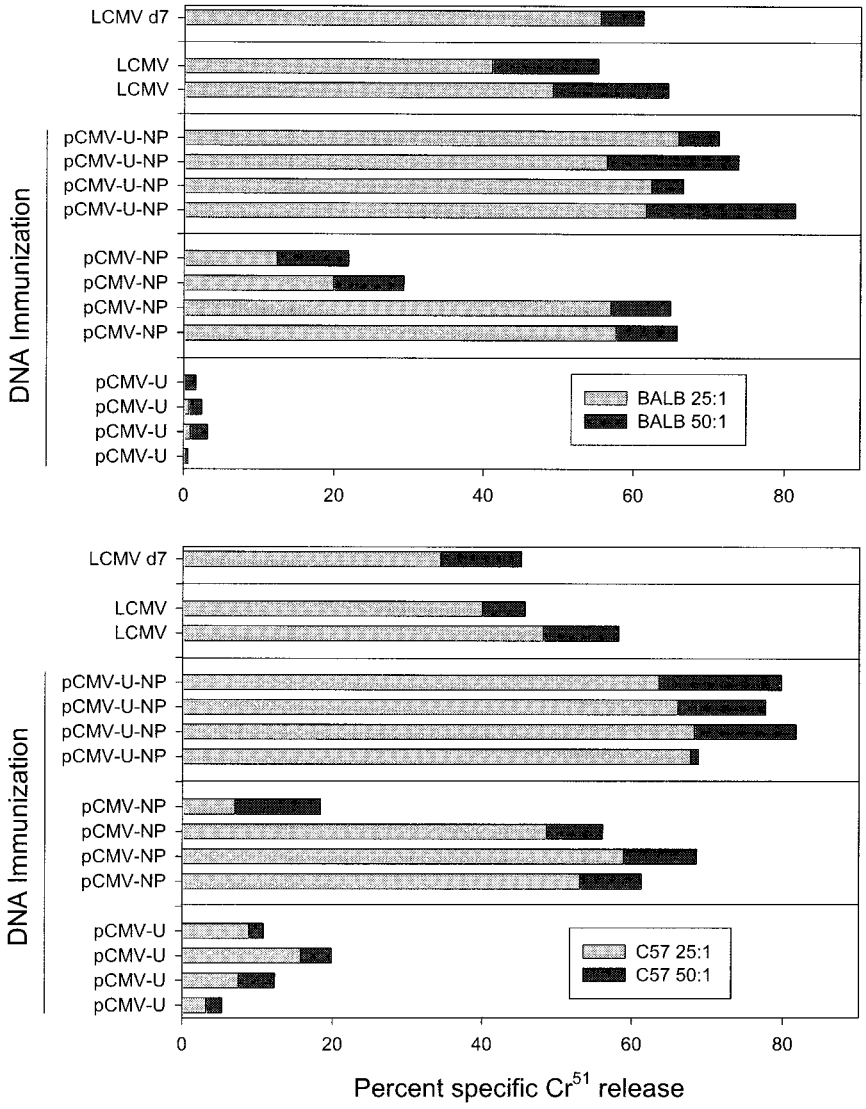

FIG. 5. Enhanced CTL induction by ubiquitinated NP. C57BL/6 and BALB/c mice were immunized with plasmid DNA as shown. As a positive control for successful immunization, mice were infected with LCMV (bars labeled LCMV). These mice were infected with LCMV 6 weeks later and 4 days p.i. were sacrificed; their anti-LCMV CTL activities were determined in an in vitro cytotoxicity assay. As an assay control, splenocytes from a mouse infected 7 days previously were used (bars labeled LCMV d7).

DISCUSSION

The importance of the proteasome in class I MHC presentation, and thus in the host response to invading microbes, is becoming increasingly well defined. Manipulation of the N-terminal amino acid of proteins, which alters their stability and targets them to the proteasome (33), enhances their presentation by MHC class I (31, 32). Furthermore, proteasome inhibitors such as lactacystin and ALLN interrupt antigen presentation by MHC class I (3, 7). Thus, the importance of the proteasome in most class I antigen processing and presentation is in little doubt. In contrast, the role of ubiquitin remains somewhat controversial. Studies using a hamster cell line temperature sensitive in ubiquitin function indicated that antigen presentation was severely limited at the nonpermissive temperature (20). Surprisingly, similar studies using the same cell line, as well as an additional, murine, line, contradicted these findings, and the authors concluded that there was no evidence that ubiquitination was important in antigen processing (5). More recent data indicate that the importance of ubiquitination appears to depend on the nature of the protein; efficient degradation of native protein may require ubiquitination, while the same protein in a denatured state may be degraded without ubiquitination (21).

We undertook this study to evaluate the role of ubiquitination in immune responses induced by DNA immunization. We wished to enhance class I antigen presentation, and thereby CTL induction, by cotranslational attachment of ubiquitin to LCMV NP. This method differs from that more commonly used, which exploits the N-end rule to alter protein stability. We show here that the approach appears extremely effective in increasing protein degradation in mammalian cells. Ubiquitinated NP is barely detectable under normal circumstances but is easily visualized in the presence of a proteasome inhibitor, indicating a rapid turnover of the ubiquitinated product and

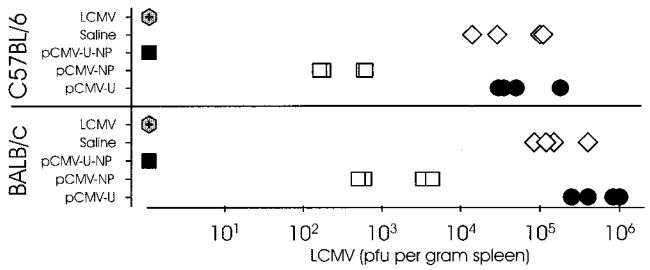

FIG. 6. Enhanced LCMV clearance in mice immunized with ubiquitinated LCMV NP. BALB/c and C57BL/6 mice were immunized as shown (with LCMV, saline, pCMV-U-NP, pCMV-NP, or pCMV-U). Six weeks later, mice were challenged with a nonlethal dose of LCMV (2×10^5 PFU i.p.); 4 days later, the mice were sacrificed and LCMV titers in the spleens were determined. For each vaccine, four mice of each strain were immunized. Results are presented for all mice, on a log scale, as PFU per gram of spleen.

implicating the proteasome in degradation of the ubiquitin-tagged protein. The enhanced degradation of ubiquitin-NP product leads to higher levels of CTL-mediated lysis of transfected target cells when compared to lysis of cells transfected with NP alone (Fig. 3A), and lysis of transfected cells is almost entirely blocked by incubation with the proteasome inhibitor (Fig. 3B), suggesting that the proteasome plays an important role in providing peptides for introduction into the MHC class I antigen presentation pathway. Together these results suggest that covalent ubiquitination of a plasmid-encoded protein improves the entry of its epitope peptides into the class I MHC pathway.

Most importantly, what are the in vivo effects of covalent ubiquitination? We measured the immunogenicity of the plasmid-borne proteins using three criteria, CTL induction, antibody induction, and protective efficacy, and the latter was evaluated in two ways, by peripheral LCMV challenge and by normally lethal i.c. LCMV challenge. In previous studies, we (38–40) and others (23, 41) have found that approximately 50 to 75% of animals immunized with pCMV-NP develop CTL. CTL induction by ubiquitinated NP appeared improved compared to that by NP alone, as all eight mice immunized with pCMV-U-NP showed high levels of lysis following secondary stimulation, while only five of eight mice immunized with pCMV-NP showed similar levels of lysis, and one mouse showed no significant lytic activity (Fig. 5). This enhancement of CTL induction parallels the improved CTL recognition shown in Fig. 3 and is consistent with improved antigen presentation. CTL are critical components of vaccine-induced protection against many viruses, including LCMV (1, 10, 14, 17, 35), and animals immunized with the ubiquitinated NP showed enhanced protection. Vaccinated mice showed more effective control of virus replication; virus was undetectable in all eight mice immunized with pCMV-U-NP, while all mice immunized with pCMV-NP still had 2 to 4 logs of virus at 4 days postchallenge (Fig. 6). Furthermore, survival following a normally lethal virus challenge was enhanced; 100% of mice immunized with plasmid encoding the ubiquitinated protein survived i.c. challenge (Fig. 7), compared to 75% of mice immunized with pCMV-NP. This is the most effective DNA vaccine that we have yet designed. Therefore, covalent ubiquitination may be a feasible approach to enhancing CTL induction and vaccine-induced protection, by DNA immunization or by other means (e.g., by recombinant viral delivery systems).

CTL are not the sole determinants of vaccine-induced immunity; antibodies also are important. For some virus families—in particular the picornaviruses—and for most bacterial infections, antibodies are vital for clearance and protection. We show here that ubiquitinated NP fails to induce antibodies (Fig. 4), despite being able to induce CTL and protection. It appears likely that this is the in vivo reflection of what we found in tissue culture; degradation of the ubiquitinated NP is so rapid and complete that there is insufficient native protein, or appropriate protein fragments, to induce humoral responses capable of recognizing the intact NP. Thus the enhanced ability of covalently ubiquitinated proteins to induce CTL may be to some extent countermanded by a reduced ability to induce humoral responses. This is of obvious potential relevance to vaccine design.

Our findings also address a major question regarding intramuscular DNA immunization; what mechanism underlies CTL induction? Muscle cells (in which most of the protein expression takes place following intramuscular inoculation of plasmid DNA) are rich in ubiquitin, and the ubiquitin-proteasome pathway is extremely active in myocytes of various species, including rodents (2, 24, 28–30). We therefore consider it likely that the rapid degradation seen in several tissue culture lines would be mirrored in muscle cells in vivo. However, a direct role of myocytes in CTL induction is doubted by many, as mycocytes express little if any class I MHC, or costimulatory molecules, and recent studies indicate that antigen-presenting cells (APCs) play a critical role in inducing T cells following DNA immunization (4, 13). There are at least two possible ways in which APCs could be pivotal to the success of DNA immunization. (i) Some APCs (e.g., macrophages) appear able to introduce exogenous protein into the MHC class I pathway (25). Perhaps, as suggested by others (4), transfected myocytes act as a source of soluble protein, which can be taken up by APCs and introduced into the MHC class I pathway. However, we consider it unlikely that CTL are induced by uptake of intact ubiquitinated protein released into solution by myocytes because, as stated above, ubiquitinated NP is probably very rapidly degraded in vivo; and if the level of intact protein released by myocytes is insufficient to induce antibodies, it is probably too low to induce CTL. (ii) APCs might take up the inoculated DNA, and the encoded protein would be synthesized and presented by these cells. This hypothesis is consistent with our observations. We therefore favor the hypothesis that

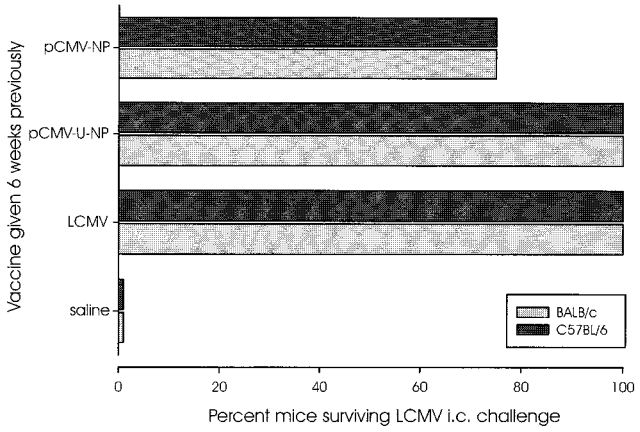

FIG. 7. Ubiquitination of NP enhances protection against normally lethal LCMV challenge. BALB/c and C57BL/6 mice were immunized as shown (with LCMV, saline, pCMV-U-NP, or pCMV-NP). Six weeks later, mice were challenged with a normally lethal dose of LCMV (20 LD_{50} i.c.) and were observed twice daily for 14 days. The percent surviving mice is shown. All deaths occurred between 6 and 8 days p.i.

the mechanism underlying DNA immunization is uptake of DNA, rather than soluble protein, by APCs and that when this DNA encodes a ubiquitinated protein, CTL induction is enhanced whereas antibody induction is obviated.

In conclusion, we have shown that covalent ubiquitination has dramatic effects on the stability and immunogenicity of the attached protein. This technique may be useful in a variety of circumstances. We are currently evaluating approaches which may retain the enhancement of CTL induction while avoiding the concurrent reduction in humoral response.

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