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Identification of a CD4$^+$ T Cell–stimulating Antigen of Pathogenic Bacteria by Expression Cloning

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Summary

Identifying the immunogenic proteins that elicit pathogen-specific T cell responses is key to rational vaccine design. While several approaches have succeeded in identifying major histocompatibility complex (MHC) class I bound peptides that stimulate CD8$^+$ T cells, these approaches have been difficult to extend to peptides presented by MHC class II molecules that stimulate CD4$^+$ T cells. We describe here a novel strategy for identifying CD4$^+$ T cell–stimulating antigen genes. Using *Listeria monocytogenes*-specific, lacZ-inducible T cells as single-cell probes, we screened a *Listeria monocytogenes* genomic library as recombinant *Escherichia coli* that were fed to macrophages. The antigen gene was isolated from the *E. coli* clone that, when ingested by the macrophages, allowed generation of the appropriate peptide/MHC class II complex and T cell activation. We show that the antigenic peptide is derived from a previously unknown listeria gene product with characteristics of a membrane-bound protein.

CD4$^+$ and CD8$^+$ T cells recognize peptide/MHC complexes on the surface of APCs and are normally required for protective immunity. The peptide antigens presented by MHC class II and recognized by CD4$^+$ T cells are of particular interest in that CD4$^+$ T cells have been shown to play a central role in immunity to bacterial pathogens as well as in autoimmune diseases (1–3). The existence of thousands of different peptides bound to the MHC class II molecules on the cell surface, however, makes it very difficult to identify the unique antigenic peptide that is recognized by any one particular T cell (4–6).

In contrast to successful identification of several antigen/MHC class I complexes recognized by CD8$^+$ T cells (7–11), available methods for identifying CD4$^+$ T cell–stimulating antigens have had little success. The difficultie result primarily from conventional methods for generating peptide/MHC class II complexes in APCs and for detecting these APCs by T cell activation assays. In conventional bulk assays, CD4$^+$ T cell responses are usually detected only when APCs are incubated with purified proteins in the nano- to micromolar concentration range. With the exception of the abundantly expressed ribosomal L9 protein that was recently identified as the source of a peptide/E^k complex recognized by tumor-specific CD4$^+$ T cells (12), antigenic proteins from complex extracts have been difficult to obtain in amounts and purity sufficient for their identification. Likewise, antigenic peptides eluted from MHC class II are difficult to purify to homogeneity because only small amounts are expressed by the APCs and they are heterogenous with respect to size (amino acids 13–25) (references 4–6, 13). Alternative antibody-based strategies suffer from the additional disadvantage that only proteins that share B and T cell epitopes can be identified, and it is possible that the proteins that elicit protective T cell immunity are different from those that induce a humoral immune response.

We describe here a novel and simple strategy for genetic identification of CD4$^+$ T cell–stimulating antigens. First, instead of generating peptide/MHC class II complexes by the conventional method of adding proteins exogenously to APCs, we obtained these complexes in macrophages fed with recombinant bacteria that expressed the antigen genes. Second, we detected the expression of these peptide/MHC class II complexes with an exquisitely sensitive single T cell assay (14, 15). Together, these methods allowed us to identify a previously unknown CD4$^+$ T cell–stimulating antigen expressed by the Gram-positive pathogen *Listeria monocytogenes* (LM)[1].

Materials and Methods

Cell Lines, Antibodies, and Mice. All mammalian cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Irvine Scientific, Irvine, CA), 2 mM glutamine, 1 mM pyruvate, 50 μM 2-mercaptoethanol, 200 U/ml penicillin, and 200 μg/ml streptomycin at 37°C in a 5% CO_2/air atmosphere incubator. The BWZ.36 and BWZ.36 CD4$^+$ fusion partners have been described elsewhere (16). The B cell lymphoma LK35.2 (H-2^{kxd}) and the hybridomas 10.2.16 (anti-A^k) and 14.4.4S (anti-E^k) were

[1] *Abbreviations used in this paper:* CPRG, chlorophenol red-β galactoside; HKLM, heat-killed LM; IPTG, isopropyl-β-D-thiogalactopyranoside; LM, *Listeria monocytogenes*; rpm, revolutions per minute; X-GAL, 5-bromo-4-chloro-3-indolyl β-galactopyranoside.

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from American Type Culture Collection (Rockville, MD). The mAb 3.155 (anti-CD8) was kindly provided by Dr. J.P. Allison (University of California, Berkeley). The polyclonal goat anti-rat antibodies were obtained from Southern Biotechnology Associates (Birmingham, AL). Male and female CBA/J ($H-2^k$) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were used between the ages of 2–10 mo.

LM. LM strain 85E0-1167 was an isolate from the California State Health Department kindly provided by Dr. Karen Grant (Department of Public Health, University of California, Berkeley). Initially, LM was streaked out on brain heart infusion broth 1.5% agar plates, and a single clone was isolated and frozen in 10% DMSO. LM was grown in liquid culture in brain heart infusion broth for all other experiments. Heat-killed LM (HKLM) was prepared as follows (17). LM cells from overnight cultures were pelleted for 15 min at 4,000 rpm, resuspended to 10^9 per ml in PBS, and incubated in an 80°C bath for 1 h. The cells were washed once with PBS to remove soluble LM proteins and then resuspended to the same volume in PBS. After verifying the completion of killing by absence of growth on brain heart infusion broth/agar plates, the HKLM was aliquoted and stored at 4°C.

LM-specific T Cells. For immunizations, CBA mice were immunized intraperitoneally with 10^5 LM suspended in PBS. 6 d after immunization, spleens from immunized animals were harvested and depleted of B cells and macrophages by passage over nylon wool columns as described (17, 18). Cells were enriched for $CD4^+$ T cells by panning over anti-CD8 (mAb 3.155)–coated petri dishes. Nonadherent cells were collected and incubated with 10^7 HKLM/ml and 5×10^5/ml irradiated (1,400 rads) syngeneic spleen cells in a medium containing 100 μg/ml gentamicin to enrich in vitro for antigen-specific T cells. After 5 d, cells were harvested, washed, and cultured in complete RPMI + 100 U/ml recombinant human IL-2 for 3 d and fused with the BWZ.36 or BWZ.36 $CD4^+$ fusion partner using polyethylene glycol (PEG 1500; Boehringer Mannheim Biochemicals, Indianapolis, IN), as described (16). Hybrids were screened for specificity using syngeneic CBA splenic cells as APC and HKLM as the antigen.

Antigen/MHC–Specific T Cell Stimulation Assays. 10^5 T cell hybrids were cocultured overnight with the appropriate APC (peritoneal macrophages, syngeneic irradiated spleen, or LK35.2 lymphoma) in medium alone or in the presence of the indicated concentration of antigen. Peritoneal macrophages, used as APC for particulate bacterial antigens, were elicited by intraperitoneally injecting CBA mice with aged thioglycollate 3–4 d before the experiment, as described (18–20). The animals were killed, and the macrophages were collected by washing the peritoneum with PBS; they were plated in 96-well plates at a density of 10^5 cells per well. Plates were centrifuged for 1 min at 1,000 rpm and then incubated at 37°C to allow adherence of macrophages. After 2 h, nonadherent cells were removed by washing; the remaining cells were incubated overnight in medium containing 100 U/ml IFN-γ (Genzyme, Boston, MA). The next day, cells were washed and placed in antibiotic-free medium. The indicated number of bacteria, also in antibiotic-free medium, were added to the activated macrophages. Plates were spun and incubated at 37°C to allow phagocytosis of bacteria. After 1 h, bacteria were washed off and T cells were added in medium with the antibiotic gentamicin (200 μg/ml) to assay the expression of peptide/MHC complexes.

T cell activation was measured as lacZ expression in single cells or in bulk cultures with 5-bromo-4-chloro-3-indolyl β-galactopyranoside (X-GAL; Sigma Immunochemicals, St. Louis, MO) or chlorophenolred-β galactoside (CPRG; Calbiochem, San Diego, CA) substrates, respectively (14–16). Briefly, for single T cell assays, individual cultures were washed once with PBS and the cells were fixed with cold 2% formaldehyde/0.2% gluteraldehyde for 5 min at 4°C. Cells were washed again in PBS and then overlaid with a 100 μl solution containing 1 mg/ml X-GAL, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$ in PBS. The cultures were examined microscopically for the presence of $lacZ^+$ blue cells after 6–18 h at 37°C. For bulk T cell assays, cultures in the microtiter plates were washed once with PBS and then lysed by addition of 100 μl PBS buffer containing 100 μM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP40, and 0.15 mM CPRG. After 4–6 h at 37°C, 50 μl stop buffer (300 mM glycine and 15 mM EDTA in water) was added, and the absorption of each well was determined at 595 nm using a 96-well plate reader. Results of representative experiments are shown as average of replicate cultures, with standard deviations (<10%) omitted for clarity.

Genomic Expression Library from LM. Genomic LM DNA was prepared as described, partially digested with Sau3A, and size fractionated on an agarose gel to isolate 1–4-kb DNA fragments (21). DNA was purified by electroelution and ligated to dephosphorylated BglII cut pTrcHis C vector (Invitrogen, San Diego, CA). The ligated plasmids were transformed into the TOP10F′ strain of *Escherichia coli* by electroporation and plated onto selective (100 μg/ml ampicillin) L broth/agar plates to quantitate transformation efficiency.

Screening of LM Expression Library. Transformed bacteria were resuspended in selective media to give ~100–300 transformants per ml, and 0.1 ml were plated into each well of 96-well microtiter plates. The plates were grown overnight at 37°C with shaking. Replicate cultures were made by splitting the overnight cultures 1:10 into fresh plates and storing the original as the master plate at 4°C. Freshly split plates were grown for 30–45 min at 37°C, after which protein expression was induced by the addition of isopropyl-β-D-thio-galactopyranoside (IPTG; Sigma) to a final concentration of 1 mM. After 2 h of induction, the plates were centrifuged for 2 min at 2,000 rpm, the medium was removed, and the bacteria were resuspended in an equal volume of antibiotic-free medium. 25–75 μl bacteria were added to IFN-γ–activated macrophages. To allow for phagocytosis of bacteria, the macrophages were washed and resuspended in complete medium after 1 h. T cells were added to the cultures, and after overnight incubation, $lacZ^+$ cells were visualized by staining with the X-GAL substrate, as described above.

Analysis of Clone 3A.1.1. The 0.6-kb deletion construct, R10, was made by deleting the 1-kb fragment between the internal- and the flanking vector–derived EcoRI sites in clone 3A1.1. The C7 deletion construct was made by ligating the 1-kb EcoR1 fragment of 3A1.1 into EcoR1 cut and dephosphorylated pTrcHis C vector. This plasmid, C7, contained the 1-kb 3′ fragment in the orientation opposite that in the original 3A1.1 clone. The 815.9 fusion construct was generated by first digesting the 3A1.1 plasmid with Ava1 that removed the DNA between the 5′ Ava1 site in the polylinker and the Ava1 site 3′ to the promoter elements (see Fig. 5). Blunt ends generated by mung-bean nuclease were religated to yield an in-frame fusion of the p3A1 residues with the NH_2-terminal polyhistidine residues in the vector (see Fig. 4 *A*). For analysis of the antigenic protein, overnight cultures of 815.9 bacteria were diluted 1:100 into 1 liter of medium, grown until $OD_{600} = 0.5$, and induced with 1 mM IPTG for 5 h. Total protein extracts were prepared and the metal binding proteins were purified by passage over Ni-NTA resin (Qiagen, Chatsworth, CA), according to the manufacturer's instructions. The eluted proteins were desalted by centrifugation through 5-kD cutoff filters (Milli-

pore, Bedford, MA), and the retentates were used as exogenous antigens with LK35.2 APC, as described above.

Results and Discussion

Generation and Characterization of lacZ-inducible, LM-specific T Cell Hybrids. LM has been extensively studied as a model for immunity to intracellular pathogens (2, 22). Optimal protective immunity to LM is T cell mediated and requires both $CD4^+$ and $CD8^+$ T cells (23–25). Whereas the mechanisms of LM virulence have been studied extensively, only two antigens recognized by $CD8^+$ T cells have been identified recently (8, 26), and relatively little is known about the antigens that elicit LM-specific $CD4^+$ T cell responses (2, 27). To obtain β-galactosidase (lacZ)-inducible, LM-specific T cell hybrids, $CD4^+$ T cells from LM-immunized CBA (H-2^k) mice were fused with the BWZ.36 T cell fusion partner that contains the lacZ gene under transcriptional control of the NFAT element of the IL-2 enhancer (16, 17). As demonstrated previously with other antigens (11, 16), the resulting T cell hybrids expressed lacZ activity specifically upon stimulation with HKLM and syngeneic spleen cells as APC. The LM-specific hybrids were either A^k (LMZ30.4, LMZ23.1) or E^k (LMZ22.2, LMZ25.4) restricted, as indicated by specific inhibition with either 10.2.16 (anti-A^k) or 14.4.4S (anti-E^k) mAbs (Fig. 1). Thus, the LM-specific $CD4^+$ T cell response was heterogeneous.

Isolation of the T Cell–stimulating LM Antigen Gene. To identify the LM antigens recognized by these $CD4^+$ T cells, an expression library was constructed by ligating 1–4 kb LM genomic DNA fragments into the bacterial expression vector pTrc HisC (see Materials and Methods). Expression of recombinant LM proteins was induced in *E. coli* by growing pools of transformed bacteria in 96-well plates and treating the cells with IPTG for 2 h. Aliquots of IPTG-induced bacteria were transferred to another 96-well plate containing syngeneic peritoneal macrophages that were allowed to phagocytose the bacteria for 1 h. After washing off excess bacteria, expression of the antigen/MHC complexes on the macrophage surface was determined by probing with the lacZ-inducible LM-specific T cell hybrids. Finally, the cultures were fixed and stained with the X-GAL substrate to visualize the activated $lacZ^+$ "blue" T cells (15, 16).

With LMZ30.4 (LM/A^k) T cells, bacterial pools were screened for the presence of the corresponding antigen at a complexity of $\sim 10^3$ recombinants per 96-well plate. Macrophages fed with one of the bacterial pools, 3A1, stimulated LMZ30.4 T cells, as indicated by the presence of 50–100 $lacZ^+$ blue cells, over a background of 0–5 blue cells in other wells (Fig. 2, *A* and *B*). The same bacterial pool failed to generate the peptide/MHC complexes recognized by another LM/A^k–specific T cell, LMZ23.1, suggesting that this pool contained only the antigen recognized by LMZ30.4 (Fig. 2 *D*). By repeating the assay with individual colonies derived from pool 3A1, a single bacterial colony, 3A1.1, was obtained that yielded a dramatically higher number of $lacZ^+$ LMZ30.4 T cells (Fig. 2 *C*).

Characterization of Clone 3A1.1 and Its Gene Product. The response of LMZ30.4 T cells to 3A1.1 bacteria was both antigen specific and A^k MHC restricted, as demonstrated by the dose-dependent stimulation of LMZ30.4 but not LMZ23.1 (LM/A^k) T cells and by the specific inhibition of

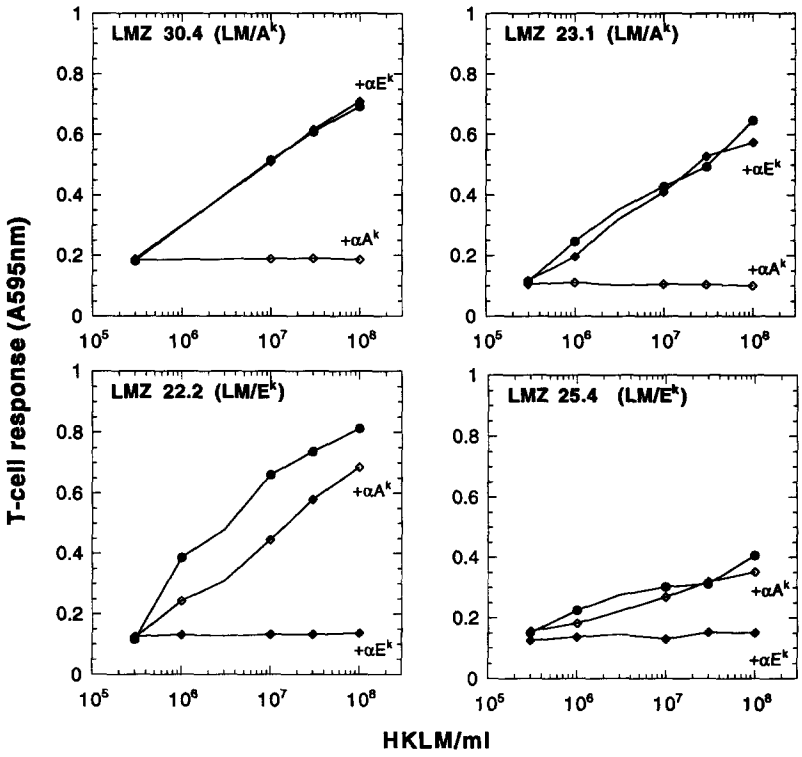

Figure 1. Generation of LM/A^k– and LM/E^k–specific T cell hybrids. 10^5 of the indicated T cell hybrids were cultured overnight with 5×10^5 CBA (H-2^k) spleen cells as APC and varying concentrations of HKLM with medium alone (●) or in the presence of either the anti-A^k antibody (10.2.16) (◇) or the anti-E^k antibody (14.4.4S) (◆). T cell response was measured as induced lacZ activity by using the CPRG substrate.

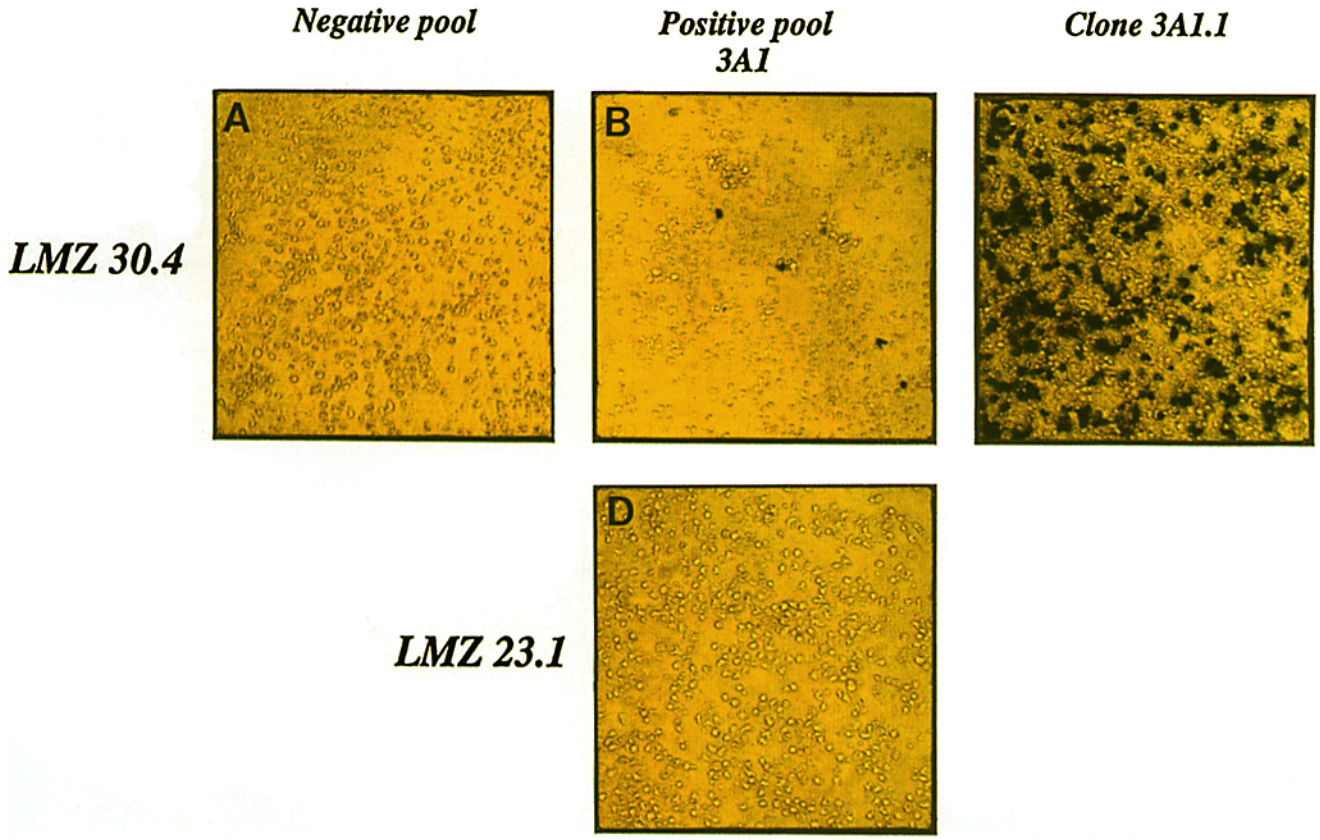

Figure 2. Isolation of the 3A1.1 antigen gene from the LM expression library using the LMZ 30.4 T cell hybrid as a single-cell probe for the cognate peptide/MHC complex. Peritoneal CBA (H-2^k) macrophages were fed pools of recombinant bacteria expressing a genomic LM library. 10^5 LMZ 30.4 T cells were added to the cultures and 24 h later were assayed for presence of $lacZ^+$ cells with the X-GAL substrate (16). The blue $lacZ^+$ cells, indicating the presence of the antigen, are easily visible over the unstained background representing APC and unactivated T cells. The panels show photo micrographs of the LM/A^k–specific LMZ 30.4 T cells responding to (*A*) a negative pool of recombinants, (*B*) the positive pool 3A1, and (*C*) the single colony 3A1.1 isolated from pool 3A1. In (*D*), a different LM/A^k–specific T cell (LMZ 23.1) was not stimulated by the same 3A1 bacterial pool. ×25.

this response with 10.2.16 (anti-A^k) but not 14.4.4S (anti-E^k) mAbs (Fig. 3). The 3A1.1 plasmid therefore encoded the LM gene that allowed expression of the peptide/A^k complex recognized by LMZ30.4 T cells.

The 3A1.1 plasmid DNA was analyzed to identify the antigenic LM protein termed p3A1. The size of the DNA insert in 3A1.1 was 1.6 kb (Fig. 4 *A*). An internal EcoR1 restriction site, ~0.6 kb from the 5′ end of the insert in 3A1.1, as well as an EcoR1 site in the 3′ end of the vector polylinker were used to generate the R10 and C7 deletion constructs of the cloned 3A1.1 DNA (Fig. 4 *A*). The T cell–stimulating activity was retained within the 1-kb DNA insert of C7,

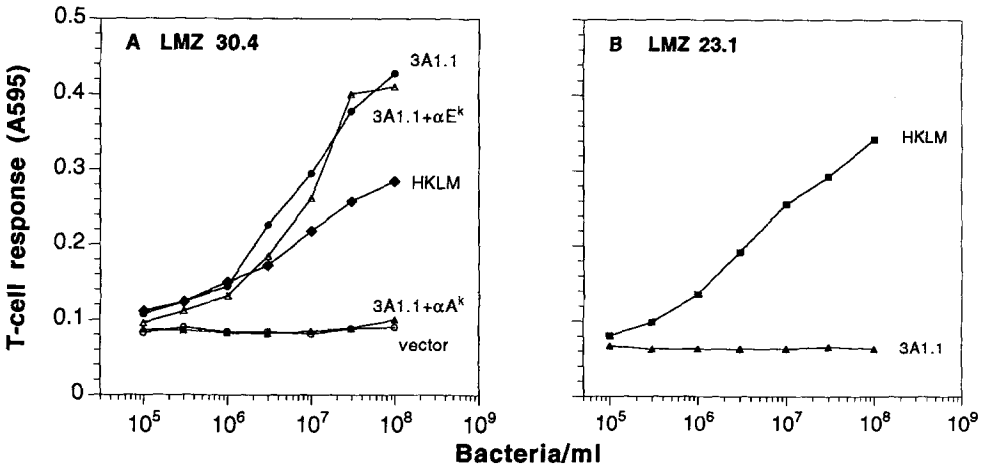

Figure 3. The LMZ 30.4 response to 3A1.1 is antigen specific and A^k restricted. Peritoneal macrophages from CBA mice (H-2^k) were incubated with varying numbers of IPTG-induced bacteria expressing the vector alone (○) or 3A1.1 plasmid in medium (●) or in the presence of the anti-A^k (▲), the anti-E^k (△) mAbs or HKLM (◆) (17). After 1 h, excess bacteria were washed off and the macrophages were incubated with either (*A*) LMZ30.4 or (*B*) LMZ23.1 T cells. T cell response was assayed by measuring the induced lacZ activity with the CPRG substrate (16).

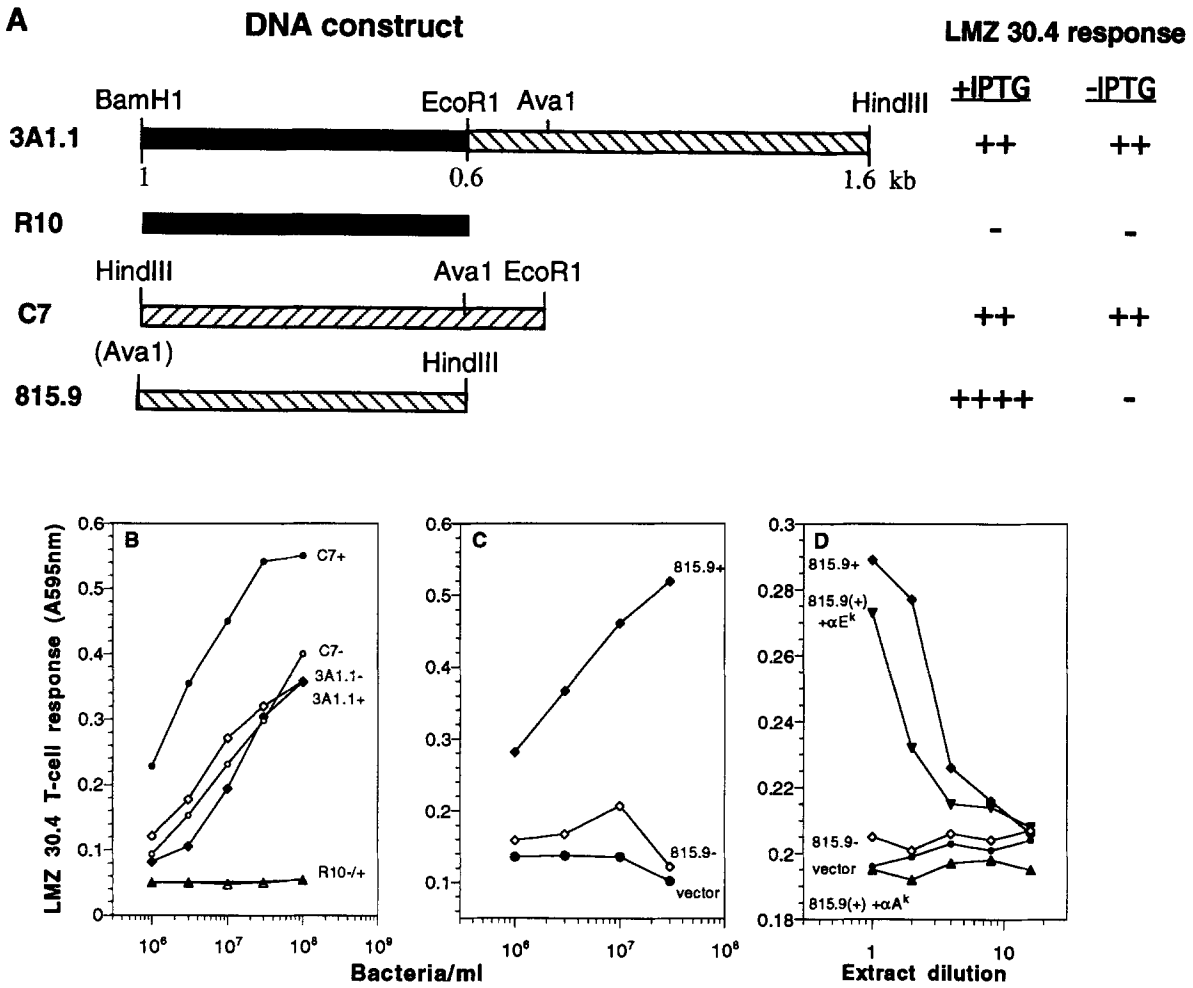

Figure 4. (*A*) Schematic representation of the 1.6-kb 3A1.1 clone, its derivative DNA constructs, and their ability to stimulate the LMZ30.4 T cell response. The DNA fragments are filled in or shaded to indicate their relationship to the original 3A1.1 clone. All constructs were prepared in the bacterial expression vector pTrcHis that allows IPTG-inducible transcription of DNA inserts and expression of in-frame fusion proteins with the metal binding polyhistidine tag. The internal EcoRI, AvaI, and the vector-derived HindIII sites are indicated to show relative orientation of the insert with respect to the 5′ vector–encoded promoter elements. (*B* and *C*) The LMZ30.4-stimulating activity of clone 3A1.1 maps to the 3′ 1-kb DNA fragment and within the p3A1 protein encoded by this fragment (*A*). Bacteria expressing the clone 3A1.1, its deletion constructs C7 or R10 (*B*), or the p3A1 fusion protein 815.9 (*C*) were incubated with (+, *closed symbols*) or without (−, *open symbols*) IPTG for 2 h, and then assayed for their ability to generate the peptide/A^k complex recognized by LMZ30.4 T cells, as in the legend to Fig. 2. (*D*) Extracts of bacteria expressing 815.9 fusion protein contain the LMZ30.4–stimulating antigen. Bacteria expressing the fusion construct 815.9 or the vector alone were treated with (+, *closed symbols*) or without (−, *open symbols*) IPTG for 5 h. Bacterial extracts were passed over a Ni-resin column to enrich the fusion protein, and the eluted proteins were added to 3×10^4 LK35.2 (H-2^{kxd}) cells as APC. The anti-A^k mAb, 10.2.16, or the anti-E^k mAb, 14.4.4, were included in the cultures as indicated.

despite its opposite transcriptional orientation with respect to the IPTG-inducible promoter in the vector (Fig. 4 *B*). Indeed, strong LMZ30.4 T cell responses were observed with either 3A1.1 or its C7 deletion construct, regardless of whether the bacteria were pretreated with IPTG, suggesting that the cloned DNA insert contained constitutive promoter elements for the p3A1 antigen gene (Fig. 4 *B*). This was directly confirmed upon analysis of the nucleotide sequence that revealed the presence of a prokaryotic promoter, and a ribosome binding site preceding the open reading frame encoding 253 residues that extended into the vector to yield a protein of 268 amino acids (Fig. 5). No significant similarities were found for either the DNA sequence or for the predicted p3A1 protein in the sequence databases. A consensus prokaryotic lipid attachment motif (ILGAC) (28, 29) was present within the amino acid sequence (Fig. 5), however, suggesting that the 3A1 protein may be a membrane-bound lipoprotein.

To establish conclusively that this predicted p3A1 protein was the source of the antigenic peptide, the AvaI/HindIII DNA fragment, lacking the promoter elements, was used to generate an IPTG-inducible fusion protein 815.9, with an NH_2-terminal metal binding polyhistidine tag (Fig. 4 *A*). In contrast to the LMZ30.4 response to 3A1.1 and C7 constructs that did not require IPTG induction, the response to bacteria expressing the 815.9 fusion protein was obtained only upon IPTG induction (Fig. 4 *C*). SDS-PAGE analysis of bacterial extracts passed over a Ni-resin column to en-

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                                                  ↓
     GTTTTGACAGAAAGCTCTGACTAACATTATATCTCTCGGGAGGGGTTTTAAGGTGAAAAAGCGTACATTTGCTTTAGCAC
  1  ---------+---------+---------+---------+---------+---------+---------+---------+   80
                                                      V  K  K  R  T  F  A  L  A  L

     TATCAATGATTATTGCTTCTGGCGTTATCCTAGGTGCTTGTGGTTCGAGTAGCGACGATAAAAAAAGTAGCGATGATAAG
 81  ---------+---------+---------+---------+---------+---------+---------+---------+  160
       S  M  I  I  A  S  G  V  I  L  G  A  C  G  S  S  S  D  D  K  K  S  S  D  D  K

     AGCAGTAAAGATTTTACAGTAGCAATGGTTACAGATACTGGTGGCGTTGATGACCGTTCGTTTAACCAATCAGCATGGGA
161  ---------+---------+---------+---------+---------+---------+---------+---------+  240
     S  S  K  D  F  T  V  A  M  V  T  D  T  G  G  V  D  D  R  S  F  N  Q  S  A  W  E

     AGGCTTACAAAAATTTGGTAAAGCTAACGACATGGAAAAAGGTACAGATGGTTACAACTACTTGCAATCAGCTTCTGAAG
241  ---------+---------+---------+---------+---------+---------+---------+---------+  320
      G  L  Q  K  F  G  K  A  N  D  M  E  K  G  T  D  G  Y  N  Y  L  Q  S  A  S  E  A

     CAGACTACAAAACAAACTTAAACACTGCTGTTCGTAGCGATTATGATTTAATTTATGGAATTGGTTACAAACTGAAAGAT
321  ---------+---------+---------+---------+---------+---------+---------+---------+  400
       D  Y  K  T  N  L  N  T  A  V  R  S  D  Y  D  L  I  Y  G  I  G  Y  K  L  K  D

     GCAATTGAAGAAGTTTCTAAACAAAAACCTAAAAATCAATTCGCTATTGTTGATGACACAATTGATGACCGTGATAATGT
401  ---------+---------+---------+---------+---------+---------+---------+---------+  480
     A  I  E  E  V  S  K  Q  K  P  K  N  Q  F  A  I  V  D  D  T  I  D  D  R  D  N  V

     AGTAAGTATTGGATTTAAAGATAACGATGGTTCTTATCTAGTTGGTGTAGTAGCTGGCTTAACAACAAAAACAAATAAAG
481  ---------+---------+---------+---------+---------+---------+---------+---------+  560
      V  S  I  G  F  K  D  N  D  G  S  Y  L  V  G  V  V  A  G  L  T  T  K  T  N  K  V

     TTGGATTTGTTGGTGGAGTAAAAGGAACTGTTATCGACCGTTTTGAAGCTGGTTTCACTGCTGGTGTAAAAGCTGTTAAT
561  ---------+---------+---------+---------+---------+---------+---------+---------+  640
       G  F  V  G  G  V  K  G  T  V  I  D  R  F  E  A  G  F  T  A  G  V  K  A  V  N

     CCTAACGCACAAATTGATGTACAATATGCAAACGATTTCGCTAAAGCAGACAAAGGACAACAAATCGCTTCTTCCATGTA
641  ---------+---------+---------+---------+---------+---------+---------+---------+  720
     P  N  A  Q  I  D  V  Q  Y  A  N  D  F  A  K  A  D  K  G  Q  Q  I  A  S  S  M  Y

     TTCGAGTGGAGTTGACGTAATTTTCCATGCTGCTGGTGGTACTGGTAACGGTGTCTTTGCAGAAGCTAAAAATCTGAAGA
721  ---------+---------+---------+---------+---------+---------+---------+---------+  800
      S  S  G  V  D  V  I  F  H  A  A  G  G  T  G  N  G  V  F  A  E  A  K  N  L  K  K

     AAAAAGATCTGCAGATGGTACCATATGGGAATTCGAAGCTTGGCTGTTTTGGCGGATGA
801  ---------+---------+---------+---------+---------+---------+---------  859
       K  D  L  q  m  v  p  y  g  n  s  k  l  g  c  f  g  g  *
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Figure 5. The nucleotide and predicted amino acid sequence of the antigenic LM protein that is recognized by LMZ30.4 T cells. The p3A1 open reading frame is preceded by consensus sequences of the prokaryotic promoter and the ribosome binding site shown in bold letters. The lipid attachment site characteristic of membrane lipoproteins is underlined (28, 29). The 253 amino acids encoded by the insert are in uppercased single-letter code; those encoded by the vector are in lowercase. The vertical arrow indicates the 5′ end of the fragment used to construct the 815.9 fusion protein.

rich the fusion protein revealed a faint band of 33 kD that corresponded to its predicted size (data not shown). Finally, only the extracts of IPTG-induced 815.9 bacteria were active in stimulating the A^k MHC–restricted response of LMZ30.4 T cells when added exogenously to LK35.2 (H-2^{kxd}) lymphoma cells as APC (Fig. 4 *D*). This result demonstrates formally that the antigenic activity was due to the p3A1 protein itself rather than to an indirect effect of the ingested bacteria on some other cellular gene. Taken together, these results establish that the peptide presented by A^k MHC class II molecules to LMZ30.4 T cells is derived from the p3A1 protein.

The p3A1 protein stimulated the LM/A^k–specific hybrid LMZ30.4 but none of five other LM/A^k– or LM/E^k–specific hybrids that were tested (Fig. 3 *B* and data not shown), indicating the existence of heterogeneity in the $CD4^+$ T cell responses to pathogenic LM bacteria. This result confirms previous results based on fractionation and analysis of complex bacterial extracts (30, 31). Efforts to identify these other LM antigens are in progress. Future studies on the role of this and other LM antigens in eliciting protective immunity to live LM as well as their role in the life cycle of the pathogen will yield additional insights into the mechanism of virulence and immunity to intracellular pathogens.

In conclusion, we have established a novel expression cloning strategy for identifying antigens presented by MHC class II molecules that are recognized by $CD4^+$ T cells. Because antigenic proteins of either prokaryotic or eukaryotic origin can be expressed in *E. coli* and allow generation of peptide/MHC class II complexes (20, 32), we expect this strategy to be widely applicable. Extension of these methods are likely to allow the identification of other $CD4^+$ T cell–stimulating antigens that provide immunity to pathogenic microorganisms such as *Mycobacterium tuberculosis* or *Mycobacterium leprae* as well as those involved in autoimmune disorders.

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