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Optimal immunity to the Gram-positive pathogen Listeria monocytogenes (LM) requires both CD8+ and CD4+ antigen-specific T cell responses. Understanding how CD4+ T cells function in an immune response to LM and how bacterial proteins are processed to peptide/MHC class II complexes in infected cells requires identification of these proteins. Using LacZ-inducible, LM-specific CD4+ T cells as probes, we identified two immunogenic LM proteins by a novel expression cloning strategy. The antigenic peptides contained within these proteins were defined by deletion analysis of the genes, and their antigenicity was confirmed with synthetic peptides. The nucleotide sequences of the genes showed that they encode previously unknown LM proteins that are homologous to surface proteins in other bacterial species. Consistent with their surface topology, mild trypsin treatment of LM protoplasts ablated T cell recognition of these Ags. These findings establish a general strategy for identifying unknown CD4+ T cell Ags and demonstrate that LM surface proteins can provide the peptides for presentation by MHC class II molecules that are specific targets for CD4+ T cells during murine LM infection.

While numerous studies have addressed the function of CD4+ T cells during LM infection, little is known about the bacterial Ags that elicit these T cells. The large number of intra- and extracellular proteins expressed by a bacterium such as LM raises the question of which bacterial proteins have access to the MHC class II processing pathway and can therefore be presented on the cell surface as peptide/MHC class II complexes to CD4+ T cells. Studies using fractionated LM extracts and LM expression libraries have indicated that a large number of proteins may be antigenic for CD4+ T cells (15–17). However, the nature of these Ags remains poorly understood, and it is presently unclear which of these proteins is capable of eliciting protective CD4+ T cell responses. Furthermore, to understand how the location of the Ag within the bacteria, the location of the bacteria within an infected APC, and the bactericidal capabilities of the APC together determine which LM proteins access the MHC class II processing pathway, it is first essential to identify the Ags recognized by the CD4+ T cells during LM infection.

Identifying antigenic peptides presented by MHC class II, and their source proteins, derived from complex Ags, has been extraordinarily difficult (18, 19). To date, only two LM proteins, the secreted hemolysin listeriolysin-O (LLO) (20) and the 3A1.1 protein (21), have been conclusively identified as targets of CD4+ T cells. Of these, the peptides presented by the MHC class II molecules have been defined only for the LLO protein. Here, we first identify the antigenic peptide of the 3A1.1 protein that is presented by the Aβ MHC class II molecule and recognized by the LM-specific CD4+ T cell hybrid LMZ30.4. Further, by using the ability of peritoneal macrophages to phagocytose and present Ags that are expressed in recombinant Escherichia coli as peptide/MHC class II complexes, we screened an LM DNA library and isolated another novel Ag gene, termed 12A4.G7. Deletion analysis of this gene led to the identification of the T cell-stimulating peptide presented by the Aβ MHC class II molecule. Sequence analysis revealed that both 3A1.1 and 12A4.G7 are homologous to prokaryotic lipoproteins that are targeted to the bacterial surface. Consistent with their proposed surface localization, mild trypsin treatment of LM protoplasts ablated the T cell response to the 3A1.1 and 12A4.G7 Ags. These findings demonstrate that surface proteins of LM are targeted for immune recognition by CD4+ T cells.

Materials and Methods

Cell lines, Abs, and mice

All mammalian cells were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 1 mM pyruvate, 50 μM 2-ME, 200 U/ml penicillin, and 200 μg/ml streptomycin (complete RPMI) at 37°C in a 5% CO2/air atmosphere. The LacZ-inducible, LM-specific T cell hybridomas...
LMM22.2, LMM23.1 and LMM30.4 have been described previously (21, 22). The B cell hybrid LKM35.2 (H-2k) and the mAb-secreting hybridomas 10.2.16 (anti-Ak) and 14.4.4S (anti-Ek) were obtained from American Type Culture Collection (Rockville, MD). Male and female CBA/J (H-2s) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used between the ages of 2 and 10 mo.

Listeria monocytogenes

Strain S5E0-1167, used to prepare the LM genomic expression library, was an isolate of the California State Health Department provided by Dr. Karen Grant (Department of Public Health, University of California-Berkeley). All other experiments were performed with strains 43250 and 43251 purchased from the American Type Culture Collection (Rockville, MD). All strains were grown in liquid culture in brain heart infusion broth. LM protoplasts were prepared by growing the bacteria overnight in a 25-ml culture in complete RPMI, pelleting by centrifugation at 1900 g, resuspending the protoplasts in 5 ml equal parts of PB (1.6% nutrient broth, 1.4% tryptone, and 1.2% yeast extract) and 2× SMM (1 M sucrose, 0.01 M MgCl₂, 0.04 M maleic acid, and 0.02 M Tris, pH 6.8). The cells were then resuspended in 5 ml equal parts 2× SMM and 1× protoplast broth containing equal parts of PB (1.6% nutrient broth, 1.4% tryptone, and 1.2% yeast extract) and 2× SMM (1 M sucrose maleic acid (SMM) and 1× protoplast broth containing equal parts of PB (1.6% nutrient broth, 1.4% tryptone, and 1.2% yeast extract) and 2× SMM) for 30 min at 37°C.

Ag/MHC-specific T cell stimulation assays

T cell hybrids (10⁶) were cocultured for 18 to 24 h with the appropriate APC (peritoneal macrophages, fresh splenocytes, or LK 35.2 cells) in medium alone or with the indicated concentration of Ag. Peritoneal macrophages were elicited by i.p. injection of CBA/J mice with aged thioglycolate (Difco, Detroit, MI). The mice were killed 3 or 4 days later, and the macrophages were harvested by peritoneal lavage with PBS, plated out in 96-well plates at a density of 10⁶/well in complete RPMI, and incubated at 37°C to allow adherence. After 2 to 5 h, nonadherent cells were washed away, and the remaining cells were overlaid with complete RPMI and 100 U/ml recombinant murine IFN-γ (Genzyme, Boston, MA). The following morning the cells were washed with PBS and plated out with antibiotic-free RPMI. The indicated number of bacteria, also in antibiotic-free RPMI, were added, and the cultures were spun for 2 min at 850 × g before being incubated for 1 h at 37°C to allow phagocytosis of bacteria by macrophages. Bacteria were then washed off, and 10⁴ T cells/well were added in RPMI and 100 μg/ml gentamicin. The cultures were incubated at 37°C for at least 18 h before T cell activation was assayed by adding chlorophenol red-β-D-galactopyranoside (CPRG; Calbiochem, San Diego, CA). The cultures were washed once with 100 μl of PBS and then lysed by the addition of PBS buffer containing 100 μM 2-ME, 9 mM MgCl₂, 0.125% Nonidet P-40, and 0.15 mM CPRG (23). After 6 to 10 h at 37°C, 50 μl of stop buffer (300 mM glycine and 15 mM EDTA) was added, and the absorbance of each well at 595 nm was measured using a 96-well plate reader. The results of representative experiments are shown as the average of replicate cultures, with SDs (<10%) omitted for clarity.

LM expression library screening

E. coli strain Top10F’ cells (Invitrogen, San Diego, CA) transformed with the LM genomic library were resuspended in LB and 100 μg/ml ampicillin to yield about 100 transformants/ml, and 0.1 ml/well was plated out in round-bottom 96-well plates and grown overnight at 37°C in a shaker. Replicate plates were made, and the originals were stored as master plates at −70°C. The transformants were grown at 37°C with shaking for 45 to 60 min before recombinant protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG; Boehringer Mannheim, Indianapolis, IN) to a final concentration of 1 mM. After 2 h of induction, bacteria were pelleted by centrifugation at 1900 × g for 5 min, the medium was removed, and the cells were resuspended in 125 μl of antibiotic-free RPMI. One hundred microliters of each sample was added to IFN-γ-activated peritoneal macrophages, which were used in a T cell stimulation assay as described above.

Plasmid DNA and synthetic peptides

The pTrHisC E. coli expression vector was purchased from Invitrogen (San Diego, CA). The LM expression library was made by isolating and ligating 2- to 4-kb fragments of LM genomic DNA that had been partially digested with the restriction enzyme Sau3A1 into the BglII site of the pTrHisC vector. The 12A4 G712A construct was made by digesting 12A4 G7 DNA with the indicated restriction enzyme, removing the DNA between the unique EcoRI or HindIII sites, and ligating to yield the desired deletion constructs. The GST-12A4.G7 construct as well as all GST fusion constructs used in identification of the 12A4.G7 and 3A1.1 antigenic peptides were made using PCR to amplify a region of the 12A4.G7 or 815.9 (His₆-tagged 3A1.1) (21) plasmid that contained the indicated regions of the 12A4.G7 or 815.9 inserts and the upstream BamHI site of the pTrHisC multiple cloning site. The amplification products were then digested with BamHI and the appropriate fragments were isolated and ligated into the pGex3X GST fusion vector (Phar- macia, Uppsala, Sweden) that had been digested with BamHI and Smal. Synthetic peptides were prepared by automated solid phase synthesis using the Applied Bioscience model 421 synthesizer (Foster City, CA).

Protein analysis of 12A4.G7

Overnight cultures of 12A4.G7 were diluted 1/10 into LB medium and grown with shaking at 37°C for 1 h before expression of the recombinant 12A4.G7 protein was induced by the addition of IPTG to a final concentration of 1 mM. The bacteria were then grown with shaking for 5 h at 37°C. 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 de- salted by centrifugation through 5-kDa cut-off filters (Millipore, Bedford, MA), and the retentates were used as exogenous Ags with LK35.2 cells as APC as described above.

Southern blots and isolation of 12A4.G7 and 3A1.1 genomic fragments

Ten micrograms of LM genomic DNA was digested with either EcoRI or HindIII as indicated, and the resulting fragments were separated on 1% agarose gels. The gels were soaked in 0.25 M HCl for 15 min followed by 0.4 M NaOH for 15 min before DNA was transferred overnight onto Zeta- Probe (Bio-Rad, Hercules, CA) filters. After transfer, the filters were baked at 80°C for 30 min before they were probed with the indicated 32P-labeled DNA fragments according to standard procedures. The 12A4.G7 probe corresponds to the 1.7-kb EcoRI/Khol fragment of the 12A4.G7∆EcoRI construct, while the 3A1.1 probe corresponds to the 0.8-kb EcoRI/BamHI of the GST-3A1.1 construct.

LM genomic DNA was digested with either EcoRI or HindIII, and 1- to 2-kb EcoRI fragments as well as 3- to 6-kb HindIII fragments were isolated electrophoretically. Libraries were constructed by ligating these fragments into either the EcoRI or HindIII site of pTrHisC. These libraries were transformed into the Top10F’ strain of E. coli, and transformants were plated out on LB agar containing 100 μg/ml ampicillin. Colonies were lifted onto nitrocellulose filters and lysed according to standard procedures. The 1- to 2-kb EcoRI library was probed with the 32P-labeled 3A1.1 probe described above, and the 3- to 6-kb HindIII library was probed with the 32P-labeled 12A4.G7 probe. Hybridizing colonies were subsequently cloned, and their inserts were sequenced.

Results

Identifying the antigenic peptide recognized by the LM-specific hybridoma LMMZ30.4

We previously isolated the LM gene, termed 3A1.1, that encodes the antigenic peptide presented by A₃ MHC class II molecules to the LM-specific CD₄⁺ T cell hybrid, LMMZ30.4 (21). To identify the precise antigenic peptide within the 3A1.1 gene, a series of 3’ deletion fragments were prepared in the prokaryotic expression vector pGex3X (Fig. 1A). Bacteria expressing each deletion construct were tested for their ability to stimulate LMMZ30.4 T cells following phagocytosis by CBA/J (H-2²) peritoneal macrophages (Fig. 1B). T cell activation assays with the first series of deletions that eliminated residues C-terminal to amino acids (aa) 190, 127, and 65 revealed that the antigenic activity was dependent upon residues 128 to 190. The second series of deletions showed that the antigenic activity was contained within aa 148 (Δ148E construct), but was lost when residues C-terminal to aa 141 were deleted (Δ141E construct). To definitively establish the antigenicity of the inferred amino acid sequence, a set of three N-terminally nested peptides (aa 128–148; Fig. 1C) was synthesized, and each peptide was tested for its ability to stimulate LMMZ30.4 T cells upon addition to the B cell hybrid LK35.2 (H-2k) used as APC. Each synthetic peptide was a potent stimulator of LMMZ30.4 T cells (Fig. 1D).
but the 13-aa peptide is slightly less active than the 17- or 21-aa peptides. We therefore conclude that the 3A1.1 peptide containing aa 132 to 148 was sufficient for maximal T cell stimulation and therefore defines the region of the 3A1.1 protein that is processed and presented by the Ak MHC class II molecule during LM infection.

Expression cloning a novel LM Ag gene presented as a peptide/MHC class II complex

The anti-LM CD4\(^+\) T cell response involves the recognition of multiple Ags as demonstrated by lack of reactivity of the LM/Ak-specific LMZ23.1 T cell hybrid to the 3A1.1 protein (21). The secreted hemolysin LLO has also been shown to contain Ak-restricted T cell epitopes, and it has been suggested that LLO is an immunodominant CD4\(^+\) T cell Ag (24). However, the LMZ23.1 T cell responds equally well to both LLO\(_1\) and LLO\(_2\) LM strains (data not shown), demonstrating that this T cell is not LLO specific, but instead recognizes a different LM protein.

To identify the LMZ23.1 Ag, we used an expression cloning strategy developed in our laboratory and schematically illustrated in Figure 2 (21). A genomic LM library was prepared by ligating 2- to 4-kb partially digested Sau3A1 chromosomal DNA fragments into the \(Bgl\) II site of the IPTG-inducible, prokaryotic expression vector pTrcHisC. This \(Bgl\) II site lies within a short open reading frame (ORF) that encodes a polyhistidine tag and is under the transcriptional control of the inducible Trc promoter. Each recombinant plasmid thus potentially encodes a polyhistidine-tagged LM fusion protein. The library was transformed into \(E. coli\), and pools containing approximately 10 CFU each were grown in 96-well plates overnight. The cultures were replica plated into new 96-well plates, and recombinant protein expression was induced by adding IPTG. Aliquots of each pool were then cocultured with CBA/J peritoneal macrophages, which phagocytose the recombinant bacteria and present bacterial protein Ags as peptide/MHC class II complexes (21, 25). Ag-containing pools were identified by adding LMZ23.1 T cells, which express LacZ upon Ag recognition, to each well. T cell activation was assayed by lysing cultures and measuring the hydrolysis of the colorimetric LacZ substrate CPRG. After screening approximately 1200 pools (\(\approx 12,000\) CFU), one pool was found that upon coculture with CBA/J peritoneal macrophages, which phagocytose the recombinant bacteria and present bacterial protein Ags as peptide/MHC class II complexes (21, 25), Ag-containing pools were identified by adding LMZ23.1 T cells, which express LacZ upon Ag recognition, to each well. T cell activation was assayed by lysing cultures and measuring the hydrolysis of the colorimetric LacZ substrate CPRG. After screening approximately 1200 pools (\(\approx 12,000\) CFU), one pool was found that upon coculture with CBA/J macrophages, generated the peptide/MHC complex recognized by LMZ23.1 T cell (Fig. 3A). This pool was then fractionated, and several identical clones that stimulated LMZ23.1 were isolated, one of which, termed 12A4.G7, was used in subsequent experiments (Fig. 3B).

Characterization of the 12A4.G7 clone

The 12A4.G7 clone contained a 4.7-kb insert (Fig. 4A). The unique HindIII and EcoRI restriction sites within the insert were used to generate 3’ deletion constructs of the 12A4.G7 clone. \(E. coli\) transformed with either the 12A4.G7\(_3\) HindIII or 12A4.G7\(_3\) EcoRI construct retained the ability to stimulate LMZ23.1 T cells. Furthermore, the T cell response to either the original 12A4.G7 or its deletion constructs was dependent upon treating the bacteria with...
IPTG (data not shown), suggesting that 12A4.G7 encoded a polyhistidine-tagged antigenic fusion protein. The nucleotide sequence of the 5' end of the 12A4.G7 insert revealed a 75-aa ORF fused in-frame with the vector-encoded polyhistidine tag. To determine whether this ORF encoded the antigenic peptide, we prepared a construct (12A4.G7 5' ORF) containing only the 5' 231 nucleotides of the 12A4.G7 insert, which encoded the 75-aa ORF, fused in-frame with GST in the prokaryotic expression vector pGex3X. E. coli expressing this construct retained the ability to stimulate LMZ23.1 cells, strongly suggesting that the antigenic peptide was encoded within this ORF (Fig. 4B). To formally demonstrate that the antigenic activity was contained within the polyhistidine tagged fusion protein, we purified this protein from lysates of IPTG-induced 12A4.G7 bacteria by elution from a nickel resin. SDS-PAGE analysis of purified proteins revealed the presence of a band at approximately 13 kDa, the predicted molecular mass of the fusion protein (data not shown). This purified protein was a potent stimulator of the LMZ23.1 T cells. Furthermore, inclusion of anti-Ak, but not the irrelevant anti-Ek Abs in the cultures blocked this response, conclusively demonstrating that this short 75-residue ORF contained the antigenic epitope recognized by LMZ23.1 T cells.

To identify the LMZ23.1-stimulating peptide encoded within the 12A4.G7 gene, a PCR-mediated deletion analysis was performed on the 12A4.G7 5' ORF similar to that used to identify the LMZ30.4-stimulating peptide. After two rounds of deletions, the C-terminal boundary of the antigenic peptide was determined to lie between residues 51 and 42 of the 75-aa 12A4.G7 5' ORF (Fig. 5, A and B). To conclusively establish this assignment, a set of three N-terminally nested peptides was synthesized and tested for their ability to stimulate LMZ23.1 T cells with LK35.2 cells as APC (Fig. 5, C and D). All three synthetic peptides were active, and maximal responses were obtained with a peptide corresponding to aa 31 to 50 of the 12A4.G7 5' ORF. We conclude that this or a closely related peptide/Ak complex was generated during the LM infection and was immunogenic for CD4+ T cells.

Cloning and characterization of 3A1.1 and 12A4.G7 genomic fragments

Both 3A1.1 and 12A4.G7 were initially cloned as truncated gene fragments. The 3A1.1 clone encodes 253 aa but lacked the 3' translational termination codon (21), and the 12A4.G7 clone contained only a short ORF encoding the C-terminal 75 aa of the antigenic protein. To gain insight into the possible function and intracellular locations of these immunogenic proteins, we cloned larger genomic fragments containing these genes. First, Southern blot analysis of LM DNA was performed using 3A1.1 and 12A4.G7 clones as probes. The 3A1.1 probe hybridized to a unique 1.2-kb EcoRI fragment (Fig. 6A), which was then isolated from an LM library of EcoRI fragments. The 12A4.G7 probe hybridized to two distinct genomic fragments in both HindIII- and EcoRI-digested DNA (Fig. 6A; data not shown). It is possible that the second band corresponds to another gene closely related to 12A4.G7. Alternatively, because only 231 bp in the 1.7-kb probe represented the 12A4.G7 gene itself, the second band could also be due to hybridization to this yet uncharacterized region. The 1.7-kb probe was then used to screen a library of LM HindIII fragments,

FIGURE 2. Schematic representation of the strategy used to isolate the LM-specific CD4+ T cell-stimulating Ag gene. A genomic LM library was made in the E. coli expression vector pTrcHisC. The library was divided into pools, which were grown in replica plates, and protein expression was induced with 1 mM IPTG. Aliquots of each pool were then cocultured with IFN-γ-activated, thioglycolate-elicited peritoneal macrophages from CBA/J mice. After 1 h of coculture, the bacteria were washed off, and LMZ23.1 T cells were added. T cell activation was assayed after 18 to 24 h. The bacterial colony containing the Ag gene was obtained by limiting dilution from the corresponding well of the original master plate.
and a 3.6-kb HindIII fragment was isolated and, as shown below, contained the 12A4.G7-coding region.

The nucleotide sequence of the cloned EcoRI fragment revealed the complete ORF encoding the 3A1.1 protein. This gene encodes a 357-aa protein with a consensus prokaryotic lipoprotein signal sequence at its N-terminus (Fig. 6B). BLAST searches (26) and protein sequence alignments showed that the 3A1.1 protein is 57% identical with the yufN lipoprotein discovered within the complete sequence of the \textit{Bacillus subtilis} genome (27). The yufN protein, based upon its sequence features, has been assigned a putative ABC transporter function. The 3A1.1 sequence is also 40% identical with the TmpC lipoprotein of the spirochete \textit{Treponema pallidum}, a 35-kDa surface protein recognized by anti-\textit{T. pallidum} Abs (28).

The cloned 3.6-kb HindIII fragment is truncated at the 5' end of the 12A4.G7 gene, but encodes a total of 387 aa (Fig. 6C). The protein sequence of this region of 12A4.G7 is 32% identical with the oligopeptide binding protein appA of \textit{Bacillus subtilis} (29). The appA protein is a member of the prokaryotic cluster 5 extracellular solute binding protein family (30). Based on this sequence similarity, we propose that 12A4.G7 may also bind extracellular ligands and deliver them to ABC-type transporters for translocation across the bacterial membrane. In Gram-positive bacteria, all members of this protein family contain the same N-terminal lipoprotein signal sequence as 3A1.1.

In Gram-positive bacteria such as LM, a lipoprotein signal sequence directs the cotranslational insertion of a protein into the bacterial membrane before it is cleaved off the protein, and lipid moieties are added to an N-terminal cysteine residue. These attached lipids are then believed to tether the lipoprotein to the extracellular face of the bacterial membrane (31, 32). Therefore, both 3A1.1 and 12A4.G7 could be targeted to the bacterial surface with access to the class II MHC Ag processing pathway in infected cells.

**Discussion**

We have developed a novel expression cloning strategy for identifying CD4\(^+\) T cell Ags. This strategy is based upon the ability of peritoneal macrophages to phagocytose recombinant \textit{E. coli}, leading to the processing and presentation of bacterial Ags on MHC class II molecules for recognition by LacZ-inducible T cell hybridomas. Using this strategy, we have identified two antigenic proteins derived from the pathogenic bacterium LM. This strategy has several advantages over existing methods for CD4\(^+\) T cell identification. It leads to the direct identification of the CD4\(^+\) T cell Ags.
cell-stimulating Ag gene, bypassing the need for any protein or peptide purification. While peptide (34) and protein (35) purification have been successfully used to identify MHC class II-restricted Ags, this expression cloning method may be particularly suitable for the identification of rare Ags that are expressed at low copy numbers or are otherwise difficult to purify. Other strategies for expression cloning CD4+ T cell Ags have relied on Ab screening (36, 37) or epitope tagging followed by affinity purification of the recombinant proteins (38). By directly feeding recombinant E. coli to macrophages, we have eliminated these procedures, which may bias the expression library to proteins that are also B cell Ags or proteins that retain their epitope tags during manipulations. This feature proved useful in the initial identification of the 3A1.1 Ag gene, which was found not as an IPTG-inducible polyhistidine-tagged fusion protein as was expected, but instead contained its own LM-derived promoter that allowed expression of the antigenic protein in E. coli host cells (21). The utility of bacterial expression libraries for identifying T cell Ags has also been demonstrated by the recent identification of the LM epitope recognized by H2-M3-restricted CD8+ T cells (39).

The availability of the Ag gene provides a relatively simple method for identifying the processed antigenic peptide that is presented by MHC class II molecules to T cells. Two methods have been traditionally used to identify CD4+ T cell-stimulating peptides. The first is based on the proteolytic digestion of purified antigenic proteins followed by HPLC fractionation of the resulting peptides to isolate and sequence those that retain antigenic activity. However, this type of analysis does not lend itself well to proteins that are difficult to isolate in sufficient quantity. T cell-stimulating peptides have also been identified by making a series of overlapping peptides for the entire protein sequence and testing each for antigenic activity. For large antigenic proteins, this is an expensive and inefficient method. The availability of the 12A4.G7 and 3A1.1 Ag genes allowed us to generate a series of deletion constructs, which led to the rapid identification of the relevant peptides responsible for T cell stimulation. The activity of synthetic peptides corresponding to these regions confirmed the results of the deletion analysis. Recently, it has been reported that Ak uses an aspartic acid residue as a primary anchor in peptide binding (40). Both the 3A1.1 and 12A4.G7 antigenic peptides contain several aspartic acid residues, but which of these serve as Ak binding anchor(s) is not yet known.

Both the 3A1.1 and 12A4.G7 proteins are similar in sequence to proteins from the closely related bacterium Bacillus subtilis with proposed functions in ABC transporter systems on the cell surface. Complete sequencing of the B. subtilis genome has revealed the existence of 77 potential ABC transporter genes (27). It has therefore been suggested that these transporter systems are a major protein component of the surface of B. subtilis, and the same may be true for LM as well. The surface localization of these proteins may explain how they access the class II MHC Ag processing pathway and are therefore targeted for specific immune recognition. Based on the average prokaryotic gene density of one gene per 1.1 kb of DNA revealed by the sequencing of a number of bacterial genomes (27, 41–46), the 3.15-megabase LM genome (47) encodes approximately 2800 proteins, all of which could potentially serve as CD4+ T cell Ags. While fractionation of LM extracts (17) and protective immunizations with heat-killed LM (48) have demonstrated that both secreted and cell-associated bacterial proteins are recognized during murine infection, before the identification of 3A1.1 and 12A4.G7 proteins, the only known CD4+ T cell epitopes of LM were derived from the secreted LLO protein (20). Therefore, the identity of the cell-associated proteins capable of accessing the MHC class II Ag-processing pathway was
unknown. For instance, for proteins localized to the bacterial cytoplasm to be processed, the cell wall and cell membrane of the bacterium must be breached in a compartment that is part of the MHC class II-processing pathway. The likelihood of this occurring depends on the bactericidal capabilities of the APC, the distribution of MHC class II molecules within that cell, and the nature and life cycle of the invading bacterium. In vitro experiments using recombinant *E. coli* (49) and *Salmonella typhimurium* (50) expressing a defined lysozyme epitope demonstrated that intracellular localization of the Ag had little effect on its presentation by peritoneal macrophages. Unlike LM, however, upon entry into the APC these Gram-negative organisms remain in the phagosome. In contrast, after entry of LM into host cells, many bacteria rapidly escape from the phagosome into the cytosol through the action of the secreted hemolysin LLO and therefore exit the primary MHC class II Ag-processing pathway. Concordant with this altered localization, LLO activity inhibits the presentation of MHC class II Ags in vitro (51). In addition, live LM alter the trafficking and delay the maturation of the phagosome (52, 53). Each of these events may profoundly affect which bacterial proteins are made available for presentation by MHC class II. We demonstrate here for the first time that proteins targeted to the LM surface access the MHC class II-processing pathway and are recognized by CD4 T cells during murine LM infection. Consistent with this idea, other studies of intracellular pathogens, both bacterial and protozoan, have indicated that surface proteins could be targets of CD4 T cells (34, 54, 55). The role of this class of Ags in eliciting protective immune response to virulent and avirulent LM strains can now be addressed by analyzing the relative frequency of peptide-specific CD4 T cells.

**FIGURE 6.** 12A4.G7 and 3A1.1 Southern blots and sequences. **A**, LM genomic DNA was digested completely with EcoRI (3A1.1 panel) or HindIII (12A4.G7 panel). The fragments were separated electrophoretically, transferred to nitrocellulose filters, and probed with 32P-labeled fragments of the 3A1.1 and 12A4.G7 genes. The size of each band is indicated in the margin. **B**, Complete sequence of the 3A1.1 gene with its predicted translation product. The lipid attachment sequence is in italics and underlined. The LMZ30.4-stimulating peptide is in boldface and underlined. **C**, Available sequence of the 12A4.G7 ORF within the 3.6-kb HindIII fragment. The LMZ23.1-stimulating peptide is in boldface and underlined.
The identification of LM proteins that enter the MHC class II presentation pathway could also be useful in developing recombinant LM as a vaccine vector. Protective immune responses to a number of pathogens, including *Leishmania major* and *Plasmodium falciparum*, are associated with the generation of pathogen-specific Th1 CD4+ T cells (56). Because LM induces a strong CD4+ T cell response polarized toward the Th1 phenotype, LM is an attractive candidate for the production of recombinant vaccines against these and other infectious agents. For this vaccination strategy to work, however, it is essential that the relevant Ag(s) access the MHC class II processing pathway when immunized with recombinant LM. The surface proteins identified here as the natural targets of LM-specific CD4+ T cells may indeed serve this function.

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**References**


