Membrane Topology of the L6 Antigen and Identification of the Protein Epitope Recognized by the L6 Monoclonal Antibody*

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The murine monoclonal antibody (mAb) L6 recognizes an integral membrane glycoprotein that is highly expressed on lung, breast, colon, and ovarian carcinomas and is referred to as the L6 antigen. This antigen is an attractive target for therapeutic intervention due to its high level expression on malignant cells. We have previously reported the isolation of a cDNA encoding the human L6 antigen (H-L6). Here, we report the isolation of a cDNA clone encoding the murine L6 antigen (M-L6). This cDNA contains one long open reading frame, which encodes a 220-amino acid polypeptide that is 78% homologous to H-L6. This protein contains short NH2- and COOH-terminal hydrophilic domains and four hydrophobic regions, each long enough to span the plasma membrane. Each of these hydrophobic domains is separated by a hydrophilic domain, the longest of which contains one possible N-linked glycosylation site and is located between the third and fourth hydrophobic domains. We have previously demonstrated that the murine L6 mAb recognizes a protein epitope expressed on human tumor-derived cell lines. Now, using chimeric cDNA constructs encoding human-murine L6 antigen hybrids in conjunction with monoclonal antibody binding experiments, we show that the 42-residue hydrophilic domain of the L6 antigen, located between the third and fourth hydrophobic domains, is outside the cell and that residues in the NH2-terminal region of this domain are critical for the binding of the murine L6 mAb to H-L6.

We have previously reported the isolation of a cDNA clone encoding a human tumor antigen defined by the murine mAb1 L6 (1) and referred to as the human L6 antigen (2). Characterization of this clone indicated that the H-L6 is an integral membrane protein with a predicted molecular mass of ~22 kDa. Examination of the hydrophilicity profile of its predicted amino acid sequence suggests that it spans the plasma membrane four times. Its lack of an NH2-terminal hydrophobic domain further suggests a membrane orientation in which both the NH2 and COOH termini are cytoplasmic. This membrane orientation would give rise to two hydrophilic extracellular domains, one between the first and second hydrophobic domains and the other between the third and fourth hydrophobic domains. Comparison of the predicted amino acid sequence of H-L6 with that of other reported proteins indicated that H-L6 is a member of a large family of integral membrane proteins, which includes the leukocyte antigens CD9, CD37, CD53, CD63, and TAPA-1, the carcinoma associated antigen CO-029, and the Schistosoma mansoni worm antigen SM23 (3-10). COS cell transfection and mAb binding studies indicated that the H-L6 cDNA could direct the expression of the L6 antigen in a heterologous cell (2). Immunoprecipitation studies of in vitro translated L6 transcripts in the presence or absence of microsomal membranes indicated that incorporation into a membrane is required for the recognition of H-L6 by this mAb (11). Furthermore, N- and O-glycanase treatment followed by immunoprecipitation studies of in vitro translated H-L6 indicated that the mature H-L6 antigen contains N-linked carbohydrate, which is not involved in the interaction between H-L6 and the L6 mAb (11).

H-L6 is highly expressed in a number of carcinomas, including lung, breast, colon, and ovary, and, thus, has attracted attention as a target for cancer therapy. In a phase 1 clinical trial, unmodified murine L6 mAb was administered to patients with recurrent cancer of either the breast, colon, lung, or ovary. This study showed that the antibody was well tolerated and effectively localized to the tumor in vivo (12). Furthermore, one patient with recurrent breast cancer underwent complete, albeit temporary, remission following treatment. An additional in vivo study in which patients with metastatic breast carcinomas were given 121I-labeled chimeric (mouse-human) L6 showed delivery of sufficient amounts of radioactivity to tumors to cause partial remissions in approximately one-third of the patients (13).

Although these studies suggest that the L6 mAb may be useful for the treatment of some tumors, several issues remain to be addressed. Chief among these concerns is that the human L6 antigen is expressed, albeit at low levels, by several normal human tissues, endothelial cells in particular (14).2 This indicates that nude mice models, which are routinely used in preclinical studies to evaluate the efficacy of passive immunotherapy, would be misleading if used to evaluate the L6 mAb, since the L6 mAb does not bind to normal murine tissues. Thus, meaningful studies on passive immunity to the L6 antigen cannot be carried out in mice, unless the murine counterpart of H-L6 is identified.

The aim of this study was 2-fold as follows: first, to isolate and characterize a cDNA clone encoding the murine L6 antigen; second, to use this murine cDNA and the cDNA encoding H-L6 to prepare human-mouse L6 hybrid antigens. These chimeric proteins were then used in conjunction with the L6 mAb

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L15429.

1 The abbreviations used are: mAb, monoclonal antibodies; H-L6, human L6 antigen; M-L6, murine L6 antigen; PCR, polymerase chain reaction; kb, kilobase(s).

2 J. R. Rillema and P. Wallace, personal communication; J. S. Marken, unpublished results.
in binding studies to determine the orientation of the L6 antigen in the plasma membrane and to identify the region of the protein recognized by the L6 mAb. This information would allow the future synthesis of H-L6 peptides, which could be used to generate additional murine anti-human L6 antigen mAbs.

MATERIALS AND METHODS

Preparation of Par cDNA Library—Poly(A) mRNA was purified from the murine melanoma K1735 clone referred to here as Par (109 cells) by proteinase-K/SDS digestion followed by selection with oligo(dT) Sepharose beads (Boehringer Mannheim) as previously described (15). First strand cDNA was synthesized from 5 mg of mRNA using avian myeloblastosis virus-reverse transcriptase (Life Sciences Inc., Petersburg, FL) and oligo(dT) primer (15-mer, Boehringer Mannheim). Second strand cDNA synthesis was performed using DNA polymerase and RNaseH (Boehringer Mannheim). The cDNA was fractionated by electrophoresis on seaplaque low melting agarose (FMC Bioproducts, Rockland, ME). Complementary DNA >1 kb was packaged using the Gene Clean kit (Bio-101, La Jolla, CA) according to manufacturer’s instructions. EcoRI adapters containing an internal XhoI site (5’-AAATTCCTCGAG-3’, 5’-CTCGAGGG-3’) were ligated to the cDNA, which was subsequently ligated to λ-Zap phage arms previously digested with EcoRI (Stratagene, La Jolla, CA). The phage library cDNA was packaged with Giga Pack Gold packaging extracts (Stratagene) according to manufacturer’s instructions. Approximately 5 × 108 phage were plated from each of 106 independent colonies.

Par cDNA Library Screening—Plaques were transferred to duplicate hybond-N nylon membranes (Amersham Corp.) as previously described (16). The membranes were screened with a 32P-labeled random-prime generated using the random prime kit (Boehringer Mannheim) according to manufacturer’s instructions. EcoRI adapters containing an internal XhoI site (5’-AAATTCCTCGAG-3’, 5’-CTCGAGGG-3’) were ligated to the cDNA, which was subsequently ligated to λ-Zap phage arms previously digested with EcoRI (Stratagene, La Jolla, CA). The phage library cDNA was packaged with Giga Pack Gold packaging extracts (Stratagene) according to manufacturer’s instructions. Approximately 5 × 108 phage were plated from each of 106 independent colonies.

Par cDNA Library Screening—Plaques were transferred to duplicate hybond-N nylon membranes (Amersham Corp.) as previously described (16). The membranes were screened with a 32P-labeled random-prime probe generated using the random prime kit (Boehringer Mannheim). The adapted cDNA was fractionated on a potassium acetate velocity gradient. cDNA, >1 kb, was ligated into the CDM8 plasmid (19), which was subsequently ligated to A-Zap phage arms previously digested with EcoRI (Stratagene, La Jolla, CA). The phage library cDNA was packaged with Giga Pack Gold packaging extracts (Stratagene) according to manufacturer’s instructions. Approximately 5 × 108 phage were plated from each of 106 independent colonies.

DNA Sequence Analysis—Dideoxynucleotide termination sequencing reactions (16) were carried out in the presence of 32P-labeled dCTP with Sequenase-2 (Stratagene) on double-stranded templates. Both strands of the cDNAs were sequenced. Bluescript and CDM8 templates were prepared by alkaline lysis/neutralization followed by phenol/chloroform extraction (16). Synthetic oligonucleotide primers used in the sequencing reactions were synthesized as needed.

DNA blot Analysis—Total RNA was purified from the murine fibroblast cell line P3T3, the murine histiocytic lymphoma P819, the murine T-cell lymphoma EL4, and the murine thymic epithelial cell lines TE71 and 2172 by the overlap extension polymerase chain reaction (PCR) method as previously described (22). To reduce the chance of unwanted amino acid substitutions during amplification, plaque-forming unit polymerase (Stratagene) was used. A three-step method was used to replace the entire 41-amino acid third hydrophilic domain (119–161) of the murine L6 with the corresponding human sequence (ML6HL6-1). Initially, sequences encoding the human hydrophilic domain III and the corresponding murine 5’- and 3’-flanking sequences were amplified separately by PCR. Then the full-length chimera was generated by two rounds of overlap extension PCR. The ML6HL6-4 (K120L, S122L, A124S, H125L, V127Q), ML6HL6-5 (N142D, S143T, M145S, K148E, V150T), and ML6HL6-6 (H152N, T163S) human-murine L6 antigen chimeras were generated by PCR using internal oligonucleotide primers and sequences encoding the above mutations to generate 5’ and 3’ halves that were joined by overlap extension PCR. The ML6HL6-3 (K120L, N142D, K148E) chimera was generated similarly except that an additional chimera (N142D and K148E) was prepared and used as a PCR template for the final substitution (K120L). The chimera encoding HL6ala-1, in which amino acids 34–41 of the human L6 antigen were replaced with alanines, was constructed as follows: amino acids 34–37 and 38–41 were mutated to alanines by PCR as 5’ and 3’ DNA fragments, respectively. The two chimeric halves were joined by ligation to an EcoRI restriction site, which was included in the internal oligonucleotides used for the PCR reaction.

L6 mAb Binding to the Human-Murine L6 Chimeras—Cos cells were transfected with 2 μg/ml plasmid DNA by the DEAE/dextran plus chloroquin phosphate method as described previously (18). Seventy-two hours post-transfection, cells were incubated at room temperature with 10 μg/ml murine anti-human L6 mAb (IgG2a) or rat antimurine L6 mAb for 1 h in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum. After 3 h, the cells were washed with Dulbecco’s modified Eagle’s medium and incubated for 1 h at room temperature with 2 mg/ml fluorescein conjugated, affinity-purified, goat anti-mouse IgG (Cappel, Organon Teknika Corp., West Chester, PA). The cells were washed three times with RPMI and examined on a UV fluorescent microscope (Zeiss). Mock-transfected COS cells were used as a control for nonspecific antibody binding. Similarly transfected COS cells were lifted from the plastic dishes using phosphate-buffered saline containing 0.5 mm EDTA stained with the murine anti-human L6 mAb followed by fluorescein.
The murine and human L6 antigens are closely related. Residues conserved between the human L6 antigen (H-L6) and the murine L6 antigen (M-L6) are shown in shaded boxes, the possible sites of N-linked glycosylation are shown boxed. Each of the three hydrophilic domains located between two putative hydrophobic transmembrane domains are labeled I, II, and III. The four putative hydrophobic transmembrane domains are underlined.

Membrane Topology of the L6 Antigen

Comparison of the deduced amino acid sequence of this clone with the deduced amino acid sequence of H-L6 showed 78% identity with both polypeptides being of equal length (Fig. 2). The murine protein contained 16 Cys residues, all of which are conserved in the human polypeptide, which contains two additional Cys residues. H-L6 contains two potential N-linked glycosylation sites (2), one of which is conserved in the murine clone. Inspection of the hydrophobicity profile of this clone showed that it, like its human counterpart, contains a short NH2-terminal hydrophilic domain followed by three short and closely spaced hydrophobic domains, a 45-amino acid hydrophilic domain (hydrophobic domain III), a fourth hydrophobic domain, and a short COOH-terminal hydrophilic domain. Based on these observations, we propose that this clone encodes the murine counterpart of the human L6 antigen.

RNA Blot Analysis—Total RNA samples were prepared from a number of human tumor cell lines, the murine RNA samples prepared from murine skin, lung, and kidney were found to express high levels of mRNA encoding L6 (Fig. 3A). The lymph nodes and kidneys were found to express moderate levels of this mRNA, while no mRNA encoding the L6 protein was seen in spleen, liver, and gut. RNA from the murine thymic stromal cell lines Z172 and TE71 and the murine fibroblast cell line P3T3 contain mRNA transcripts encoding murine L6, while the T cell line EL4 and the mastocytoma cell line P819 expressed no detectable L-M6 mRNA (Fig. 3B). As had been observed in RNA blot analysis of samples prepared from a number of human tumor cell lines, the murine RNA samples prepared from murine skin, lung, and kidney contained two species that hybridized to the M-L6 probe. One corresponds to a species of ~1.8 kb, which appears to be the more abundant species in murine skin, and the other corresponds to a species of ~1.1 kb, which is present at low levels in all L6-expressing tissues. We presume that the ~1.1-kb fragment corresponds to the isolated cDNA. It is possible that the ~1.8-kb species corresponds to either an immature or alterna-
FIG. 4. Description of the human-murine L6 chimeric antigens and the human L6 antigen mutant. A, the deduced amino acid sequence of the human L6 antigen (H-L6) and the murine L6 antigen (M-L6) in the third hydrophobic domain (III) located between the third and fourth hydrophobic domain is shown above the deduced amino acid sequence of the corresponding sequences of human-murine L6 chimeric antigens (ML6HL6-1, ML6HL6-3, ML6HL6-4, ML6HL6-5, and ML6HL6-6). The amino acids of H-L6, which replace the corresponding amino acids in M-L6, are shown in boldface underline type. B, the predicted amino acid sequence of the human L6 antigen (H-L6) in the first hydrophilic domain (I) located between the first and second putative hydrophobic transmembrane domains is shown above the amino acid sequence of the corresponding sequences of the human L6 antigen mutant antigen in which the residues from this domain have been changed to alanine (HL6ala-1).

Membrane Topology of the L6 Antigen

![Diagram]

| A | ML6HL6-1 | ML6HL6-3 | ML6HL6-4 | ML6HL6-5 | ML6HL6-6 |
|---|---|---|---|---|---|
| GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS |
| GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS |
| GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS | GLAEGLPLCDLQGWNYTFASTEQYLLDTSWSECTFKHEVWNVSLFS |

Supports our earlier proposal that the L6 antigen is oriented in the cytoplasmic membrane such that its NH₂ and COOH-terminal domains are located intracellularly, a membrane orientation analogous to the one proposed for TAPA-1 (28). Expression of M-L6 in COS transfectants was verified using a rat anti-M-L6 mAb (Fig. 5, IB). Region of the L6 Antigen Recognized by the L6 mAb—In order to identify the protein epitope recognized by the murine anti-human L6 mAb, additional human-murine chimeric L6 genes were prepared as described in Fig. 4. Although the human and murine L6 proteins are highly homologous, there are a number of differences that could account for the specificity of the interaction of the murine L6 mAb, which binds to H-L6. Our finding that replacement of hydrophobic domain III of M-L6 with the corresponding sequences from the human clone resulted in the ability of this chimera to bind the L6 mAb suggested that this region of the protein contained the epitope recognized by the mAb. Perhaps the most striking difference in this region between the deduced amino acid sequences of H-L6 and M-L6 is the nonconservative replacement of L120, D142, and E148 in the mouse clone with the corresponding residues in the human antigen generating the ML6HL6-3 chimera. Transfection of this chimera into COS cells did not result in the expression of protein that could bind the L6 mAb (Fig. 5f). It follows that these changes are insufficient to account for the differential reactivity of the L6 mAb with the human and murine proteins.

Further inspection of the differences between the human and murine clones showed that the amino acid differences between the proteins encoded by the two clones were located at either end of hydrophobic domain III. This observation led us to replace the NH₂-terminal region of this domain in the murine clone with the equivalent region from the human clone. This change resulted in the replacement of K120, S122, A124, H125, and Y150 in the mouse clone with the corresponding residues L, I, S, L, and Q in the human clone, generating the ML6HL6-4 chimera. Likewise, replacement of N142, S143, M145, K148, and Y150 in the COOH-terminal region of hydrophobic domain III in the murine protein with the corresponding residues in the human clone D, T, E, T, respectively, generated the ML6HL6-5 chimera. Furthermore, one of the two potential
N-linked glycosylation sites in H-L6 is not present in M-L6. To examine the role of this site in the interaction between H-L6 and the L6 mAb, H159 in the murine clone was replaced with N, thereby restoring this N-linked glycosylation site and generating the ML6HL6-4 chimera. DNA corresponding to each of these chimeras was transfected into COS cells, and their ability to express the L6 mAb and a fluorescein-conjugated anti-mouse antibody as described under "Materials and Methods." II, COS cells transfected as described above were analyzed by flow cytometry following staining with the rat anti-M-L6 mAb (12A8) as described under "Materials and Methods." A total of 10,000 cells were analyzed in each experiment.

The potential contribution of this domain to antibody binding by substituting residues 34–41 of this domain of the human clone with alanines. This resulted in the generation of the mutant cDNA clone HL6ala-1 (Fig. 4), which, when transfected into COS cells, directed the expression of a protein that reacts with the L6 mAb (Fig. 5, II) suggesting that residues in this domain do not play a critical role in the binding of the L6 mAb.

The isolation of a cDNA clone encoding M-L6 is an important step in studying the in vivo effects of passive and active immunotherapy directed against the L6 antigen in a murine tumor model. In addition, the determination of the membrane orientation of the L6 antigen and the identification of residues critical for the binding of the L6 mAb should allow us to generate additional anti-L6 antigen mAb that could be used in a clinical setting for diagnosis and/or therapy.

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