Studies of the Structure of the Metastasis-Associated 67 kDa Laminin Binding Protein: Fatty Acid Acylation and Evidence Supporting Dimerization of the 32 kDa Gene Product To Form the Mature Protein†

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ABSTRACT: The level of expression of the 67 kDa high-affinity laminin binding protein (LBP) correlates with the progression of many solid tumors. The cDNA clone for the 67 kDa LBP is sufficient to encode a polypeptide of only 32 kDa, and there is no readily identifiable mechanism for membrane association. We have overexpressed the transfected 67 kDa hamster LBP in quantities that have enabled us to analyze the membrane-bound form of the protein. Treatment of the purified LBP with methyl transesterification reagents, follwed by GC−MS, identified the covalently bound fatty acids palmitate, stearate, and oleate. The fatty acid modification may provide a mechanism for membrane association. Molecular mass determination by MALDI-TOF MS demonstrated the true molecular mass of the protein to be 66.7 kDa, compatible with the SDS−PAGE observation of 67 kDa. Treatment of the LBP with neuraminidase, *O*-glycanase, or Endo-F glycosidase has no detectable effect on the apparent molecular mass of the protein, and the MALDI-TOF MS did not show evidence of mass heterogeneities typically observed with glycosylated proteins. Reduction with dithiothreitol or β-mercaptoethanol had no effect on the apparent molecular mass on SDS-PAGE or on the relative quantities of molecular mass species on MALDI-TOF MS. The experimentally determined amino acid composition, however, was found to be consistent with the 67 kDa form being a homodimer of the 32 kDa precursor. Preliminary experiments also suggest that the high-affinity laminin binding characteristic of the protein may be modulated by an, as yet, unidentified membrane accessory molecule.

To be successful in metastasis, cancer cells must adhere to and invade extracellular basement membranes. This adhesion and invasion is accomplished using cell surface receptors specific for basement membrane components [for reviews, see Aznavoorian et al. (1993) and Stetler-Stevenson et al. (1993)]. Several laminin binding receptors have been identified, one of which is a high-affinity laminin binding protein that migrates with an apparent molecular mass of 67 kDa on reducing SDS−PAGE. The 67 kDa high-affinity laminin binding protein was initially isolated from a highly metastatic breast carcinoma by immunoprecipitation with a function blocking antibody (Barsky et al., 1984). Using this antibody as a probe, a partial cDNA sequence was identified in a human endothelial cell expression library. The sequence was found to completely overlap that of a mRNA which was preferentially overexpressed in highly metastatic colon carcinoma as compared to adjacent normal colonic epithelium (Yow et al., 1988).

The 67 kDa high-affinity laminin binding protein (LBP)[1] has since been shown to be expressed at high levels on lung carcinoma (Satoh et al., 1992), melanoma (Vacca et al., 1993), breast carcinoma (Martignone et al., 1992), and several other invasive solid tumors. The 67 kDa LBP is expressed at relatively low levels in benign and normal tissues, and its level of expression correlates very well with the metastatic phenotype of the tumor (Mafune et al., 1990). Since adhesion to the basement membrane is considered to be a critical step in the metastatic cascade, the laminin binding function of this protein makes it a potential target for therapeutic intervention in metastatic disease as well as a useful prognostic indicator.

The full-length nucleotide sequence of the high-affinity laminin binding protein is sufficient to encode a protein with a predicted molecular mass of approximately 32 kDa (Yow et al., 1988), while the laminin affinity isolated protein migrates with an apparent molecular mass of 67 kDa on reduced SDS−PAGE. The mechanism of this shift of the apparent molecular mass is not readily apparent, and is somewhat controversial. Although the original data have not been published, one group has reported that an *Escherichia coli* expression product of a full-length clone fails to bind to a laminin affinity column [discussed in Mecham (1991)]. The 67 kDa LBP, when isolated from tumor cell

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[1] Abbreviations: LBP, laminin binding protein; CMF, calcium and magnesium free; FBS, fetal bovine serum; ATCC, American Type Culture Collection; dhfr, dihydrofolate reductase; HT, hypoxanthine and thymidine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; EHS, Englebreth−Holm−Swarm; HPLC, high-performance liquid chromatography; GC, gas chromatography; GC−MS, gas chromatograph−mass spectrometer; MALDI-TOF, matrix-assisted laser desorption time of flight; KLH, keyhole limpet hemocyanin; PVDF, poly(vinylidene difluoride); DTT, dithiothreitol; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

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membrane extracts, initially binds to a laminin affinity column with high affinity (Barsky et al., 1984). However, following laminin affinity purification, the protein does not rebind to laminin with the same high affinity (M. Sobel and H. Kleinman, personal communication, and this paper). More recently, another group has reported that a fusion protein of the 67 kDa LBP cDNA with TrpE, which was expressed in *E. coli*, did bind to a laminin−Sepharose affinity column (Siyanova, 1992). However, it is not clear whether the TrpE secretory signal sequence was present or not, or what molecular weight the affinity-isolated product displayed. Taken together, these data would imply that there are unidentified structural characteristics of the laminin binding protein which play a critical role in its adhesion to laminin.

Rao et al. have reported the existence of a 37 kDa precursor in mouse NIH3T3 cells which can be chased into a 67 kDa product (Rao et al., 1989). These proteins were immunoprecipitable with an antibody raised to a synthetic peptide corresponding to the amino-terminal region of the cDNA sequence. However, these authors were unable to identify the mechanisms underlying the molecular weight shift. Although there are 14 serine and 23 threonine residues in the putative amino acid sequence of the cDNA clone which might be modified with O-linked glycosylation, there are no consensus sites for N-linked carbohydrates. Analysis of the predicted amino acid sequence of the protein reveals no simple hydrophobic domain characteristic of a transmembrane region, leading to some speculation that the protein may not actually be a cell surface receptor (Grosso et al., 1991). Several groups, in independent and often unrelated studies, have identified proteins in yeast (Davis et al., 1992), hydra (Keppel & Schaller, 1991), and *Drosophila* (Melnick et al., 1993), with extensive sequence similarity to the 67 kDa LBP. These proteins are not cell surface proteins, but are apparently components of the ribosomal translational machinery. While the cDNA sequence homology of these proteins, which derive from phylogenetically divergent species, with the mammalian LBP is very high in the amino-terminal half of the molecule, the carboxyl-terminal domain (which has previously been identified to contain the region important for association with laminin) is less highly conserved. In the present paper, we hypothesize that the 67 kDa LBP protein has two functions in mammalian cells. One function may be intracellular, and perhaps related to translation. The second function may be dependent on posttranslational modifications responsible for surface localization and laminin binding characteristics.

Recently, many proteins have been identified with post-translational modifications in which lipid moieties are covalently attached to the protein [for reviews, see Schmidt (1989) and McIlhinney (1990)]. These acylproteins have varied functions in the cell, including playing key roles in adhesion and signalling. Several mechanistic roles for lipid modifications of proteins have been proposed, including localization of proteins to lipid bilayer membranes. Lipid modifications have also been shown to modify protein−protein interactions, and acyl modifying groups may be active in generating a second messenger in signal transduction cascades [reviewed in Magee (1990) and Chow et al. (1992)].

We report here that the 67 kDa high-affinity LBP is acylated by the fatty acids palmitate, oleate, and stearate. These fatty acids appear to be covalently associated via ester or thioester linkages and, in the absence of other known mechanisms, are likely to be responsible for targeting the protein to the cell surface, where it can participate in cell adhesion to the extracellular matrix. We established the true molecular mass of the membrane form of the protein at 66.7 kDa using MALDI-TOF mass spectrometry, and suggest that it may form a homodimer of the 32 kDa polypeptide modified by fatty acid chains. From our results, we also postulate that associations with other membrane molecule(s) assist in mediating high-affinity laminin binding.

MATERIALS AND METHODS

Expression of the Laminin Binding Protein. A cDNA clone of the high-affinity laminin binding protein, isolated from a hamster expression library, was kindly provided by Dr. James Strauss of the California Technical Institute in the pcDNA I/neo vector (Invitrogen) (pcLR). This expression vector utilizes a CMV promoter and G418 selectable marker (Wang et al., 1992). An expression vector for the dihydrofolate reductase (dhfr) gene, under the control of an SV40 promoter, was obtained from ATCC (Rockville, MD). Both plasmids were simultaneously transfected by calcium phosphate precipitation (Chen & Okayama, 1988) into DG44CHO cells which were obtained from Dr. Lawrence Chasin of Columbia University. These cells are double negative mutants for the dhfr gene, and rely on the presence of exogenous hypoxanthine and thymidine (HT) in the culture media for proliferation (Urlaub & Chasin, 1980). Cotransfection of the pcLR and dhfr plasmids was carried out at 5:1 and 20:1 ratios of pcLR to dhfr with a total of 20 μg of DNA per 10^6 cells. Untransfected cells were grown in αMEM (Sigma) containing 10% FBS (Intergen) and supplemented with HT, 5 μg/mL bovine insulin, 10 mM L-glutamine, and 10 units/L each of penicillin and streptomycin. Selection of transfectants was carried out in the same medium without HT, and containing 10% dialyzed FBS and 400 μg/mL G418 (Gibco/Life Technologies). Methotrexate treatment of dhfr/pcLR transfected cells was initiated at a concentration of 0.03 μM, and increased by 0.02−0.05 μM increments approximately every fifth cell passage. Amplification of pcLR expression was monitored by Northern blots and FACScan analysis using the sequence-specific antibody described below (Landowski et al., 1995). In order to gain insight into the proportion of the expressed protein which was presented at the cell surface, we directly conjugated the sequence-specific anti-LBP antibody described below to fluorescein, and used it in FACScan studies to compare the quantity of surface to total cellular immunoreactive protein in the methotrexate amplified pcLR transfected DG44CHO cells. Cells were harvested using minimal trypsin exposure followed by rapid trypsin inactivation in serum-containing medium. The cells were then washed in a large excess of serum-free medium and resuspended, and one aliquot was directly stained with antibody. A second aliquot was antibody-stained after being permeabilized with 50 μg/mL lysolecithin treatment for 5 min at 0 °C. Lysolecithin treatment was carried out in the presence of an excess of bovine serum albumin to promote the maintenance of cell structural integrity.

For metabolic labeling of glycosylphosphatidylinositol (GPI)-modified proteins, [^{14}C]ethanolamine (NEN) was added to the culture medium at a concentration of 0.1 μCi/mL (specific activity of 2 μCi/μmol). Labeling of cells was carried out for 16 h at 37 °C.

Laminin Binding Protein Isolation. The laminin binding protein was extracted by the method of Wewer et al. (1986) with minor modifications. DG44CHO cells, selected for expression of the pcLR and dhfr plasmids, were harvested by rinsing twice with Puck's CMF Saline G solution, approximately 10 min each at 37 °C, and a third time with the same solution containing 0.01 mM EGTA until the cells detached. This harvesting technique was used to avoid the use of trypsin and preserve membrane protein integrity. Cells were centrifuged for 10 min at 500*g*; the pellet was resuspended in 10 volumes of CMF−Dulbecco's PBS (v/v) and centrifuged again under the same conditions. The protease inhibitors PMSF (50 μg/mL), *N*-ethylmaleimide (1 mM), and benzamidine (5 mM) were included in this and all subsequent buffers. Cells were suspended in 2 volumes of ice-cold 25 mM Tris/0.3 M sucrose, pH 7.4 (v/v), and sonicated with (4−5) × 5 s bursts on ice using a Fisher Sonic Dismembrator, Model 50, at 50% power. Nuclei, cytoskeletal proteins, and unbroken cells were pelleted by centrifugation at 500*g* for 10 min at 4 °C, and the supernatant was collected. The pellet was resuspended in 5 volumes of Tris/sucrose buffer, sonication was repeated, and the supernatant was again collected and combined with the first supernatant. These steps were repeated 3 times, or until the vast majority of the cells appeared lysed under microscopic examination. The membrane fraction was then collected on a sucrose cushion by centrifugation at 143000*g* for 90 min at 4 °C, and adjusted to a protein concentration of approximately 1 mg/mL (protein concentration determined using the Bio-Rad dye reagent) with 25 mM Tris/150 mM NaCl/1 mM $CaCl_2$/3 mM $MgCl_2$, pH 7.4, buffer. An equal volume of the same buffer containing 1% NP-40 detergent was added, and the solution was rotated end-over-end for 12−16 h at 4 °C. Insoluble material was pelleted by centrifugation at 200000*g* for 60 min. The supernatant was applied to a laminin-Sepharose column which had been preequilibrated with 10 column volumes of 25 mM Tris/150 mM NaCl/1 mM $CaCl_2$/3 mM $MgCl_2$ + 0.05% NP-40 followed by the same buffer containing 400 mM NaCl. Laminin−Sepharose affinity columns were prepared by coupling EHS laminin (Gibco/Life Technologies) to CNBr-activated Sepharose 4B at a concentration of 0.5 mg/mL according to the procedure recommended by the manufacturer (Pharmacia). The column eluates were monitored at 214 nm, where the peptide bond has high absorbance, in an effort to reduce interference from other membrane components. After application of the detergent extract, the column was washed with 50 mM Tris/0.1 M NaCl, pH 7.4, until the UV absorbance at 214 nm returned to base line. Approximately 1 column volume of 50 mM Tris/1.0 M NaCl, pH 7.4, was added and the column allowed to stand 15−20 min prior to elution of the high-affinity binding protein with the latter elution buffer.

Isolated laminin binding protein was labeled with ^{125}I by the lactoperoxidase method using Enzymobeads (Bio-Rad), and labeled protein was separated from free iodine by chromatography on a BioGel P6 column with 1 M NaH_2PO_4, pH 7.4. The specific activity of the labeled protein was 1.5 × 10^6 cpm/mg.

Antibody Production and Western Blotting. A peptide corresponding to residues 205−229 of the predicted amino acid sequence of the 67 kDa LBP (RDPEEIEKEEQAAAEKAVTKEEFQG) was synthesized using standard Fmoc chemistry on a Milligen 9050 automated peptide synthesizer. Following purification by reverse phase HPLC and assessment of purity with electrospray mass spectrometry, the peptide was conjugated to KLH (Sigma) by cross-linking with 0.2% glutaraldehyde for 2 h at room temperature. The solution was dialyzed against Dulbecco's PBS for 48 h with 4 changes of dialysate to remove glutaraldehyde and free peptide prior to immunization. New Zealand white rabbits were injected with 1 mg of peptide−KLH and Freund's complete adjuvant at multiple subcutaneous sites, and boosted every 2 weeks with peptide−KLH and incomplete Freund's adjuvant. Thirty days later, a test bleed was obtained and the anti-peptide activity titered by ELISA using KLH, peptide−KLH, and peptide conjugated to ovalbumin as target antigens. The antiserum was purified on a protein A column (Pierce), and the majority of the anti-KLH activity was subsequently removed using a KLH−Sepharose column.

Affinity-isolated laminin binding protein was electrophoresed on a 10% reducing SDS−PAGE gel and transferred to a PVDF membrane (Bio-Rad) using a semi-dry blotter (Ellard Instrumentation). The 67 kDa LBP did not adhere well to the PVDF membrane; therefore, the filter was fixed in Dulbecco's PBS containing 0.02% glutaraldehyde for 2 h at room temperature prior to washing and blocking with 5% non-fat dry milk and 0.1% Tween-20 in PBS. To ensure that the glutaraldehyde fixation was not introducing false positive results, fixation in 10% acetic acid/20% methanol was also used, and found to give identical results. Antibody detection was performed using alkaline phosphatase conjugated goat anti-rabbit antibody (Bio-Rad) and BCIP/NBT substrate (Kirkegaard Perry Inc.). Because of its poor adherence of the LBP to the support matrix, insufficient LBP was retained on Western blots of the crude unpurified membrane extract for antibody detection. Therefore, duplicate gels of the crude and affinity-purified material were silver-stained to assess the efficiency of the laminin affinity isolation. The specificity of antibody staining was established by the ability of the immunizing peptide ($LBP^{205-229}$) to block antibody binding in Western blots, and by the failure of the antibody to recognize bovine serum albumin and several other test proteins loaded with the laminin binding protein in control Western blots (e.g., Figure 1b, lane 2).

Determination of Molecular Weight. Determination of the molecular weight of the affinity-isolated laminin binding protein was performed by matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) on a Perseptive/Vestec BenchTop II instrument. One microliter aliquots of 0.5 mg/mL LBP solution in 50 mM Tris/1.0 M NaCl were applied to sample wells, followed by 1 μL of a saturated solution of the UV sensitizer, sinapinic acid in water (Aldrich). Samples were air-dried and subjected to 5 ns flashes from a 337 nm nitrogen laser. Flight times were measured using a centroid algorithm in the GRAMS (Galactic Industries) software, and external mass calibration was carried out with cytochrome *c*, bacteriorhodopsin, and ovalbumin.

Amino Acid Composition. Analysis of the amino acid composition of the LBP was determined using the method of Heinrikson and Meredith (1984). Amino acid standards and other reagents were of Sequanal grade (Pierce). Aliquots of lyophilized receptor were dissolved at a concentration of 5 mg/mL in 200 μL of 6 N HCl, and norleucine was added as an internal standard at 2.5 μmol/mL. After being sealed

in vacuo, the samples were placed at 110 °C for 24, 48, or 72 h, after which time the HCl was removed under vacuum. Dried samples were dissolved in 100 μL of coupling buffer (acetonitrile/pyridine/triethylamine/H_2O, 10:5:2:3) and dried by rotary evaporation to remove of all traces of HCl. Residual amino acids were redissolved in 100 μL of coupling buffer, to which 5 μL of phenyl isothiocyanate (PITC) (Pierce) was added. The mixture was incubated for 5 min at room temperature with occasional mixing, before evaporation to dryness (approximately 2 h under high vacuum). For HPLC analysis, the PITC-amino acids were dissolved in 250 μL of water/acetonitrile (7:2), filtered, and immediately placed on ice. Reverse phase HPLC was performed on a C18 column (25 × 4 mm) maintained at 52 °C with a water jacket and monitored using a detector wavelength of 255 nm. The mobile phase was the following: buffer A, 0.05 M ammonium acetate in water, pH 6.8; buffer B, 0.1 M ammonium acetate, 44% acetonitrile, and 10% methanol, pH 6.8. Amino acid peaks obtained from the hydrolyzed receptor preparations were identified by comparison to a mixture of Sequanal grade amino acid standards (Pierce) which were similarly labeled.

Lipid Characterization. Affinity-purified 67 kDa LBP was dialyzed against deionized H_2O, and lyophilized to dryness. Fifty micrograms of protein (dry weight) was dispersed in 200 μL of Methyl Prep II (Alltech) and transesterified at 65 °C for 60 or 90 min. The Methyl Prep II reagent converts esterified fatty acids to methyl esters, and any nonesterified fatty acids that may be present partition into the aqueous phase as *m*-(trifluoromethyl)phenyl]trimethylammonium salts (McCreary et al., 1978). The reaction was quenched by the addition of 200 μL of double-distilled H_2O and 100 μL of methanol containing 20 μg/mL butylated hydroxytoluene, and the fatty acid methyl esters were extracted with hexane. The hexane-extracted fatty acid methyl esters were dried down under a stream of argon, and redissolved in 20 μL of isooctane. Gas chromatographic analyses were performed with a Hewlett-Packard Model 5890 Series II gas chromatograph coupled to a Hewlett-Packard Model 5971 mass spectrometer operated in the electron impact mode using a 12 m HP-1 capillary column. Temperatures used were as follows: injector, 250 °C; column, 100−300 °C at 5 °C/min. The affinity isolation buffer plus NP-40 was similarly treated as a control for external lipid contamination. In separate assays, lyophilized LBP samples were first washed with methylene chloride, and the wash was treated with Methyl Prep II and analyzed by gas chromatography as described above. The methylene chloride extraction was carried out to test for the presence of noncovalently associated lipids.

Glycosidase Treatments. Affinity-purified 67 kDa LBP was treated with Endo-F (Boehringer-Mannheim), neuraminidase (Boehringer-Mannheim), and *O*-glycanase (Genzyme) under conditions recommended by the manufacturers. Endo-F treatment was carried out at 37 °C in 10 mM $NaHPO_4$ with 1 unit of enzyme/mg of protein. For *O*-glycanase treatment, ^{125}I-labeled 67 kDa LBP was dialyzed into 10 mM calcium acetate/20 mM sodium cacodylate buffer. Duplicate samples of 50 μL each (0.5 mg/mL) were denatured with 0.1% SDS at 80 °C for 5 min, and a 6-fold excess of NP-40 was added to reduce the concentration of SDS prior to treatment with 0.35 unit of neuraminidase at 37 °C for 2 h. *O*-Glycanase was added to a concentration of 0.1 unit/μg of protein, and the incubation was carried out for an additional 2 h or overnight. Glycosidases were checked for lack of contaminating proteases by evaluating their ability to degrade ^{125}I-labeled irrelevant unglycosylated proteins, and the glycosidase activity for each enzyme was monitored using fetuin as a target substrate.

Triton X-114 Solubility. To asses the amphipathic nature of the 67 kDa LBP, the method of Bordier (1981) was used to determine its Triton X-114 solubility. Affinity-purified, ^{125}I-labeled LBP was dialyzed to 10 mM Tris/150 mM NaCl, pH 7.4, and dissolved in precondensed 1.0% Triton X-114 (Sigma) in the same buffer at 0 °C. The samples were then warmed to 30 °C for 5 min and the phases separated by centrifugation at 10000*g* on a 6% sucrose cushion at room temperature. The upper aqueous phase was removed and brought to 0.5% Triton X-114, cleared on ice, and again brought to 30 °C for 5 min. This sample was reapplied to a 6% sucrose cushion, and centrifuged to separate the phases. Aqueous and detergent-rich phases were analyzed by electrophoresis on 10% SDS−PAGE, and the radioactive components were visualized on a Molecular Dynamics phosphorimager using ImageQuant software.

Reconstitution of Laminin Binding. Affinity-purified ^{125}I-labeled LBP was dialyzed back into the laminin affinity column binding buffer (25 mM Tris/150 mM NaCl/3 mM $MgCl_2$/1 mM $CaCl_2$, pH 7.4). Aliquots of this solution were brought to 0.05% NP-40 and reapplied directly to a laminin−Sepharose column. Equivalent aliquots were also reconstituted with fractions 1 and 4 of the 50 mM Tris/0.1 M NaCl wash from previous LBP affinity purifications. Fraction 1 contains the majority of the NP-40-soluble membrane-associated proteins which do not bind to laminin directly and wash through the affinity column. Fraction 4 includes a "shoulder" which was consistently noted in the effluent peak from the 0.1 M NaCl wash of the laminin column, and which may include components which bind to laminin with intermediate affinity. Reconstituted solutions were brought to 0.05% NP-40, and reapplied to a laminin−Sepharose affinity column equilibrated as for the initial purification. Column elutions of the reconstituted mixtures were carried out exactly as for the *de novo* isolation procedure for the high-affinity LBP. The data shown in Figure 8 are from the integrated peak volumes for the 214 nm absorption tracings from the affinity column elutions.

RESULTS

Expression and Identification of the Laminin Binding Protein. As detailed in the introduction, other labs have reported difficulties in obtaining a high-affinity laminin binding product in bacterial expression systems. We therefore postulated that posttranslational modifications may be required to obtain a fully functional protein. Since posttranslational modifications may be species-specific, we chose to transfect hamster CHO cells with the hamster cDNA clone for the laminin binding protein. The hamster protein was identified by Wang et al. as a cell surface receptor for the Sindbis virus (Wang et al., 1992). The hamster cDNA shows 99% identity with the mouse cDNA (Rao et al., 1989) and 96% identity with the human cDNA (Yow et al., 1988).

Northern analysis of transfected cell populations demonstrated two mRNA products: one an endogenous product which was also found in untransfected controls, and a second

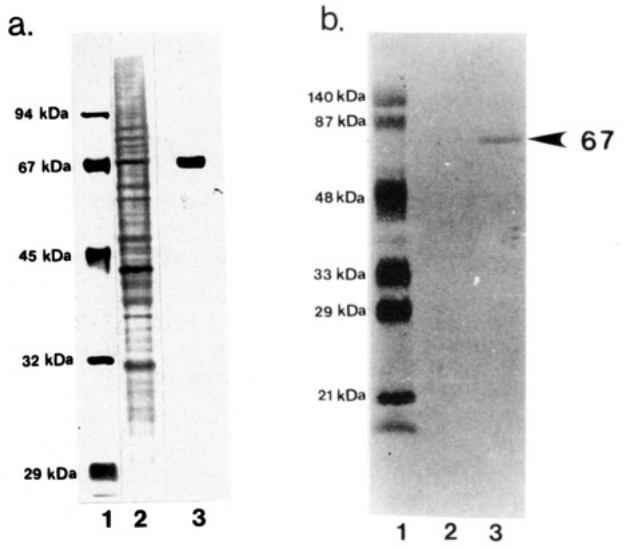

FIGURE 1: (a) Silver-stained SDS−PAGE demonstrates purification of the 67 kDa LBP by elution from a laminin−Sepharose column with a high ionic strength buffer. Lane 1, molecular mass standards (Pharmacia); lane 2, starting membrane detergent extract that was applied to the laminin−Sepharose column; lane 3, high-salt eluate of membrane detergent extracts of DG44CHO pcLR/dhfr cells applied to laminin−Sepharose. (b) Polyclonal antiserum raised to a synthetic peptide derived from the deduced amino acid sequence of the 67 kDa LBP, as detailed in the text, recognizes the affinity-isolated product on a Western blot. Lane 1, prestained molecular mass standards (Bio-Rad); lane 2, bovine serum albumin loaded as a sample negative control protein; lane 3, affinity-purified 67 kDa LBP probed with the sequence-specific anti-peptide 205−229 antibody.

at a slightly higher molecular weight consistent with the expected product of the transfected plasmid (Landowski et al., 1995). Although the mechanism is not well understood, it has been shown that methotrexate causes reduplication of transfected dhfr plasmids and simultaneous amplification of any cotransfected plasmid (Kaufman & Sharp, 1982). Using this protocol, we were able to achieve amplification of the transfected gene, and to increase expression of the transfected plasmid by approximately 10-fold as measured by quantitative densitometry. The protein product, isolated by laminin affinity from these transfected cell lines, was also increased by approximately 10-fold, resulting in a yield of approximately 300 μg of purified LBP per 8 mL of packed cell volume (Figure 1a). The identity of the LBP was confirmed by Western blot using a sequence-specific polyclonal antiserum (Figure 1b). This antibody stained the 67 kDa laminin affinity-isolated protein from the transfected cell lines, and the laminin affinity-isolated proteins from the EHS tumor and B16BL6 mouse melanoma cells. Antibody binding on Western blots could be completely inhibited by the presence of 1 mg/mL immunizing peptide. The antiserum failed to react with bovine serum albumin, ovalbumin, or any other protein tested (Figure 1b, lane 2). This sequence-specific antipeptide antibody was also used to determine the proportion of the expressed LBP which was present on the cell surface compared to the total cell content of immunoreactive protein. This was determined using FACScan analysis of antibody binding to unfixed cells and to unfixed but permeabilized cells. As shown in Figure 2, a much greater abundance (at least 30-fold) of protein was found to be intracellular in location, even though the surface expression is considerably elevated compared to untransfected cells (Landowski et al., 1995).

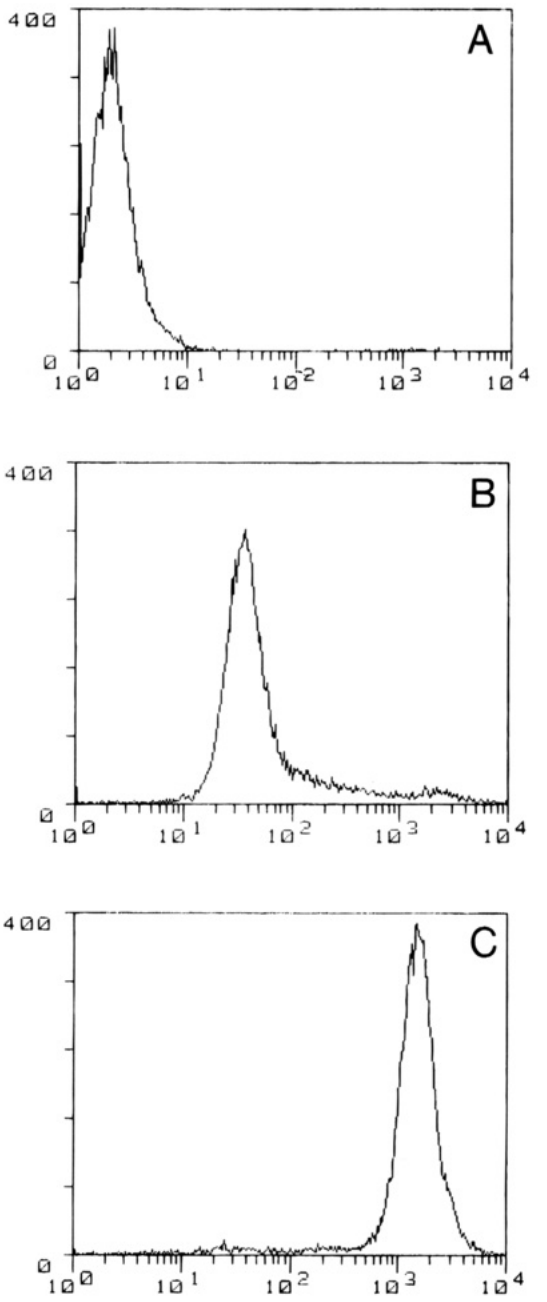

FIGURE 2: FACScan analysis of the surface and total expression of the 67 kDa LBP in methotrexate-amplified pcLR-transfected CHO cells. log fluorescence is plotted versus number of events. Panel A shows control, untreated cells. Panel B shows labeling of impermeable cells and demonstrates only surface staining. Panel C shows permeabilized cells and demonstrates both surface and cytoplasmic staining. Cells were stained with the sequence-specific antibody against the laminin binding protein described in the text.

The MALDI-TOF MS Measurement of Molecular Weight Is Compatible with the Apparent MW Observed by SDS−PAGE. Since SDS−PAGE provides apparent molecular weights, and mobility artifacts are common with membrane proteins, the true molecular weight of the affinity-isolated material was determined by MALDI-TOF mass spectrometry. Mass spectra of the protein indicated the major species at a mass of 66.7 kDa. Minor peaks were identified at apparent molecular masses of 33, 133, and 201 kDa (Figure 3). The TOF mass spectrometer was calibrated with the external standards listed under Materials and Methods, and the accuracy of the molecular weight determination is expected to be in the range of ±0.2% (Aitken, 1992). To determine

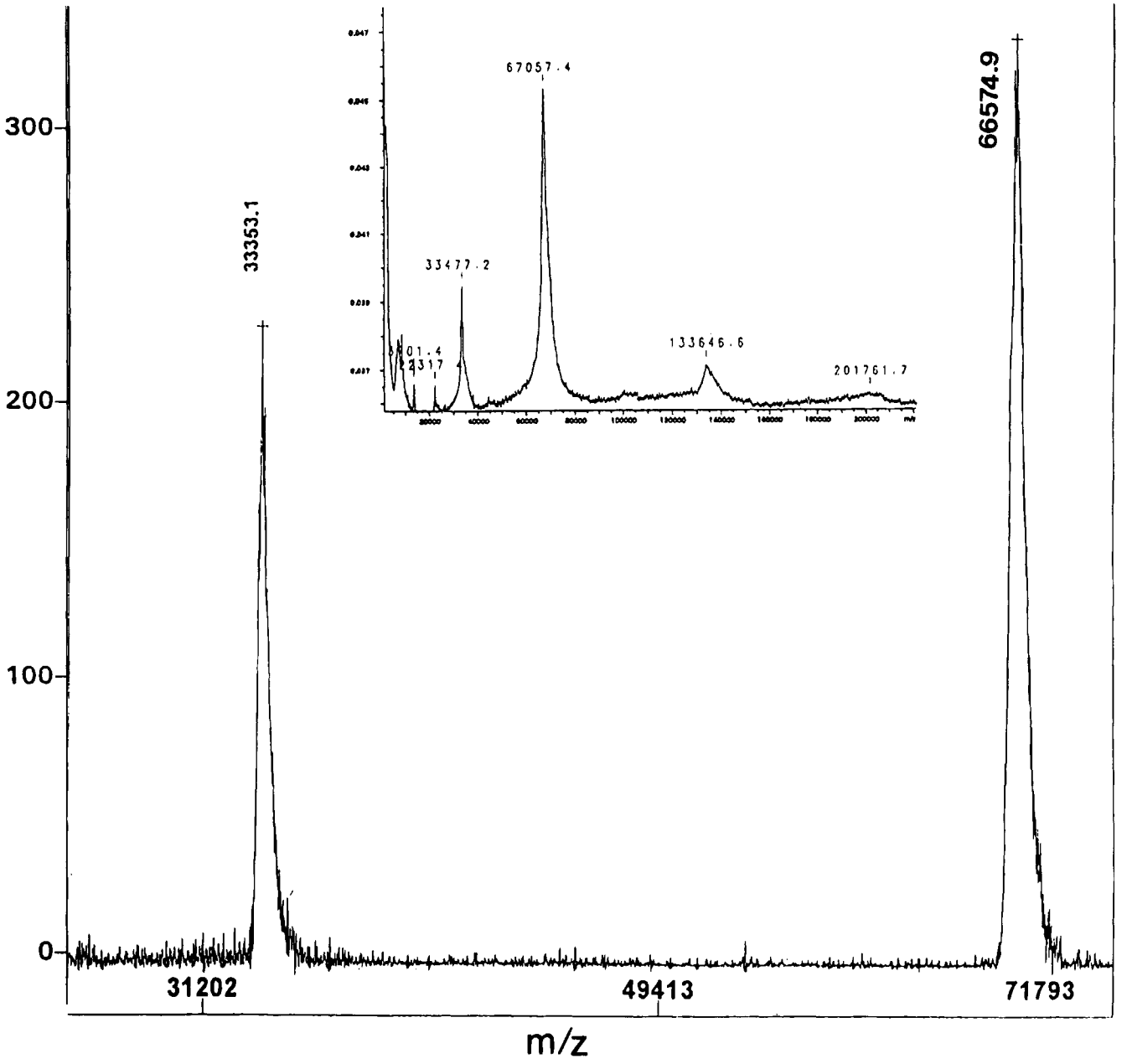

FIGURE 3: Affinity-purified 67 kDa LBP was subjected to MALDI-TOF mass spectrometry for accurate molecular weight determination. The major species was found at a mass of 66.7 kDa. The accuracy of this method is ±0.2% with external calibration carried out using cytochrome *c*, bacteriorhodopsin, and ovalbumin. The insert demonstrates the relative quantities of the minor species seen at 33, 133, and 201 kDa.

if disulfide bonding is involved in the shift in molecular mass from the cDNA-predicted 32 kDa to the 67 kDa apparent mass on standard reducing SDS−PAGE, the isolated protein was treated with 10 mM DTT prior to molecular weight determination by MALDI-TOF MS. The DTT treatment did not significantly change the relative quantities of the four peaks obtained (data not shown). In addition, ^{125}I-labeled LBP was subjected to strong reduction with 1 M DTT at 80 °C for 1 h. This treatment did not reduce the apparent molecular mass of the protein on SDS−PAGE (Figure 4a). Our findings on disulfide reduction are consistent with the report by Rao et al. (1989). The small increase in apparent molecular mass which is seen following treatment of the protein with disulfide reducing agents (Figure 4a) is probably due to the reduction of the single internal disulfide bond, as has been previously reported by these authors (Rao et al., 1989).

The Amino Acid Composition of the Isolated 67 kDa Laminin Binding Protein Is Compatible with a Homodimer of the 32 kDa Gene Product. The amino acid composition determined after acid hydrolysis of the isolated 67 kDa LBP is very close to that deduced for the cDNA-predicted 32 kDa protein (Table 1). Small differences in the percent amino acids found and predicted are well within discrepancies commonly found in amino acid analysis of highly purified proteins. While such a result could, in theory, be due to an association between two different proteins of similar amino acid composition, this is quite unlikely. The results are consistent with the 67 kDa LBP being a homodimer of the 32 kDa protein.

The 67 kDa Laminin Binding Protein Is Covalently Associated with Lipids, but Does Not Appear to Be Modified by Carbohydrates. The treatment of affinity-isolated 67 kDa LBP with *O*-glycanase, or *O*-glycanase and neuraminidase,

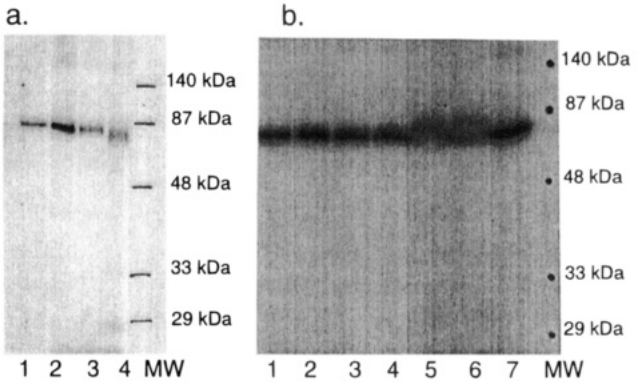

FIGURE 4: (a) Reduction of 67 kDa LBP with β-mercaptoethanol or dithiothreitol does not reduce the apparent molecular weight of the protein on 10% SDS−PAGE. ^{125}I-Labeled LBP, isolated by laminin−Sepharose affinity chromatography, was used in all cases. Lane 1, β-Mercaptoethanol treatment for 2 h at 80 °C. Lanes 2 and 3, DTT treatment for 2 h at room temperature and 80 °C. Lane 4, unreduced. (b) Molecular mass shift from a cDNA-predicted polypeptide of 32 kDa to the observed 67 kDa of affinity-isolated LBP does not appear to be mediated by glycosylation. Treatment with neuraminidase, or neuraminidase and *O*-glycanase, has no effect on the apparent molecular mass of affinity-purified 67 kDa LBP. ^{125}I-Labeled LBP is used in all cases, and experiments were analyzed by SDS−PAGE. Lanes 1 and 2, neuraminidase treatment for 60 min (1) or for 2 h (2); lanes 3 and 4, neuraminidase followed by *O*-glycanase treatment for 2 h (3) or for 16 h (4); lanes 5 and 6, 67 kDa LBP denatured at 80 °C with 0.1% SDS to control for detergent effects; lane 7, untreated starting material. The positions of prestained molecular mass standards were marked with ^{14}C ink prior to exposure of the phosphorimaging screen.

Table 1: Comparison of the Predicted Amino Acid Composition for the 32 kDa LBP Gene Product with That Determined for the Hydrolyzed Affinity-Isolated 67 kDa LBP

amino acid residue	predicted mol % (Yow et al., 1988)	mol % value found for isolated 67 kDa LBP[b]
Ala	12.50	12.80
Arg	5.06	6.53
Asp	5.09	5.22
Cys	ND[c]	ND[c]
Glu	8.48	6.27
Gly	5.09	5.85
His + Thr	9.54	8.91
Ile + Leu	12.55	15.88[a]
Lys	3.39	3.02
Met	2.37	3.88
Phe	3.39	4.14
Pro	6.78	6.89
Ser	4.75	5.75
Tyr	2.37	3.34
Val	6.44	6.74

[a] Peak showed partial overlap with the norleucine internal standard and may be overestimated. [b] Experimental data shown are for the 48 h hydrolysis values. [c] ND, not determined.

that were shown to be active with positive controls, had no affect on the apparent molecular mass of ^{125}I-labeled protein (Figure 4b). Consistent with the absence of consensus sites for N-linked glycosylation, no molecular mass shift was observed when the affinity-isolated protein was treated with Endo-F (data not shown).

To determine whether the 67 kDa LBP is modified by fatty acids, affinity-isolated LBP was dialyzed against double-distilled H_2O to remove buffer salts and reduce detergent remaining from the extraction procedure and lyophilized. SDS−PAGE analysis of the lyophilized material showed the same apparent molecular weight as the starting material (data not shown). Samples of 50 μg of protein (dry weight) were transesterified with the alkaline methanolic reagent Methyl Prep II. The transesterified mixture was hexane-extracted to obtain the fatty acid methyl esters which were subjected to GC and GC−MS. Comparison of peaks observed in electron ionization GC−MS with a library of mass spectrometry fragmentation patterns enabled us to unambiguously identify three lipid moieties: palmitate, stearate, and oleate (Figure 5a,b). Semiquantitative comparison of the lipids extracted from the 67 kDa LBP with external standards indicated approximately 0.05 μg of palmitate, 0.04 μg of stearate, and 0.02 μg of oleate per 50 μg protein sample. Transesterification at room temperature resulted in a significantly lower yield of palmitate, and the stearate and oleate were not detected in all experiments under these conditions. Organic solvent extraction (methylene chloride) and transesterification of the extracts of the isolated protein yielded no detectable fatty acid esters. Analyses of lyophilized laminin affinity column elution buffer containing NP-40 were entirely negative for fatty acids, as were methylene chloride extracts of affinity column washes.

Lipids Identified with the 67 kDa Laminin Binding Protein Do Not Appear to Be Associated via a Glycosylphosphatidylinositol (GPI) Structure. Lipid modifications of plasma membrane-bound proteins are sometimes attached by a GPI moiety. Two separate assays were used to test whether the lipids we identified were present as part of a glycosylphosphatidylinositol structure. Temperature-induced phase separation in Triton X-114 showed that the ^{125}I-labeled LBP partitioned primarily into the aqueous phase (Figure 6a). This finding is compatible with the predicted hydrophobicity index of the amino acid sequence deduced from the cDNA (Rao et al., 1989), but is unlikely for a GPI-tailed protein (Hooper et al., 1987; Hooper & Turner, 1989). When we attempted to label the 67 kDa LBP with [^{14}C]ethanolamine, detectable levels of radioactivity were not found associated with the affinity-isolated LBP product by phosphorimage analysis of SDS−PAGE gels. Extended exposure of the phosphorimage screen indicated that, while the specific activity of the labeling was low, it was sufficient to detect at least some labeled protein products, and a small [^{14}C]ethanolamine-labeled protein was readily identified in the low ionic strength elution of the laminin affinity column (Figure 6b, lanes 1−3). No [^{14}C]ethanolamine-labeled proteins were detected in the material eluted from the laminin column at high ionic strength (Figure 6b, lanes 4−6).

High-Affinity Laminin Binding of the LBP Appears to Be Modulated by Accessory Factors. When affinity-purified and ^{125}I-labeled LBP was reapplied to a laminin−Sepharose column under conditions identical to its initial purification, most of the protein failed to bind to the affinity column (Figures 7 and 8). Approximately half of the applied activity was recovered in the unbound column effluent, while the majority of the rest of the ^{125}I-labeled LBP was eluted from the column during the wash with low ionic strength buffer (Figures 7 and 8). During the initial purification of the 67 kDa LBP, the same low ionic strength buffer is used, under identical conditions, to remove all loosely associated material, and is found to contain many proteins with a wide range of molecular weights. The high-affinity products are then eluted with a high ionic strength buffer to obtain fractions such as shown in Figure 1a, lane 3. As shown in Figures 7 and 8, upon repurification, the affinity of the ^{125}I-labeled 67 kDa

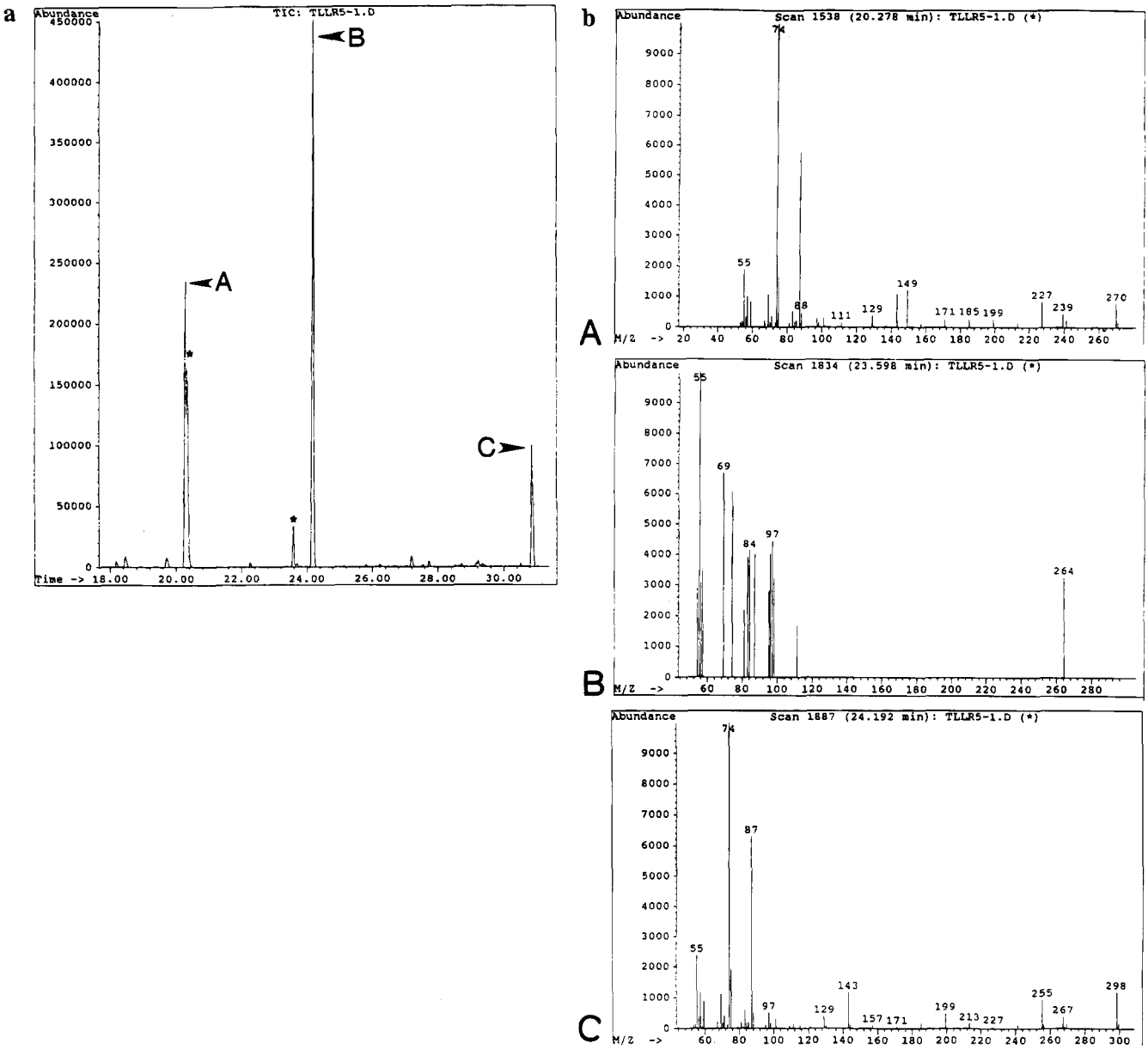

FIGURE 5: Affinity-isolated 67 kDa LBP was exposed to alkaline methanolysis transesterification conditions; the fatty acid methyl esters were extracted with hexane, dried down under argon, and redissolved in isooctane for GC analysis. Peak A was identified as palmitate (Figure 5b, panel A). Peak B was identified as oleate (Figure 5b, panel B), and peak C was shown to be stearate (Figure 5b, panel C). The two small peaks indicated by asterisks were shown to be plasticizers.

protein for the laminin column was greatly decreased so that, while a small absorption peak was detected at 214 nm, none of the protein could be detected by phorphorimaging in fractions eluted by the high ionic strength buffer (Figure 7, lane 4). The binding of the purified ^{125}I-labeled LBP to the laminin affinity column was partially restored by the addition of fractions 1 and 4 of the low-affinity eluate to the purified 67 kDa LBP preparation. The reconstituted material was applied to a freshly prepared laminin affinity column and resulted in a binding and elution pattern similar to that seen on the initial isolation. After reconstitution with fractions 1 and 4, a much larger fraction of the purified protein was retained on the column and appeared in the high ionic strength elution buffer as is shown in Figure 8. The average ratios of low-affinity to high-affinity binding materials are given in Figure 8 for (a) primary affinity isolations of the 67 kDa LBP, (b) reapplication of purified 67 kDa LBP to the affinity column in the absence of other membrane fractions, and (c) reapplication of purified 67 kDa LBP to the affinity column in the presence of membrane fractions 1 and 4 from the initial purification. The reconstitution experiments were carried out using ^{125}I-labeled 67 kDa LBP to facilitate following the protein, and in all cases the identity of the material eluting at 214 nm was checked by silver-stained SDS−PAGE. Although it is possible that the radioiodination process could lead to some loss of laminin binding capacity, the reconstitution experiments showed that a substantial fraction of the ^{125}I-labeled material was active.

It should be noted that the low ionic strength eluates for the three cases shown in Figure 8 are not strictly equivalent because of the nature of the experiments. The low ionic strength eluate for the primary isolates contains many membrane components which extract into the detergent but which show modest or no binding to laminin. Similarly, the low ionic strength eluate from the reconstituted experiments contains many of the same membrane components,

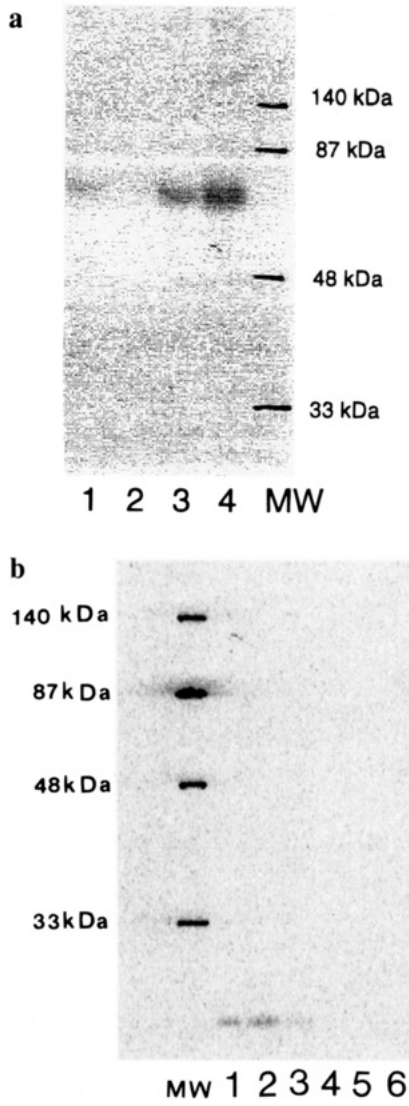

FIGURE 6: (a) Affinity-purified LBP partitioning into the aqueous phase of a two-phase Triton X-114 assay suggests that it is not likely to be modified by a glycosylphosphatidylinositol tail. ^{125}I-Labeled protein was subjected to temperature-induced phase separation at 0 and 30 °C and separated on a sucrose cushion as described under Materials and Methods. Lane 1, first detergent phase; lane 2, second detergent phase; lane 3, aqueous phase; lane 4, untreated protein. M, prestained molecular mass markers (Bio-Rad) marked with ^{14}C ink. (b) Lack of [^{14}C]ethanolamine-labeled proteins at 67 kDa in the high ionic strength elution of membrane detergent extract purified on a laminin affinity column. [^{14}C]-Ethanolamine was used to metabolically label proteins with a glycosylphosphatidylinositol tail. Fractions from the low ionic strength elution buffer (lanes 1−3) and fractions from the high ionic strength elution buffer (lanes 4−6) were separated using SDS−PAGE, and label was visualized on a Molecular Dynamics Phosphorimager. MW, prestained molecular mass marker positions indicated using ^{14}C ink.

while the low ionic strength eluate for the purified 67 kDa LBP contains only the LBP. On the basis of optical density readings at 214 nm, approximately 4 times as much protein was recovered in the high-affinity eluate when the purified 67 kDa laminin binding protein was reconstituted with the low-affinity fractions, than was recovered from equivalent loadings of the LBP without reconstitution.

DISCUSSION

The 67 kDa laminin binding protein has been shown to play an important role in the adhesion and extravasation of

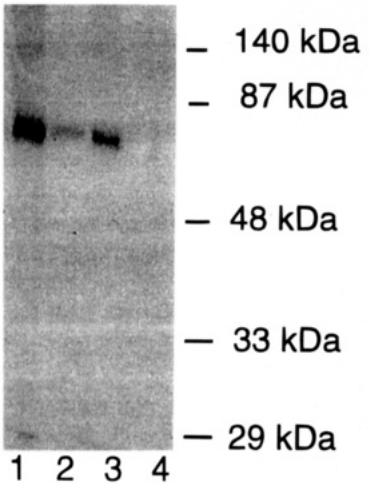

FIGURE 7: Fractions collected from a laminin−Sepharose affinity column following application of purified, ^{125}I-labeled 67 kDa LBP. The majority of the purified material did not rebind, or showed clearly lowered affinity during the salt elutions. All samples were analyzed by SDS−PAGE. Lane 1, unbound fraction; lane 2, first fraction of low ionic strength column wash; lane 3, fourth fraction of low ionic strength column wash; lane 4, high ionic strength column eluate. Molecular mass standards are as marked.

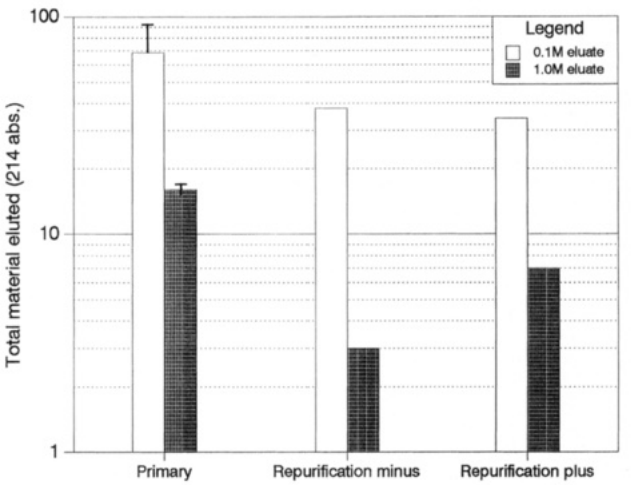

FIGURE 8: Comparison of the relative amounts of 214 nm absorbing material eluting in the low ionic strength wash (open bars) and the high ionic strength was (filled bars) when purified 67 kDa LBP is reapplied to a laminin−Sepharose affinity column. The left-hand data set (primary) shows the average elution pattern for four independent *de novo* isolations for reference. The middle data set (repurification minus) shows the elution pattern for the purified 67 kDa LBP reapplied to the laminin affinity column without any reconstitution with low-affinity membrane fractions. The right-hand data set (repurification plus) shows the elution pattern for the purified 67 kDa LBP reapplied to the column after reconstitution with low-affinity membrane fractions.

tumor cells during the metastatic dissemination of cancer [reviewed in Sobel (1994)]. However, neither the structure, the ligand binding mechanism, nor possible factors cooperating with this protein are understood in any detail. Considerable confusion in the literature has been generated by the fact that a number of laminin binding proteins of similar apparent molecular weight have been described, and frequently assumed to be the same molecule [reviewed in Mecham (1991)]. Subsequent sequencing has identified some of these proteins as 5′-nucleotidase (Misumi et al., 1990), aspartactin (Clegg et al., 1989), and a splice variant of β-galactosidase (Hinek et al., 1993). Antibodies to these various laminin binding proteins have been shown to cross-

react with each other, possibly due to common epitope structures (Risse et al., 1989).

The isolation of a cDNA clone for the 67 kDa metastasis-associated laminin binding protein failed to clarify the issue, as the cDNA clone was sufficient to encode only a 32 kDa polypeptide (Yow et al., 1988). Furthermore, the deduced protein sequence contained no consensus sites for N-linked glycosylation which could account for the molecular weight discrepancy. The protein clearly functions on the surface of metastatic cells, yet no obvious transmembrane region is apparent from the sequence (Yow et al., 1988). Classical biochemical analyses such as peptide mapping and direct amino acid sequencing have been limited by insufficient material for standard assays and idiosyncratic behavior of the protein. Reticulocyte lysate *in vitro* translation studies (Castronovo et al., 1991; Rao et al., 1989) were reported to result in a 37 kDa protein. In those studies, anti-synthetic peptide antibodies which immunoprecipitated the 67 kDa product did not always recognize the putative 37 kDa precursor. In the current study, we have utilized a homotypic eukaryotic overexpression system to produce the 67 kDa LBP in a physiologically functional form.

Using the technique of MALDI-TOF mass spectrometry, we were able to demonstrate that the molecular weight of the protein is consistent with its SDS−PAGE migration. It is attractive to speculate that the 33 kDa species seen on the MALDI-TOF MS could represent the native gene product with the 66 kDa species representing a dimer, and the 133 and 201 kDa species representing 4× and 6× subunits, respectively. Such an interpretation is supported by the experimentally obtained amino acid composition data for the isolated protein (Table 1) which are consistent with the 67 kDa form being a dimer of the 32 kDa sequence. Treatment with disulfide reducing agents did not affect the molecular weight, either on SDS−PAGE or in the mass spectra, indicating that, if this protein does exist as a dimer or higher multimer, the association does not appear to be dependent on disulfide bonds. If the high molecular weight peaks identified on the TOF MS represent nonspecific aggregates of a 33 kDa monomer, one would also anticipate the presence of 3× and 5× multimers, which were not detected. However, it should be emphasized that with the methods employed here, it is not possible to definitively distinguish between a singly protonated monomer (MH^{1+}) of 33 kDa and a doubly protonated monomer (MH_2^{2+}) of 66 kDa which would also appear at *m/z* 33 kDa. In fact, it is commonly found in UV MALDI-TOF that (MH_2^{2+}) species are observed. The cDNA-predicted product of 32 kDa has not been unambiguously rationalized with the principal 67 kDa observed species, but the mass spectrometry data in no way conflict with this interpretation.

Castronovo et al. reported that *N*-glycosidases had no effect on the molecular weight of isolated 67 kDa LBP (Castronovo et al., 1991), and there are no consensus sites for N-linked carbohydrates. These authors were also unable to metabolically label a transfected construct in COS-7 cells with [^{3}H]-glucosamine or galactosamine. Our results, using endoglycosidase digestion of affinity-purified LBP, support the conclusion that neither N- nor O-linked glycosylation is responsible for the molecular mass shift from the cDNA predicted protein product of 32 kDa to the affinity-isolated 67 kDa LBP product. Glycoproteins typically show a considerable degree of heterogeneity in glycosylation. However, the MALDI-TOF spectra show relatively sharp peaks, with no evidence of glycosylation-induced heterogeneity. The lack of heterogeneity in molecular weight provides evidence against the 67 kDa LBP being a glycoprotein.

Acylation has recently been shown to be relevant to the structure and function of numerous mammalian proteins [reviewed in Chow et al. (1992)]. Three primary mechanisms of fatty acid attachment have thus far been described: (1) ester or thioester linkages to an internal amino acid; (2) amide linkages to an amino-terminal glycine residue; (3) a phosphodiester linkage to a glycan moiety, forming a glycosylphosphatidylinositol (GPI) tail on the carboxyl terminus of the protein. The fatty acid most commonly identified with an ester linkage is palmitate, whereas cysteine residues in a CAAX consensus sequence are frequently modified with isoprenoids through a thioester linkage. We have identified the covalent association of palmitate, stearate, and oleate with the affinity-isolated 67 kDa LBP. These lipids may provide a mechanism for membrane association of the molecule on the cell surface, as there is no standard transmembrane domain apparent within the predicted amino acid coding sequence of the cDNA.

The majority of the known acylproteins on the extracellular surface of plasma membranes are associated with the membrane by lipids covalently bound to the protein through a glycosylphosphatidylinositol linkage. The amino acid motif associated with GPI addition is often quite degenerate, and addition of a GPI tail is consistent with the predicted sequence of the C-terminal region of the 67 kDa LBP. However, using two separate assay methods, we did not find evidence for the presence of a standard GPI linkage. Triton X-114 phase separation is a technique commonly used to separate amphipathic proteins. This technique is based on the ability of the nonionic detergent Triton X-114 to partition into a detergent-rich phase and an aqueous phase at 30 °C. Amphipathic proteins with a transmembrane domain tend to partition predominantly (>80%) into the detergent-rich phase, while hydrophilic proteins generally partition into the aqueous phase. Proteins which are anchored in the membrane with GPI tails have been shown to partition into the detergent phase even when associated with a very hydrophilic protein (Hooper et al., 1987; Hooper & Turner, 1988; Hooper, 1992). Upon Triton X-114 partitioning, the 67 kDa LBP remained predominantly in the aqueous phase. In a second assay for GPI association of the identified lipids, we attempted to metabolically label the 67 kDa LBP with [^{14}C]-ethanolamine. We failed to detect any [^{14}C]ethanolamine labeling in the isolated 67 kDa LBP. Ethanolamine is a specific label for GPI moieties, but in a mammalian GPI anchor could only incorporate a maximum of three ethanolamine residues per anchor under optimum biosynthetic conditions (McConville & Ferguson, 1993). The specific activity of the [^{14}C]ethanolamine used was near the limits of detection in this system. In fact, the phosphorimaging technology used to visualize the gels is not optimized for ^{14}C, and is relatively insensitive for this radiolabel. However, during affinity isolation of the LBP, other moieties labeled with radioactive ethanolamine were detected in the low ionic strength column elutions, so failure to detect labeling due solely to low activity is considered less likely.

The temperature conditions required to release the covalently bound lipid from the protein were rather stringent, relative to those needed for standard fatty acid analyses

(McCreary et al., 1978). Transesterification under milder temperature conditions resulted in significantly lower yields of methyl esters, and the oleate and stearate were not always detectable under the milder experimental conditions. Acid hydrolysis also failed to release fatty acids, indicating that the linkage mechanism is not likely to be an amide bond (data not shown). The most common mechanism for palmitate attachment to proteins has been shown to be an ester bond with the hydroxy group on serine or threonine (McIlhinney, 1990). Our data are compatible with this chemistry. Ester linkages of stearate and oleate to proteins are also a possibility. As stearate and oleate are common components of the phospholipid bilayer, great care was taken to ensure that their presence was not due to membrane lipid contamination of the protein preparations. Given the harsh conditions required for transesterification of these fatty acids, we are confident that they are, in fact, covalently associated with the protein. However, we were unable to definitively identify the linkage chemistry.

While the data are in fairly good agreement with a 1:1 molar ratio of fatty acid to 67 kDa protein, the quantity of lipid was insufficient to account for more than a small portion of the total molecular mass shift from the cDNA-predicted 32 kDa to the observed 67 kDa. Given the harsh experimental conditions required to release the lipid, complete transesterification may not have been achieved, and the number of fatty acids per 67 kDa protein may be underestimated. An alternative mechanism which could account for a majority of the shift from 32 kDa to 67 kDa would be a dimerization of the native gene product. The dimerized product could in turn be stabilized by lipid−lipid association. Within the calibration error range of ±0.2%, the MALDI-TOF molecular weight measurement compared to the cDNA-predicted molecular weight could accommodate up to 2 fatty acids per 32 kDa molecule, or 4 fatty acids per 67 kDa molecule. Lipid modification of the protein is likely be responsible for plasma membrane association of the 67 kDa LBP in the apparent absence of a hydrophobic membrane anchor polypeptide domain. Alternatively, the lipids may contribute to a specific conformation of the protein, or promote an association with additional factors. Castronovo et al. have proposed a covalent linkage with a second protein molecule (Castronovo et al., 1991), and our results do not eliminate that possibility. Indeed, we show evidence in this paper that the high-affinity ligand binding function may be modulated by an, as yet, unidentified factor in detergent extracts of tumor cell membranes.

We show preliminary evidence in this paper that reconstitution of the laminin binding protein with other components in the detergent extracts of tumor cell membranes restores the original avid laminin binding ability of the affinity-isolated 67 kDa LBP. It is possible that plasma membrane components in the extracts provide the proper hydrophobic environment for a conformation-dependent binding. However, detergent alone was not sufficient to restore the high-affinity binding of the isolated protein, and it appears that one or more additional membrane factors are required for proper conformation or orientation.

Expression of the 67 kDa LBP has been shown to correlate with expression of the $\alpha_6\beta_1$ integrin receptor on small cell lung cancer cell lines (Pellegrini et al., 1994). These authors speculated that the 67 kDa LBP did not bind laminin directly, but functioned as an accessory molecule for $\alpha_6\beta_1$. This integrin receptor is thought to bind to the E8 fragment of laminin-1, which is located in the distal region of the long arm (Sonnenberg et al., 1990). The 67 kDa LBP has been shown to interact with a five amino acid sequence, YIGSR, located in the proximal region of the short arm of the laminin-1 β chain (Graf et al., 1987). This finding, however, was not supported by rotary shadowing experiments, which showed isolated 67 kDa LBP associated with the long arm of laminin-1 (Cioce et al., 1993). Coordinated binding by the two receptors, $\alpha_6\beta_1$ and the 67 kDa LBP, could reconcile these discrepant observations. Coordinated binding implies surface clustering of the laminin binding molecules which in turn is likely to involve common intracellular trafficking. Interestingly, exactly such a pattern has been noted for the 67 kDa LBP and the $\alpha_6\beta_1$ integrin in immunogold electron microscopy studies (Romanov et al., 1994). Furthermore, laminin was found to specifically increase this common trafficking to the surface. Since the laminins are large, multidomain proteins with multiple functions, it is also possible that two or more adhesive sites exist within the laminin molecule whose activities are modulated by accessory factors.

We suggest a working model where, in the presence of accessory factors, possibly molecules such as $\alpha_6\beta_1$, or other nonproteinaceous factors, a high-affinity laminin binding site is accessible and used by the 67 kDa LBP. In the absence of such accessory factors, perhaps the 67 kDa LBP utilizes a lower affinity binding site. The present work has implicated cofactors functioning to promote high-affinity binding of the 67 kDa LBP to laminin. Further definition of the surface form and adhesive characteristics of laminin binding proteins should clarify our understanding of their mechanisms of action and, so, facilitate the design of specific therapeutics for the treatment of metastatic disease.

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