Ligatin: A Peripheral Membrane Protein with Covalently Bound Palmitic Acid*

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The ligatin monomer is a polypeptide of Mr = 10,000 which is soluble in acidified chloroform:methanol, a characteristic similar to that of Folch-Lee proteolipid. The hydrophobicity of ligatin is also reflected by its ability to intercalate into the phosphatidylecholine bilayer as shown by a concentration-dependent change in membrane conductance. However, unlike other proteolipids the amino acid composition of ligatin is not enriched in hydrophobic amino acids (isoleucine, valine, methionine, phenylalanine, tryptophan). Instead, the hydrophobic character of ligatin could be explained, at least in part, by the covalent association of fatty acids, 1.4–1.7 mol of palmitate/10,000 g of protein, as revealed by gas chromatography mass spectrographic analyses. The post-translational addition of fatty acid may therefore be the means by which ligatin acquires an affinity for membranes.

The luminal surface of ileal enterocytes in fetal and neonatal mammals is covered by 7.5-nm square particles that are attached to a fibrillar array (ligatin). The square particles have been identified as β-hexosaminidase (1, 2) and its attachment to the filament is the formation of a complex similar to that of Folch-Lee proteolipid. The square particles were products of Sigma. All other chemicals were reagent grade.

The hydrophobicity of ligatin is also reflected by its characteristic similar to that of Folch-Lee proteolipid. Instead, the hydrophobic character of ligatin could be explained, at least in part, by the covalent association of fatty acids, 1.4–1.7 mol of palmitate/10,000 g of protein, as revealed by gas chromatography mass spectrographic analyses. The post-translational addition of fatty acid may therefore be the means by which ligatin acquires an affinity for membranes.

Experimental Procedures

Stearic acid was obtained from Mann Laboratories. Methylated fatty acid standard mixture (NIH), n-ecosanoic acid, chromatographically pure egg yolk phosphatidycholine (C16:0 34%; C18:1 35%), and bovine phosphatidylserine (C18:0 42%; C18:1 37%) were purchased from Supelco Inc. Decane was obtained from Eastman Kodak Co. Spectragrade hexane was purchased from Burdick and Jackson Laboratories Inc. Spectragrade CHCl3 was purchased from Pierce Chemical Co. Neomycin and glycophase-CPG (200-400 mesh) were products of Sigma. All other chemicals were reagent grade.

Purification—Ligatin was isolated from 109 neonatal rat intestines as described by Jakoi et al. (1). Briefly, the procedure involved isolating a crude membrane pellet from homogenates of epithelial scrapings of the ileum by centrifugation at 27,000 × g for 10 min. The crude membrane pellet was further fractionated by centrifugation through a sucrose step gradient made up of equal quantities (2 ml) of the following (w/w) percent sucrose: 45%, 36%, sample (34%), and 26%. The gradient was overlaid with HEPES buffer (10 mM HEPES, 1 mM NaCl, pH 7.6). The preparation was centrifuged in an SW41 rotor at 95,000 × g for 45 min. Material at the 26/34% and 34/36% sucrose interfaces was pooled, resuspended in HEPES buffer, and centrifuged at 27,000 × g for 10 min. The membrane pellet was treated with 10 mM CaCl2 (pH 6.0, 5 min, 4 °C) to release a sedimentable ligatin-β-hexosaminidase complex. The treated pellet was then dialyzed against HEPES-EGTA buffer (5 mM HEPES, 0.5 mM EGTA, pH 7.6) for 16 h. After dialysis ligatin no longer sedimented at 110,000 × g. Solubilized components were removed from membranes by centrifugation at 110,000 × g for 90 min and then fractionated by size chromatography on a Bio-Rad P-100 column (1.5 × 30 cm, 4 °C) equilibrated in HEPES buffer. The fractions containing ligatin were pooled and filtered through a PM-10 (Amicon) filter. Thirty μg of ligatin were routinely isolated from 50 animals.

The purified ligatin was next extracted with organic solvents in order to remove noncovalently associated lipids. The protein was suspended in 0.5 ml of water and extracted with 1.5 ml of chloroform:methanol (1:1, v/v), to which 0.5 ml of chloroform was added to generate a two-phase system. The sample was centrifuged at 1000 × g for 15 min. The organic phase was removed, dried under nitrogen (N2) and found to lack fatty acids by gas chromatographic analysis. The aqueous layer containing the ligatin polypeptide was retained and lyophilized. Since only complexed polyphosphoinositides (PIP and PIP1) would not have been removed under these conditions, the treated pellet was eluted in buffers B and C. The preparation was centrifuged in an SW41 rotor at 95,000 × g for 45 min. The 8-ml fractions were collected separately and each fraction was reinserted into a sucrose gradient made up of equal quantities (2 ml) of the following washes (w/w percent sucrose): 34%, 36%, 45%. The column was eluted with 8 ml of each of the following washes: buffer A (0.2 M NH4Ac); buffer B (chloroform:methanol:water (3:6:1, v/v) containing 0.15 M ammonium acetate; the chloroform:methanol-extracted ligatin was reinserted in 0.2 ml of first column buffer and applied to the neomycin column. The column was eluted with 8 ml of each of the following buffers: chloroform:methanol:H2O (3:6:1, v/v) containing 0.15 M ammonium acetate; the chloroform:methanol-extracted ligatin was reinserted in 0.2 ml of first column buffer and applied to the neomycin column. The column was eluted with 8 ml of each of the following buffers: chloroform:methanol:H2O (3:6:1, v/v) containing 0.15 M NH4Ac (buffer A); chloroform:methanol:H2O (3:6:1, v/v) containing 0.6 M NH4Ac (buffer B); chloroform:methanol (1:2, v/v) containing 25% concentrated NH4OH (buffer C); chloroform:methanol:H2O (3:6:1, v/v) (buffer D); and chloroform:methanol (1:2, v/v) with 0.01 M HCl (buffer E). Each 8-ml fraction was collected separately and evaporated to dryness. Fatty acids were only detected in the fraction containing the polypeptide (buffer A). Any associated PIP or PIP1 would have eluted in buffers B and C.

The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; EGTA, [ethylenbis(oxyethylenenitrilo)] tetracetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GC-MS, gas chromatography-mass spectrometry; rpHPLC, reverse-phase high performance liquid chromatography; PIP, polyphosphoinositide.
Ligatin was further purified by reverse-phase chromatography on a Beckman Ultrapore reverse-phase separation (C8) column (0.46 x 7.5 cm). The column was equilibrated with solvent A (10 mM potassium phosphate, pH 6.5, containing 5% CH3CN). The flow rate was 1.0 ml/min. Ultraviolet absorbance of the effluent was monitored at 214 nm. Only 2 µg of protein in 150 µl of solvent A could be injected onto the column/run because of the limited solubility of ligatin in neutral organic solvents. A linear elution gradient was generated as follows: within the first 5 min the percent of solvent B (CH3CN) was increased to 30%, during the next 27 min, solvent B was increased to 45% by a linear gradient, the percentage of solvent B was then increased linearly to 100% within the next 10 min. Total run time including re-equilibration in solvent A was 45 min. 

Polyacrylamide Gel Electrophoresis (PAGE)—SDS-PAGE was done according to the method of Benya et al. (6). Protein was labeled either with [14C]iodoacetic acid or with fluorescamine (7) prior to SDS-PAGE. Fluorescamine-labeled material was photographed using Kodak Wratten Gelatin Filters, No. 36 for excitation and No. 4 for transmission.

Amino Acid Analysis—Samples were hydrolyzed in 6 N HCl at 110 °C for 24 h in vacuo. Amino acid analysis was performed on a Beckman 6300 automated analyzer using ninhydrin detection.

Derivatization—Fatty acids were analyzed as their methyl esters by gas chromatography. Ligatin was incubated at 24 °C in methanolic alkali (pH 10 with 0.2 N NaOH in 50% methanol) for 17 h. After acidification with HCl, the solution was extracted three times with hexane to recover fatty acid and methyl esters. The hexane was evaporated under a stream of N2 at 24 °C. The residue dissolved in 1/8 ml of anhydrous 0.5 N HCl/methanol (Supelco) and the solution heated to 70 °C for 17 h to insure all fatty acids were converted to the methyl ester. The individual hydrolysates were pooled, 0.5 ml of H2O was added, and the samples were extracted three times with 2 ml of hexane. The hexane extracts were dried under N2, resuspended in 10 µl of hexane, and 1–2 µl aliquots were subjected to GC and GC-MS analysis.

GC and GC-MS—Fatty acid methyl esters were analyzed in a Perkin-Elmer 990 gas chromatograph using a 10% EGSS-X column (100–200 mesh, Applied Science Laboratories). An isothermal program at 150 °C was employed with helium as carrier gas. The methyl esters were identified by their retention times relative to the methyl n-eicosanoic acid (C20) internal standard which was used both for quantitation and mass spectral analysis. The amount of fatty acid present per mol of protein was based on the values obtained from these analyses and from the quantity obtained from amino acid mol/mol calculation. The GC-MS analysis was done with Hewlett-Packard Quadrupole mass spectrometers (models 5982A and 5980A) coupled to ion selector devices. The instrument was operated with an electron energy of 40 eV in the ionization mode using a 5-ft glass column (0.4 cm inner diameter) packed with 3% OV-1 (Supelco) on 80/100 Supelecort. A temperature program of 150–280 °C at 6.0 degrees/min was employed. Helium was used as carrier gas at a flow rate of 25 ml/min. The ion source and jet separator were both at 260 °C. For these experiments the mass spectrometer was scanned in a range of 50–400 atomic mass units. Data were collected with a LKB-9000 GC-MS computer system.

Bimolecular Lipid Membranes—Bimolecular or black lipid membranes were formed from phosphatidycholine (25 mg/ml decane) and phosphatidylserine (10 mg/ml decane) as described by Mueller et al. (8). The protein was added to both chambers after the membrane had thinned down to a black film. The ligatin-induced conductance was measured as described by Mueller et al. (8). It usually reached a steady state within 5 min after the addition of ligatin to the aqueous phase. All aqueous solutions were buffered 0.1 M NaCl.

RESULTS

Purification and Amino Acid Composition—Ligatin purified by gel filtration is a homogeneous species of Mr = 10,000 as shown by SDS-PAGE (Fig. 1) (2, 3). When subjected to rpHPLC, this protein was eluted as a single peak at 39% CH3CN under neutral conditions (Fig. 2). No other material absorbing at 214 nm was recovered. The recovery of ligatin in multiple runs of the same preparation from this column was found to vary from 40 to 80%. The lower yields were obtained with more concentrated samples and after prolonged storage of the protein at -70 °C. This purified polypeptide was blocked to Edman degradation.

The amino acid composition of ligatin isolated by standard purification techniques and by reverse-phase chromatography is compared in Table I. The compositions are similar in that each contains predominantly aspartic acid, glutamic acid, glycine, and serine (see Table I) but differ in the amounts of isoleucine, leucine, and phenylalanine. This increase in the amount of hydrophobic residues was consistently observed in samples subjected to reverse-phase chromatography. Acidic and hydroxyamino amino acids account for almost 50% of the total residues and no tryptophan was detected by uv spectral analysis. Calculation based on the number of residues of each amino acid suggests a polypeptide of 100 residues with a molecular weight of 10,010. This is consistent with our previous estimate of Mr = 10,000 by electrophoretic analysis (2, 3). Ligatin preparations also contain ninhydrin positive material which was eluted as three peaks that do not correspond
Ligatin: An Acyl Protein from Ileal Enterocytes

The proteins were hydrolyzed for 24 h in vacuo at 110 °C in 6 N HCl containing phenol.

| Amino acid | Ligatin | Ligatin after pHPLC |
|------------|---------|---------------------|
|            | mol/10,000 g protein |                     |
| Lys        | 2.0     | 3.4 (0.6)           |
| His        | 1.9     | 1.7 (0.2)           |
| Arg        | 1.4     | 1.9 (0.3)           |
| Axt        | 11.8    | 9.9 (1.6)           |
| Thr        | 5.7     | 4.7 (0.2)           |
| Ser        | 15.0    | 7.3 (0.3)           |
| G1x        | 15.9    | 13.3 (1.8)          |
| Pro        | 4.3     | 4.7 (0.6)           |
| Gly        | 19.0    | 21.4 (1.7)          |
| Ala        | 7.5     | 6.8 (0.8)           |
| Val        | 4.4     | 5.4 (0.8)           |
| Lys(?)b    | 1.0     | 1.0 (0.3)           |
| Ile        | 2.4     | 4.4 (0.5)           |
| Leu        | 5.3     | 9.1 (0.2)           |
| Tyr        | 2.2     | 1.9 (0.1)           |
| Phe        | 2.1     | 2.6 (0.5)           |
| Cys        | NDc     | ND                  |
| Trp        | NDc     | ND                  |

* Average of five separate determinations. Standard error is given in parentheses.
* Although methionine was detected, the protein was not sensitive to cyanogen bromide cleavage and performic acid oxidation did not destroy this compound.
* ND, not detected.

to the common amino acids, ammonia, or reagent background. Two of these peaks were eluted before threonine indicating that they are highly acidic substances. The third component is relatively basic and appears just before lysine. One of the two acidic peaks corresponds to the elution position of phosphoserine (2). The other acidic peak was eluted between aspartic acid and threonine. The identity of this acidic peak and the basic peak are currently unknown.

**Fatty Acid Acylation: GC-MS**—Ligatin's solubility in acidified chloroform:methanol and its ability to interact directly with a CN resin suggested a hydrophobic character that cannot be readily explained by its amino acid composition. Since other eukaryotic membrane-bounded proteins are known to contain covalently bound fatty acids, we investigated ligatin preparations for this chemical modification. Two types of ligatin preparations were analyzed for fatty acylation: one that had been dialyzed extensively against H2O and a second that had been extracted with organic solvents and subjected to affinity chromatography on a neomycin-glass support. The polypeptide-associated fatty acids from each preparation were subsequently removed by transesterification and analyzed by GC and GC-MS. Fig. 3 shows a typical ion tracing of the ligatin preparations. Both preparations showed the presence of methyl palmitate (Fig. 3a). The spectra and retention times obtained were identical to those of published standards. The amount of methyl palmitate was quantitated (Table II) and found to be 1–2 mol/10,000 g of protein. A minor amount (0.02–0.4 mol/mol of polypeptide) of methyl oleate was also detected (Fig. 3b).

**Interaction with Lipid Bilayers**—To investigate the interaction of ligatin with membrane lipids, we tested its ability to alter the conductance of biomolecular lipid films in vitro. Two types of bilayers were constructed: phosphatidylcholine (C16:0 34%; C18:1 35%) and phosphatidylserine (C18:0 42%; C18:1 37%). The addition of ligatin to both sides of the phosphatidylcholine membranes induced an increase in membrane conductance in a concentration-dependent manner (Fig. 4).

**DISCUSSION**

In the studies reported here, we show that ligatin is an acyl protein, composed of a polypeptide of Mr = 10,000 which is a single peak in reverse-phase high performance liquid chromatography (pHPLC). Amino acid analysis shows that ligatin has a high content of polar residues, suggesting that its amphipathic properties are not due to its protein component. A covalently bound fatty acid can be released from purified ligatin by methanol alkali. GC-MS identified the fatty acid

![Fig. 3. Mass spectra of ligatin-associated fatty acids removed by transesterification. The fatty acids were isolated and esterified as described under "Experimental Procedures." The methyl esters were separated using a column packed with 3% OV-1 on 80/100 Supelcoports and analyzed with a Hewlett-Packard mass spectrometer. The spectra (a and b) and retention times obtained were identical to that of standard methyl palmitate and methyl oleate, respectively. A.M.U., atomic mass units.](image)
Phosphatidylserine translational modification appears on a subclass of glycoproteins that have hydrophobic properties yet do not contain any hydrophobic amino acids, such as myristic acid (18, 19). Since this post-translational modification of bimolecular lipid films can be separated into two groups: 1) the proteolipids (9-12) which are soluble in organic solvents and 2) a set of proteins (14-18) which are insoluble in nonaqueous solvents. Ligatin exhibits solubility characteristics common to the proteolipids (9, 10), but differs from the arachidonylphospholipids of myelin (9-12) and sarcoplasmic reticulum (9, 10) in possessing predominantly a polar amino acid composition and less than 23% apolar residues (including His and Tyr).

Fatty acids are released from purified ligatin under mild methanolic alkaline conditions, suggesting that they are covalently bound through ester linkages. The composition of the protein-bound fatty acids seems to account for ligatin's selective location at the external leaflet of the membrane. Palmitic acid is bound to the protein in a 1-2 mol:mol ratio; oleic acid which is consistently observed is bound to the polypeptide in a submolular concentration (0.02-0.4 mol:mol polypeptide). Other acyl proteins are known to contain palmitic acid (11-16) covalently attached in an amino acid-ester linkage (11, 13-15). The potential acylation sites are found close to the postulated membrane-spanning segments of these integral membrane proteins (15-17). In contrast, ligatin's extraction in the absence of detergents suggests it lacks a transmembrane domain. These findings suggest the existence of a novel site, the EXO domain of a membrane protein for fatty acylation.

The covalent attachment of lipid to cell surface proteins which contain only an EXO domain has been described (18-20). This post-translational modification, consisting of phosphatidylinositol containing glycolipid covalently linked to the carboxyl terminus, functions as a membrane anchor (18, 20). The two fatty acids attached to phosphatidylinositol have been identified as myristic acid (18, 19). Since this post-translational modification appears on a subclass of glycoproteins that have hydrophobic properties yet do not contain any extended sequence of hydrophobic amino acids (18, 19), we are exploring the possibility that phosphatidylinositol containing glycolipid is present on ligatin and may be the site of fatty acylation.

Alternately fatty acids may be esterified to serine and threonine residues of a polypeptide chain and provide an anchorage site within the lipid bilayer (16, 21-23). In support of this hypothesis, we have found that ligatin increases the conductance across neutral phosphatidylcholine bilayers in a concentration-dependent manner. Such a change is dependent on a perturbation of the fatty acid region of the bilayer, whereas an interaction with the head group region alone does not affect the conductance (24). These results lend validity to the notion that in vitro ligatin most likely interacts directly with the lipid core and not to the head group region of the membrane phospholipids. Since ligatin has a low content of hydrophobic amino acids, the fatty acids covalently bound to ligatin may be responsible for ligatin's attachment and stabilization in membranes.

The covalent attachment of palmitic acid to ligatin distinguishes it from Ca²⁺-binding proteins such as calmodulin (25), troponin C (25), calcineurin B (21, 25), and S100 (26). These proteins are members of a group of homologous Ca²⁺-binding proteins that function as secondary messengers in signal transduction as well as the control of muscle contraction (27). In contrast, ligatin is a peripheral membrane protein because of its polar amino acid composition and also because of its release from membranes by cations and pH (4.5 and 8.0) (1) which perturb ionic interactions. Yet, ligatin inserts into black lipid films in vitro in a noncovalent hydrophobic manner characteristic of integral membrane proteins. Therefore, ligatin represents a subclass of peripheral membrane proteins, one that exhibits amphipathic properties critical for protein-lipid interactions but one that partitions into either a hydrophobic or a hydrophilic domain dependent upon pH or cations. It is postulated that pH-induced changes enable this protein either to interact with target membranes or to separate from the membrane, thereby providing a mechanism for regulating ligatin's expression at the membrane surface.

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Ligatin: An Acyl Protein

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