LIGATIN FROM EMBRYONIC CHICK NEURAL RETINA

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ABSTRACT

Ligatin, a filamentous protein previously found in suckling rat ileum, has been purified from plasma membranes of embryonic chick neural retina. The isolated plasma membranes are covered in part by 4.5-nm filaments that can be released from the membranes by treatment with Ca++. Subsequent dialysis against EGTA followed by sieve chromatography results in purification of the 10,000-dalton ligatin monomer. When labeled either with radioisotopes or with fluorescamine, the monomer is shown to electrophorese as a single discrete band in polyacrylamide gels. However, during standard fixing and staining procedures it diffuses from the gels and thus is not visualized. Ligatin's amino acid composition is distinguished by its high content of polar residues, especially Glx and Asx, and by the presence of phosphorylated serine. Upon re-addition of Ca++, purified ligatin monomers polymerize to form filaments 3 nm in Diam, identical to those formed by purified ileal ligatin. However, in both retina and ileum, the filaments observed on plasma membranes are >3 nm in Diam. In ileum, this enlargement results from ligatin's function as a baseplate for the attachment of another protein, a/3-N-acetylhexosaminidase, to the cell surface. In retina, a corresponding difference in diameter between filaments seen in vivo and those formed from repolymerized ligatin alone and the co-solubilization of other proteins with ligatin suggest that ligatin may also function there as a baseplate for other cell surface proteins. The proteins associated with ligatin in retina differ morphologically from a/3-N-acetylhexosaminidase and do not possess this enzymatic activity.

KEY WORDS  ligatin  neural retina  phosphoprotein  cell surface protein

Ligatin is a 10,000-dalton plasma membrane protein that, in the presence of Ca++, polymerizes to form filaments 3 nm in Diam. It was first isolated from suckling rat ileum by Jakoi et al. (14) where it serves as a baseplate for the attachment of /3-N-acetylhexosaminidase to the external surfaces of plasma membranes of lumenal epithelial cells. The alignment of ligatin filaments on these membranes is responsible for the regular distribution of hexosaminidase seen in electron micrographs as two-dimensional lattices of 7.5-nm particles (14, 21). These rows of particles can be detached from isolated plasma membranes by treatment with 40 mM Ca++ and yet retain their unidimensional periodicity. Subsequent dialysis against ethylene-glycol-bis(β-aminoethyl ether) N,N'-tetraacetate (EGTA) results in the dissociation of the enzyme from the ligatin filaments and the depolymerization of the filaments to form the 10,000-dalton ligatin monomer. The purified enzyme is lipid-free, but phospholipids and cholesterol are found associated with ligatin. Interestingly, when subjected to polyacrylamide gel electrophoresis, mon-
Isolation of Ligatin

Laboratories, Richmond, Calif. equilibrated with to gel filtration on a Bio-Rad P-60 column (Bio-Rad CaCl₂, incubated on ice for 10 min, and subsequently was stored at 5°C and used within a 2-wk period. HEPES, pH 8.0. The retentate was centrifuged overnight against 0.5 mM EGTA, 5 mM Co. Pittsburgh, Pa.) was neutralized with sodium hydroxide, Zinc bacitracin (40-80 μg/ml PTA solution) was used as a wetting agent (11). The PTA solution containing gels contained 7.6% acrylamide and were run at 2 mA/tube for 3 h. The fluorescamine-labeled gels were photographed by using Kodak Wratten Gelatin Filters, No. 36 for excitation and No. 4 for fluorescence transmission. Radioactive gels were frozen and divided into 1-mm slices; the slices were individually solubilized (22), and the profiles of radioactivity were determined by scintillation counting. Electrophoresis under nondenaturing conditions was by the method of Davis (6). The nondenaturing gels contained 7.6% acrylamide and were run at 2 mA/tube for 3 h. Gels were fixed by incubation overnight in either 10% acetic acid (8) or 20% trichloroacetic acid (7). Coomassie blue staining was done by the procedure of Weber et al. (22). Periodic acid-Schiff staining was by the procedure of Fairbanks et al. (8).

Amino Acid Composition

Protein (200 μg) from the included P-60 peak was dialyzed against distilled water and lyophilized in a 13 x 100 borosilicate test tube. 1 ml of 6 N HCl was added to the tube, and the tube was evacuated and sealed. The sample was heated at 105°C for 24 h, diluted to 2 ml with distilled water, and then dried under nitrogen. The sample was dissolved in 250 μl of sodium citrate buffer, pH 2.2, containing 0.2 N Na⁺ and 2% thioglycolic, and

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MATERIALS AND METHODS

Preparation of Retinal Plasma Membranes

Neural retinas were dissected from 50 10-d-old chick embryos in Ca²⁺- and Mg²⁺-free Hanks' solution or 0.9% NaCl (both buffered with 10 mM N-2-hydroxyethylpiperazine-N'2-ethane sulfonic acid [HEPES] [pH 7.2]) and kept on ice. The tissue was transferred to 20 ml of HEPES buffer (10 mM HEPES, 1 mM NaN₃, pH 7.6) and homogenized with 10 strokes of a Potter-Elvehjem homogenizer. The nuclear fraction was removed by centrifugation (500 g for 2 min). The supernate was centrifuged (27,000 g for 10 min), and the pellet was resuspended in HEPES buffer containing sucrose (33%, wt/wt). Discontinuous sucrose gradients were prepared consisting of 3-ml steps of 41, 36, 33 (containing sample), and 26%. The gradients were centrifuged at 95,000 g for 45 min. Membranes at the 36%/33% and the 33%/26% interfaces were pooled and adjusted to 33% sucrose. The discontinuous gradient centrifugation was repeated. Membranes at the 36%/33% and the 33%/26% interfaces were again pooled, diluted with HEPES buffer, and collected by centrifugation (27,000 g for 10 min). Identification of this material as predominantly plasma membranes was established by enzymatic and chemical markers (unpublished results) and by electron microscopy.

Electron Microscopy: Negative Staining

1% phosphotungstic acid (PTA) (Fischer Scientific Co., Pittsburgh, Pa.) was neutralized with sodium hydroxide. Zinc bacitracin (40-80 μg/ml PTA solution) (Burroughs Wellcome Co., Research Triangle Park, N. C.) was used as a wetting agent (11). The PTA solution was stored at 5°C and used within a 2-wk period.

Isolation of Ligatin

The plasma membranes were resuspended in 40 mM CaCl₂, incubated on ice for 10 min, and subsequently dialyzed overnight against 0.5 mM EGTA, 5 mM HEPES, pH 8.0. The retentate was centrifuged (110,000 g for 90 min), and the supernate was subjected to gel filtration on a Bio-Rad P-60 column (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with HEPES buffer. The column was calibrated with blue dextran, cytochrome c, ribonuclease A, and insulin. Protein concentrations were determined by the method of Lowry et al. (17) or by the Bradford technique (5). Assays for β-N-acetylhexosaminidase activity were by the procedures of Koldovsky and Palmieri (16).

Radioactively Labeled Ligatin

Neural retinas were dissected aseptically from 20 9- to 10-d-old embryos and incubated for 24 h at 37°C in Dulbecco's Modified Eagle's Medium (Grand Island Biological Co., Grand Island, N. Y.) containing 100 U/ml penicillin and 100 μCi/ml carrier-free ³²P-inorganic phosphate (New England Nuclear, Boston, Mass.). After the incubation, plasma membranes and ligatin were prepared as described above. Alternatively, purified ligatin was carboxymethylated with [¹⁴C]iodoacetate (New England Nuclear) by the procedure of Gurd (12). The reaction mixture (1 ml) containing 10 nM HEPES, pH 8.0, and 100 μg of retinal ligatin was incubated with 250 μCi of [¹⁴C]iodoacetate at room temperature under a nitrogen atmosphere for 30 min. The reaction was terminated with an excess of β-mercaptoethanol. The preparation was subsequently dialyzed against water and lyophilized.

Polyacrylamide Gel Electrophoresis

Ligatin (50 μg), [¹⁴C]carboxymethylated ligatin, and ligatin (20 μg) coupled with fluorescamine by the methods of Friedberg and Reynolds (9) were electrophoresed in the presence of sodium dodecyl sulfate (SDS) and urea using the procedure of Benya et al. (4). The gels contained 15% acrylamide and were run at 2 mA/tube for 3 h. The fluorescamine-labeled gels were photographed by using Kodak Wratten Gelatin Filters, No. 36 for excitation and No. 4 for fluorescence transmission. Radioactive gels were frozen and divided into 1-mm slices; the slices were individually solubilized (22), and the profiles of radioactivity were determined by scintillation counting. Electrophoresis under nondenaturing conditions was by the method of Davis (6). The nondenaturing gels contained 7.6% acrylamide and were run at 2 mA/tube for 3 h. Gels were fixed by incubation overnight in either 10% acetic acid (8) or 20% trichloroacetic acid (7). Coomassie blue staining was done by the procedure of Weber et al. (22). Periodic acid-Schiff staining was by the procedure of Fairbanks et al. (8).
centrifuged at 14,000 g for 10 min. The amino acid composition in 20 μl of sample was determined by a Durrum Model D-500 Amino Acid Analyzer (Durrum Instrument Corp., Sunnyvale, Calif.).

**Analysis for o-Phosphoserine**

Aliquots of 32P-labeled protein from the included P-60 peak were analyzed for o-phosphoserine by the procedure of Bareta and Kizer (3). Samples were dialyzed against cold distilled water, lyophilized, and hydrolyzed in 6 N HCl at 105°C for 5 h in sealed, evacuated tubes. Under these conditions, ~60% of the phosphoserine residues from hydrolyzed protein will be preserved as phosphoserine (15). The other 40% is hydrolyzed to P1 and free serine. In contrast, any 32P present in the associated lipids as phosphatidylserine will be hydrolyzed to yield free serine and P1 (1). The hydrolysates were dried under N2, resuspended in 0.4 ml of 0.1 M formic acid containing 4 μmol of carrier phosphoserine, and applied to a 1.5 x 7-cm column of Dowex 1 X-8 (Dow Corning Corp., Midland, Mich.) equilibrated with 0.1 M formic acid. After 15 3-ml fractions were eluted in 0.1 M formic acid, the collection was continued by using 0.25 M formic acid containing 0.25 M pyridine. 0.5-ml aliquots were subjected to the ninhydrin assay (20) while the remainder of the fractions were counted for 32P with a liquid scintillation system.

**Sephadex LH-20 Chromatography**

Chromatography in chloroform-methanol-HCl (2:1: 10 mM) was by the procedures of Gaetjens (10). The column was calibrated with cytochrome c, lecithin, and 32P.

**RESULTS**

**Plasma Membrane Morphology**

The retinal plasma membrane fractions were examined by electron microscopy with negative staining techniques. Low power views showed no significant amounts of mitochondria, endoplasmic reticulum, nuclear membranes, or nonmembranous materials. Yet there was some heterogeneity in the preparations. Most of the membranes were covered in part with filaments 4.5 nm in Diam; others were observed to have surfaces either completely covered by particles or completely smooth (Fig. 1). These smooth profiles may be images of "inside out" membrane vesicles.

When the plasma membranes were treated with 40 mM Ca++, individual filaments 4.5 nm in Diam were seen free of the membranes scattered over the grids (Fig. 2). However, such treatment did not release all of the particulate coatings of the membranes. After dialysis against EGTA, pH 8.0, filamentous materials were no longer seen. The release of filamentous material from plasma membranes surfaces after treatment with 40 mM Ca++ and the subsequent disappearance of filaments after dialysis against EGTA are identical to results obtained with ligatin-hexosaminidase filaments isolated from ileum (14). However, both the membrane-bound and the free filaments differ in diameter and morphology from those seen in ileal preparations.

**Isolation and Purification**

After treatment with 40 mM Ca++ and dialysis vs. EGTA, pH 8, retinal plasma membranes were separated from solubilized components by centrifugation. The solubilized components were subjected to gel filtration on a Bio-Rad P-60 column (Fig. 3). Only two protein peaks were observed, one totally excluded and the other chromatographing as a globular protein of 10,000 daltons. Approx. 100 μg of included protein were isolated from 1 g of retinal tissue.

As with ileal ligatin, the included peak exhibited an optical density at 280 nm that greatly exceeds the molar extinction coefficient of a typical protein at that wavelength. The absorbance is maximal at ~250 nm and is attributable to conjugated phospholipids associated with the protein (unpublished results).

The proteins voided from the P-60 column were assayed for β-N-acetylhexosaminidase activity (16). None was found.

**Polymerization in the Presence of Ca++**

Rechromatography of the 10,000-mol wt protein in the presence of 0.5 mM EGTA did not alter its elution profile (Fig. 4a). After dialysis against 5 mM Ca++, however, the protein was completely excluded from the P-60 column (Fig. 4b). Negative stain electron microscopy of this material showed that the protein had polymerized into filaments ~3 nm in Diam (Fig. 5a). After freeze-thawing, this material formed tactoids (Fig. 5b). The morphology of the repolymerized filaments is identical to that of repolymerized ileal ligatin.

**Polyacrylamide Gel Electrophoresis**

Aliquots from the included P-60 peak (50–100 μg of protein) were subjected to disk gel electrophoresis under both nondenaturing (6) and denaturing (SDS-urea) (7) conditions. Gels were fixed
FIGURE 1 Negatively stained preparation of the isolated plasma membranes. Most membrane surfaces are covered in part by filaments ~4.5 nm in diam; others are completely covered by particles (double arrow) or are completely smooth (single arrow). × 114,000.

FIGURE 2 Negatively stained preparation of the Ca**+-treated membrane pellet. Both smooth and particulate-coated membranes are seen. In addition, filaments ~4.5 nm in diam are seen in the background free from the membranes. × 102,000.
Several explanations seemed possible: (a) the protein did not react with either of the stains employed, (b) the protein was heterogeneous and each polypeptide was present at too low a concentration to be observed, or (c) the protein was soluble in the fixative or staining solutions and diffused from the gels. The first possibility, however, seemed unlikely because the protein in solution bound Coomassie blue G-250 (5). To examine the second and third possibilities, protein from the included P-60 peak was carboxymethylated with [14C]-iodoacetate (12). Duplicate samples of the labeled preparation were electrophoresed under denaturing conditions. One gel was frozen and sliced without fixation. Its profile of radioactivity is shown in Fig. 6a. All of the counts per minute (cpm's) applied to the gel were recovered in a single, discrete band migrating with $R_f = 0.70$. The duplicate gel was fixed and stained with Coomassie blue in methanol:acetic acid:trichloroacetic acid for 12 h. No bands were visible after destaining. The gel was then sliced and the profile of radioactivity was determined. No cpm's were present in the fixed gel. Thus, the preparation was apparently not retained in the gel with conventional fixation procedures. This interpretation was confirmed by using fluoresceamine to label the protein before electrophoresis (9). A single band of fluorescence was observed before fixation (Fig. 6b). However, during the fixation process, this fluorescence disappeared. Standards fixed similarly remained in the gel and displayed fluorescence throughout the procedure.

**Protein Phosphorylation**

When retinal tissue was incubated with $^{32}$P-inorganic phosphate for 24 h, nondialyzable radioactivity was found in the included peak after P-60 chromatography. With the addition of Ca$^{++}$, the radioactivity was voided from a P-60 column (Fig. 4b), thus suggesting an association with the polymerized protein. Although the $^{32}$P might be caused solely by phospholipids known to be bound to the protein (unpublished results), some of the cpm's could be covalently bound to the protein itself through the phosphorylation of serine residues.

To test for the presence of phosphoserine in the protein, an aliquot of the $^{32}$P-labeled peak was subjected to partial acid hydrolysis (6 N HCl, 104°C, 5 h). The hydrolysate was mixed with unlabeled carrier phosphoserine and chromato-
graphed on a Dowex 1 X-8 column (3). Of the radioactivity applied to the column, 95% was recovered, 32% of which co-chromatographed with the unlabeled phosphoserine (Fig. 7). The remainder eluted in a position corresponding to inorganic phosphate. By using a 40% correction factor for the hydrolysis of protein-associated phosphoserine under these conditions (15), we found that ~55% of the initial radioactivity could be attributed to phosphorylated serine residues in the protein.

These results were confirmed when the material was subjected to molecular sieve chromatography in chloroform-methanol-HCl on a Sephadex LH-20 column. All of the lyophilized protein and radioactivity from the included P-60 peak were readily solubilized in acidified chloroform-methanol and applied to the column. The elution profile is shown in Fig. 8. Approx. 60% of the radioactivity eluted as a peak coincident with the elution position of cytochrome c. A second peak of radioactivity was observed in the region where lecithin was found to elute. When material from the earlier peak was subjected to partial acid hydrolysis and analyzed for phosphoserine, 95% of the 32P was now attributable to phosphoserine, again with a 40% correction factor (15). Furthermore, when an aliquot of the 32P-labeled material was mixed with 20 μg of the nonradioactive, fluorescamine-coupled protein from the included P-60 peak and electrophoresed under denaturing conditions, a single peak of radioactivity with Rf = 0.70 was observed that coincided precisely with the peak of fluorescence. From these studies, we conclude that the included P-60 protein is phosphorylated.

**Amino Acid Composition**

An aliquot of the included P-60 peak containing 100 μg of protein was subjected to a 24-h acid hydrolysis and analyzed for amino acid composition. The results are shown in Table I.
The amino acid composition of this protein is distinguished by its high content of polar residues, especially Glx and Asx. A low release of ammonia during the hydrolysis (<50% of the Glx value) suggests that most of these residues were initially in the acidic form. Overall, 76% of the amino acids were polar while only 17% (excluding alanine) were hydrophobic.

**DISCUSSION**

A new protein has been isolated from plasma membranes of embryonic chick neural retina that possesses the following characteristics: (a) re-

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**FIGURE 6** (a) Profile of radioactivity of [14C]carboxymethylated ligatin after electrophoresis in SDS-urea on 15% polyacrylamide gels and before fixation with acidic or acidified organic solvents. Arrows denote positions of standards: actin (43,000 daltons); ribonuclease A (13,600 daltons); cytochrome c (12,500 daltons). After fixation, no cpm's remained in the gel. (b) Distribution of fluorescence after electrophoresis of fluorescein-ligatin. After being fixed, no fluorescence remained although standards (not shown) remained clearly visible.

**FIGURE 7** Elution profile from Dowex 1 X-8 column of 32P-labeled material from the included P-60 peak after partial acid hydrolysis. Arrow indicates change of buffer to 0.25 M formate, 0.25 M pyridine. (- - -), Cpm of 32P. (---), Absorbance at 570 nm showing the elution profile of o-phosphoserine standard.

**TABLE 1**

| Amino Acid | Mol per 100 mol of amino acid |
|------------|-------------------------------|
| Asx        | 10.6                          |
| Thr        | 4.4                           |
| Ser        | 13.1                          |
| Glx        | 22.2                          |
| Pro        | 2.6                           |
| Gly        | 14.1                          |
| Ala        | 7.6                           |
| Val        | 3.4                           |
| Met        | 0.6                           |
| Ile        | 2.4                           |
| Leu        | 4.7                           |
| Tyr        | 1.8                           |
| Phe        | 2.9                           |
| His        | 2.0                           |
| Lys        | 5.1                           |
| Arg        | 2.7                           |
| Cys        | ND                            |
| Trp        | ND                            |

ND, not determined.
moval from plasma membranes by treatment with 40 mM Ca++; (b) a monomeric molecular weight of ~10,000 daltons; (c) a high apparent molar extinction coefficient at 280 nm; (d) solubility in acidic and acidified organic solutions that results in a failure of the protein to be visualized in acid-fixed polyacrylamide gels; (e) reversible, Ca+-mediated polymerization into 3-nm filaments; and (f) an amino acid composition atypically high in acidic residues and low in hydrophobic residues and containing phosphorylated serine. Because of similarities between this protein and ligatin, a protein previously isolated from ileum (14), the retinal protein also will be referred to as ligatin. Retinal ligatin constitutes >1% of the plasma membrane protein, as more than 100 μg can be isolated from 1 g, wet weight, of retina.

The initial step in the isolation procedure for retinal ligatin, treatment of plasma membranes with Ca++, results in the release of filaments 4.5 nm in Diam. These filaments differ morphologically from the 3-nm filaments formed by re-polymerization of purified ligatin monomers. In ileum, such a difference in morphology between filaments released from membranes and those formed by re-polymerization of ligatin alone results from the decoration in vivo of ligatin filaments by β-N-acetylhexosaminidase. In retina, an analogous decoration in vivo of ligatin filaments by other membrane proteins is thus suggested. Consistent with this is the co-solubilization with ligatin of proteins that are excluded from a P-60 column after treatment with EGTA. The co-solubilized proteins in retina, however, differ in morphology from the β-N-acetylhexosaminidase associated with ileal ligatin and do not possess this enzymatic activity.

Our investigations in retina for a ligatin-like molecule were prompted by similarities between ileal ligatin and a preparation isolated by Merrell et al. (18, 19) from embryonic chick neural retina that inhibits the reassociation of single retinal cells. The inhibitory activity has the following properties: (a) it is extractable from retinal plasma membranes by using a chaotrophic agent. (b) it passes quantitatively through an Amicon PM 10 filter (Amicon Corp., Scientific Sys. Div., Lexington, Mass., nominal cut-off at 10,000 daltons) but is retained by a UM 2 filter (nominal cut-off at 2,000 daltons), (c) it is inactivated by trypsin, and (d) it is not visualized after staining polyacrylamide gels with either a protein stain or the periodic acid-Schiff method (19). Correspondingly, ileal ligatin is extractable from plasma membranes, passes through PM 10 but is retained by UM 2 filters, is cleaved by trypsin, and is not visualized in conventionally stained gels (reference 14 and unpublished results). In addition, ileal ligatin functions as a baseplate for an external cell-surface protein. An analogous function by a similar protein in retina could be responsible for the biological activity found by Merrell et al.: if proteins that mediate intercellular adhesion are bound to retinal plasma membranes via ligatin, then exogenous, soluble ligatin might compete for this material and thus inhibit adhesion rates.

To determine whether ligatin possesses biological activity similar to that of the preparation of Merrell et al. (19), retinal ligatin was included in assays for intercellular adhesion among retinal cells. With ligatin present at 20 μg/ml, a 70% inhibition of adhesion compared to controls was observed (Marchase and Jakoi, in preparation). This result and the known baseplate function of ligatin in ileum suggest that retinal ligatin may function in vivo as a baseplate for components necessary for intercellular adhesion and that the observed inhibition of adhesion may be caused by competition between exogenous soluble ligatin and cell-associated ligatin for these components.

Four proteins that fail to be visualized in polyacrylamide gels stained with Coomassie blue have been reported recently. Besides the initial report of ligatin from ileum (14) and the preparation of Merrell et al. (19), Gaetjens (10) found a non-staining phosphorylated protein in human erythrocyte ghosts. This protein was reported to have a mol wt of ~14,000 daltons and to be soluble in acidified chloroform:methanol. Also, Bárány et al. (2) extracted a phosphorylated protein of 10,000 daltons from adult frog skeletal muscle with acidified chloroform:methanol.

The inability to visualize these proteins has been suggested to be either caused by a failure to bind Coomassie blue (10, 14) or because the proteins were present at too low a concentration to be seen (10, 19). However, we propose that the proteins isolated from skeletal muscle and erythrocytes were also ligatin or ligatin-like proteins and that, as in retina, their failure to be visualized was because of their solubility in acidic fixatives. This hypothesis is supported by the findings that the muscle (2) and erythrocyte (10) proteins were soluble in acidified chloroform:methanol. In addition, the molecular weights and amino acid compositions reported for the frog
muscle (2) and human erythrocyte (10) proteins are similar to those obtained for ileal (13) and retinal ligatin. Lastly, by using the Ca**+ extraction described above, we have isolated proteins from skeletal muscle membranes and erythrocyte ghosts that possess the same characteristics as retinal ligatin (unpublished results). These similarities thus suggest that ligatin is a plasma membrane protein or family of proteins found in a wide variety of tissues and across a spectrum of vertebrate species.

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