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# A Possible Role for Ligatin and the Phosphoglycoproteins It Binds in Calcium-Dependent Retinal Cell Adhesion

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Ligatin is a filamentous plasma membrane protein that serves as a baseplate for the attachment of peripheral glycoproteins to the external cell surface. Ligatin can be released from intact, embryonic chick neural retinal cells by treatment with 20 mM $Ca^{++}$ without adversely affecting their viability. α-Glucose-1-phos phate is also effective in removing ligatin-associated glycoproteins from intact cells. After either of these treatments, the retinal cells seem not to exhibit $Ca^{++}$-dependent adhesion for one another. It is thus suggested that ligatin in neural retina may serve as a baseplate for the attachment to the cell surface of glycoproteins active in $Ca^{++}$-dependent adhesion. The finding that $Ca^{++}$ serves to protect $Ca^{++}$-dependent adhesion molecules from digestion by trypsin is discussed in relation to steric constraints on trypsin's accessibility to these adhesion molecules because of their possible binding to arrayed ligatin filaments.

**Key words: neural retina, ligatin, adhesion, phosphooligosaccharides**

Ligatin is a filamentous plasma membrane protein that serves as a baseplate for the attachment of peripheral glycoproteins to the external cell surface in a variety of tissues [1–4], including embryonic chick neural retina [5]. The addition of micromolar levels of exogenous, monomeric retinal ligatin to dissociated neural retina cells results in a significant decrease in the rate of reassociation of these cells [6]. This finding has led to the hypothesis that the peripheral glycoproteins bound by ligatin in retina may participate in the formation of intercellular adhesive bonds. Here, we report that treatment of intact retinal cells with 20 mM $Ca^{++}$ removes ligatin and its associated glycoproteins from cell surfaces. This treatment does not seem to adversely affect the cells' viability but, as the above hypothesis might suggest, does render the cells less adhesive.

Evidence is also provided that treatment of intact cells with α-glucose-1-phosphate removes glycoproteins bound to ligatin from the cell surface and, like the $Ca^{++}$ treatment, causes a decrease in retinal cell adhesiveness. This finding correlates with

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information recently attained regarding the mechanism of association between retinal ligatin and the glycoproteins it binds [7]. Affinity chromatography and chemical analyses suggest that ligatin binds glycoproteins bearing high mannose-type oligosaccharides containing phosphodiester-linked terminal glucose residues. This mechanism of association is similar to that proposed for the uptake and intracellular localization of hydrolytic enzymes to lysosomes [8,9] in that high mannose-type chains containing phosphodiester-linked terminal sugars are synthesized [10]. However, in the lysosomal systems the terminal sugar is N-acetylglucosamine [10] and is subsequently cleaved from the oligosaccharide, leaving the formerly penultimate mannose residue, still bearing the phosphate, as the biologically active localization signal. Ligatin, in contrast, recognizes proteins in which the phosphodiester-linked terminal sugar is glucose and in which the terminal sugar remains as part of the recognition signal. The presence of blocked phosphate residues seems to be characteristic of the ligatin recognition mechanism in a variety of tissues [11]. This signal may function in directing a class of glycoproteins to the cell surface and, through their binding to ligatin, may also be responsible for their continued localization there. The selective release of ligatin-associated proteins from intact retinal cells by α-glucose-1-P might thus result from the same competition seen in previous affinity column studies [7].

The decreases in adhesivity observed following treatment by either $Ca^{++}$ or α-glucose-1-P were both on a $Ca^{++}$-dependent [12–19] rather than a $Ca^{++}$-independent adhesive mechanism. In addition, $Ca^{++}$ has been observed to be necessary for the integrity of closely packed arrays of ligatin filaments in ultrastructural studies. Taken together, these data suggest a possible explanation based on steric considerations for the finding [12–17] that trypsinization in the absence of $Ca^{++}$ destroys a $Ca^{++}$-dependent adhesive mechanism while $Ca^{++}$'s presence results in preservation of this mechanism.

## MATERIALS AND METHODS

### $Ca^{++}$ Treatment of Retinal Cells

Neural retina tissue was dissected from 7- and 10-day chicken embryos in Minimum Essential Medium (MEM). Hanks balanced salt solution containing 20 mM $CaCl_2$ (HBSC) and deficient by 30 mM in NaCl was prepared. Tissues were either directly transferred to HBSC or trypsin dissociated, allowed to recover for 2 hr at 37° [12], pelleted (200g, 8 min), and resuspended in HBSC. The retina cells were incubated in the high $Ca^{++}$ solution for 10 min at 4°. After gentle trituration, tissue fragments and cells were then removed by centrifugation (200g, 10 min). The supernatant was centrifuged again to remove cell debris (2000g, 10 min) and, if necessary, concentrated to about 1 ml by Amicon UM-2 ultrafiltration. The material was then dialysed versus 0.5 mM EGTA, 10 mM bicarbonate (pH 8) to remove $Ca^{++}$, a procedure that results in depolymerization of ligatin filaments and the dissociation of ligatin and its associated glycoproteins [1,5]. Molecular sieve chromatography [5] then separated ligatin from other proteins.

### Viability Studies

Procedures have been previously described [6].

### Sugar Phosphate Incubations

Retinas were metabolically labeled with $^{32}P$ [6] and then allowed to incubate in isotope-free medium for 2 hr. The retinas were washed by centrifugation and transferred to $Ca^{++}$-$Mg^{++}$-free Hanks balanced salt solution (CMF) containing sugar phosphates at 10 mM. The retinas were incubated at 37° C for 20 min and removed by centrifugation. Aliquots of the supernatants were electrophoresed in 30 mM borate, pH 9.0 [7], while retinal pellets were counted by Cerenkov radiation to provide a means of normalization. For incubations in endoglycosidase H (endo H), $^{32}P$ associated with macromolecules in aliquots of the cell supernatants was separated from free $^{32}P$ by P-60 molecular sieve chromatography. The $^{32}P$-labelled macromolecules were dialysed versus $H_20$, lyophilized, treated with endo H as described [7], and then rerun on P-60 columns. The radioactivity in any included peak was determined.

### Adhesion Assays

The protocols followed methods previously described [12,20].

## RESULTS AND DISCUSSION

### Preparation of Ligatin and Its Associated Glycoproteins From Intact Retinal Cells

Previously published procedures for the preparation of ligatin have utilized sucrose density gradient centrifugation to prepare plasma membranes [1,5]. The plasma membranes were then treated with 10–40 mM $CaCl_2$, a procedure which, as observed by electron microscopy, results in the separation of intact filaments of ligatin and its associated glycoproteins from the membranes [1,5]. The filaments were subsequently depolymerized and ligatin, due to its monomeric molecular weight of 10,000 daltons, was separated from its associated glycoproteins by molecular sieve chromatography. In retina, however, several protein species which seem not to be associated with ligatin are coincidentally co-solubilized from the membranes by the $Ca^{++}$ procedure (unpublished results).

A modification of this procedure that results in the removal of ligatin and its associated glycoproteins from intact retinal cells without significant loss in viability has now been developed. As with membranes, higher than physiologic concentrations of $Ca^{++}$ are utilized. Hanks' balanced salt solution was prepared containing 20 mM $CaCl_2$ and deficient in NaCl by 30 mM to compensate for the increase in osmolarity. Intact fragments of retinal tissue or previously dissociated and recovered single-cell suspensions [12] were incubated in the high $Ca^{++}$ solution for 10 min at 4°.

As shown in Table I, the yield of ligatin from intact tissue was slightly greater than the yield from plasma membranes prepared from the same amount of tissue. Membranes prepared following treatment of the intact tissue with $Ca^{++}$ had only trace quantities of ligatin that could subsequently be extracted. These data thus suggest that intact retinal tissue can be used as a source for the preparation of ligatin. Polyacrylamide gel electrophoresis of the proteins co-solubilized with ligatin suggest that the preparation from intact cells may provide a more highly enriched source than membranes for the glycoproteins actually bound to ligatin compared to those coincidentally co-solubilized with it (unpublished result).

**TABLE I. Ligatin Is Extractable From Intact Retina**

| Source[a] | μg Ligatin[b] |
|---|---|
| Intact neural retina | 86 |
| Plasma membranes | 72 |
| Plasma membranes prepared from retina following treatment with calcium | 4 |

[a]Seventy-eight 10-day embryonic chick neural retina.
[b]Determined by the Lowry procedure [24].

## Cell Viability Following Treatment of $Ca^{++}$

Since retinal cells were to be utilized in adhesion experiments following treatment in 20 mM $Ca^{++}$, the effects of this treatment on the viability of dissociated and recovered retinal cells [12] were determined. Three measurements provide evidence that viability was not significantly diminished (Table II). First, there was no decrease in the $Ca^{++}$-treated cells' ability to exclude trypan blue. Greater than 95% of both treated and control cells were unstained by the dye. Second, since respiration has been implicated as the rate-limiting factor in cell adhesion studies [21], rates of oxygen consumption were monitored using an oxygen electrode. No differences were seen between control and $Ca^{++}$-treated cells. Third, the incorporation of $^{3}H$-leucine into trichloroacetic acid-precipitable material was monitored over 3 hours and found to be identical for the two populations.

## Removal of Cell-Surface Proteins by Sugar Phosphates

The recognition marker mediating the attachment of glycoproteins to ligatin has recently been investigated [7] and shown to be a high mannose-type oligosaccharide on which at least one of the penultimate mannose residues is linked at its 6-carbon via a phosphodiester bond to the 1-carbon of a terminal glucose. Evidence in support of this finding includes: 1) Man6P is a constituent of the oligosaccharides present on the proteins associated with ligatin and was found to be the most effective of monosaccharides derivatized with phosphate at C-6 in eluting bound proteins from a ligatin affinity column. 2) Alkaline phosphatase alone did not have an effect on the association of the $^{32}P$ with the oligosaccharide, or on the oligosaccharides' net negative charge, suggesting that the phosphate was not terminal. However, mild acid hydrolysis resulted in an increase in the net negative charge of the oligosaccharide and the accessibility of the phosphate present on the oligosaccharide to alkaline phosphatase. Following the mild acid hydrolysis, glucose was identified as the saccharide freed from the phosphorylated oligosaccharides. Evidence that the linkage is to the 1-carbon and is in the α-configuration comes from the efficacy of αGlc1P as opposed to βGlc1P in affinity column studies [7] and from studies suggesting a potential synthetic origin for the Glc1P, an intact transfer of the Glc1P from a UDP-glucose donor (in which the phosphate is bonded to the 1-carbon in an α-configuration, unpublished results). 3) The oligosaccharide bearing the phosphate seems to be of the high mannose-type, since the phosphooligosaccharide is susceptible to cleavage from its glycoproteins by endo H but not by endo D [7].

Because of these findings, the efficacies of various sugars and sugar phosphates in removing ligatin-associated glycoproteins from intact retinal cells was investigated. Table III gives the results of an experiment in which tissue, metabolically

**TABLE II. Treatment in 20 mM $Ca^{++}$ Does Not Affect Cell Viability**

| Criterion | Control cells | $Ca^{++}$-treated cells |
|---|---|---|
| Trypan blue exclusion | >95% | >95% |
| Oxygen consumption ($\mu l$ $O_2$/min-mg protein) | 0.18 | 0.17 |
| Leucine incorporation (cpm/hr-mg protein) | 41,000 | 40,000 |

**TABLE III. Efficacies of Sugar Phosphates in Removing $^{32}P$ Associated With Macromolecules From Retinal Cells**

| Sugar phosphate | Macromolecular $^{32}P$ solubilized (cpm per $10^8$ cells) | Endo H-sensitive $^{32}P$ |
|---|---|---|
| none | 8,200 | 4,600 |
| $\alpha$-Glucose-1-P | 45,000 | 39,000 |
| Mannose-6-P | 41,000 | - |
| Fructose-1-P | 24,000 | - |
| Fructose-6-P | 9,600 | - |
| $\alpha$-Galactose-1-P | 5,900 | - |
| Galactose-6-P | 8,200 | - |

labeled with $^{32}P$, was exposed to medium containing various sugar phosphates. The $^{32}P$-containing macromolecules released from the cell surfaces were monitored by removing the cells by centrifugation and electrophoresing the supernatants to remove small molecular weight compounds. As can be seen, $\alpha$-glucose-1-P and mannose-6-P were the most effective of the sugar phosphates tested in freeing $^{32}P$ associated with macromolecules from the cell surfaces. These results correlate well with the efficacies of the various sugar phosphates in eluting bound glycoproteins from retinal ligatin affinity columns [7].

That the majority of these $^{32}P$ macromolecules were glycoproteins associated with ligatin was suggested by determinations of the percentages of the incorporated $^{32}P$ that could be released from the macromolecules by treatment with endo H, a glycosidase specific for the intact cleavage of high mannose-type oligosaccharide chains [21] and effective in removing the phosphate-containing oligosaccharides recognized by ligatin. [7]. The endo H-susceptible $^{32}P$ released from control macromolecules solubilized by CMF was compared with that released from macromolecules solubilized by $\alpha$-glucose-1-P. The phosphorylated sugar was 8 times as effective at releasing endo H specific $^{32}P$ as the control treatment.

## Effects of High $Ca^{++}$ and Sugar Phosphates on Retinal Cell Adhesion

Since exogenous ligatin inhibits the reassociation of retinal cells [6], we have hypothesized that in retina ligatin serves as a baseplate for peripheral glycoproteins that participate in the formation of intercellular adhesive bonds. If this is the case, then treatments that remove ligatin and/or its associated glycoproteins from the cell surface might be expected to decrease the adhesivity of retinal cells. Therefore, retinal cells treated with either 20 mM $Ca^{++}$ or 10 mM $\alpha$-glucose-1-P were compared to control cells in a newly developed cell binding assay [12,20].

Five laboratories have recently reported the presence of two independent adhesion mechanisms on neural retina cells [12–19], one independent of $Ca^{++}$ and one requiring the cation. Cells that have been trypsinized and allowed to recover in nutrient medium exhibit both mechanisms [12,17]; the increase in cell binding seen when $Ca^{++}$ is added gives a measure of the $Ca^{++}$-dependent mechanism. Retinal cells were prepared in this manner and then treated with either 20 mM $Ca^{++}$, 10 mM $\alpha$-glucose-1-P, or as controls. Their binding was then measured in the absence and presence of $Ca^{++}$. As shown in Table IV, levels of $Ca^{++}$-independent binding were unaffected by the treatments but $Ca^{++}$-dependent binding was significantly decreased by both 20 mM $Ca^{++}$ and by $\alpha$-glucose-1-P. These results thus provide preliminary support to the hypothesis that ligatin in neural retina could serve as a baseplate for peripheral proteins active in the $Ca^{++}$-dependent adhesion mechanism.

## $Ca^{++}$ Protection During Trypsination of the $Ca^{++}$-Dependent Adhesion Mechanism

In investigations of the $Ca^{++}$-dependent and $Ca^{++}$-independent adhesive mechanisms, it has been shown that cells can be prepared so as to exhibit one or the other of the two mechanisms [12–17]. The $Ca^{++}$-dependent mechanism remains present on cells subjected to mild trypsinization in the presence of millimolar levels of $Ca^{++}$ but is absent if $Ca^{++}$ is removed from the trypsinizing solution. This "protection" provided during trypsinization by the presence of $Ca^{++}$ to the cell-surface molecules responsible for $Ca^{++}$-dependent adhesion is not yet understood, although Takeichi [14] suggests that the protein active in adhesion might undergo conformational or positional changes in the plasma membrane with variations in $Ca^{++}$concentration.

The dependence of the integrity of ligatin filaments on $Ca^{++}$ [1,5] juxtaposed with the data presented here suggests a possible explanation for this protection. Ligatin was first discovered on plasma membranes of suckling rat ileum [1] following electron microscopic observations of two-dimensional regular arrays of morphologically distinctive macromolecular aggregates on the external cell-surface. These arrays were shown by Jakoi et al [1] to be due to the presence of aligned ligatin filaments that served as baseplates for the attachment of 800,000 dalton peripheral enzyme complexes. When $Ca^{++}$ was removed from the membranes' bathing solution by the addition of EGTA, the enzyme complexes were observed to lose their topographic regularity [1]. An explanation for this observation is suggested by the behavior in solution of purified ligatin from both ileum and retina: In the absence of $Ca^{++}$ ligatin depolymerizes into 10,000 dalton monomers; in the presence of $Ca^{++}$ it reforms filaments 3 nm in diameter [1,5].

**TABLE IV. Twenty mM $Ca^{++}$ and 10 mM $\alpha$Glucose-1-P Inhibit $Ca^{++}$-Dependent Retinal Cell Adhesion**

| | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|
| | % Input cells bound[b] | | % Input cells bound[b] | |
| Prior treatment[a] | CMF | CMF + 3 mM $Ca^{++}$ | CMF | CMF + 3 mM $Ca^{++}$ |
| none | 9 | 26 | 7 | 22 |
| 20 mM $CaCl_2$ | 14 | 15 | 11 | 13 |
| 10 mM $\alpha$G1c1P | 15 | 18 | 12 | 11 |

[a]Only single cells were treated. Cells were incubated in MEM containing the above addition at 4° for 10 min, then washed 2× in MEM.
[b]Removal force: 200g.

Electron microscopic observations of neural retina plasma membranes from embryonic chick do not display obvious periodic arrays on their plasma membranes. Ligatin filaments can be observed [5] but are not decorated by the large, morphologically distinct complexes found in ileum. However, the addition of a nonspecific ligand, cationized ferritin, to retinal membranes or to intact retinal cells [23] results in the appearance of regular two-dimensional arrays of the ferritin (Fig. 1) with spacings similar to those seen on native ileal membranes. Once again, the removal of $Ca^{++}$ from the medium by EGTA results in randomization of the lattice. While there is no assurance as yet that the cationized-ferritin lattice is present because of binding to ligatin and/or the glycoproteins ligatin secures to the cell surface, the experimental evidence is consistent with this possibility. Interestingly, trypsinization in the presence of $Ca^{++}$ does not perturb the pattern of cationized-ferritin binding [23].

The data presented here suggest that ligatin and the glycoproteins it binds to the cell surface could participate in the $Ca^{++}$-dependent adhesion mechanism. If this is substantiated, then two correlations may exist: 1) In the presence of $Ca^{++}$, ordered lattices of cationized ferritin molecules are observed in retina (and may be due to binding to aligned filaments of ligatin and its associated glycoproteins), and the

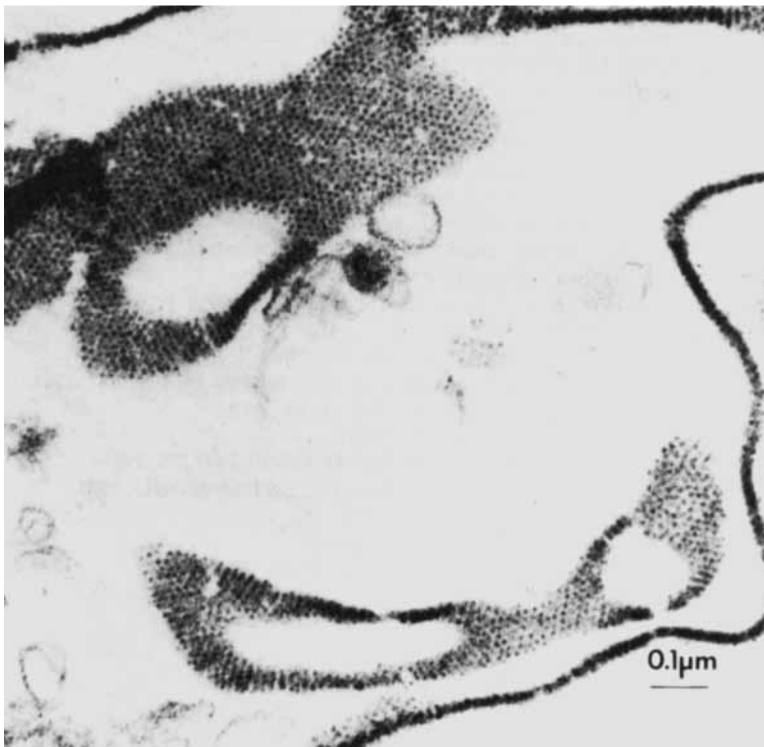


Fig. 1. Thin section of plasma membranes isolated from embryonic chick neural retina cells treated with cationized ferritin (100 μg/ml) for 1 min at 4°C. Views of the membrane surface show a two-dimensional lattice of ligand bound in rows with a center-to-center spacing of 15 nm. Prior treatments of the cells with trypsin in $Ca^{++}$-containing medium had no effect on the appearance of the lattice. Treatments with EGTA caused a randomization and dispersion of the lattice (× 79,000). Reprinted from [23].

molecules responsible for $Ca^{++}$-dependent adhesion are inaccessible to destruction by trypsin. 2) In the absence of $Ca^{++}$, the lattice of cationized ferritin molecules is randomized and dispersed, and trypsin has access to the $Ca^{++}$-dependent adhesion molecules. We thus make the tentative suggestion that the presence of ordered lattices seen on retinal cell surfaces following treatment with cationized ferritin could reflect arrays that includes $Ca^{++}$-dependent adhesion molecules bound to ligatin filaments. The tightly ordered nature of these arrays could in some manner provide steric protection to the molecules from digestion by trypsin.

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