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Identification and Purification of an Endogenous Receptor for the Lectin Pallidin from Polysphondylium pallidum

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ABSTRACT We report the identification and purification of an endogenous carbohydrate-containing receptor of pallidin, the cell surface lectin implicated in mediating cell-cell adhesion in the cellular slime mold Polysphondylium pallidum. The receptor is identified in an aqueous extract of crude P. pallidum membranes as a potent inhibitor of the hemagglutination activity of pallidin. The inhibitor is purified to apparent homogeneity by affinity precipitation with pallidin followed by fractionation of the solubilized precipitate on Sepharose 4B. The hemagglutination inhibitor (HAI) is metabolically radiolabeled, indicating that it is a biosynthetic product of the amoebae and not an ingested food substance. The HAI is released into the extracellular medium by living, differentiated amoebae. This release is markedly facilitated by the addition of D-galactose, a specific saccharide that binds to pallidin. Hence, the HAI appears to have an in situ association with pallidin at the cell surface. Exogenously added HAI promotes the agglutination of differentiated amoebae in a gyrated suspension at very low concentrations. The results are consistent with a model of cell-cell adhesion in which the HAI is a multivalent, extracellular aggregation factor that is recognized by pallidin molecules on adjacent cells. The HAI would then be analogous to the aggregation factors identified in marine sponges.

MATERIALS AND METHODS

Materials

Sepharose 4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Dextran sulfate (mol wt 8,000), heparin (Grade I), guanidine hydrochloride...
diluted 1:20, and then autoclaved for 20 min. 50-ml aliquots of bacterial suspension were added to SM agar Petri plates (100 x 20 mm). The cellswere harvested after 4 d of incubation. The cells were obtained by inoculating 10^5 spores in 0.25 ml of bacterial suspension. For the cell cohesiveness assay (see below), small preparations of the cell suspension (10^5/pans) were harvested after 5 d of incubation in the dark. At this stage, the cells were still amoeboid with a very low percentage of microcysts. For the cell cohesiveness assays (see below), small preparations of cells were obtained by inoculating 10^5 spores in 0.25 ml of bacterial suspension onto SM agar Petri plates (100 x 20 mm). The cells were harvested after 4 d of incubation in the dark.

For suspension cultures, E. coli B/r was grown to stationary phase in modified HLI5 as above. The bacteria were washed several times in SPS buffer (67.6 mM Na2H-K2HPO4, pH 6.0), adjusted to an optical density of 0.490 at OD 450 when diluted 1:20, and then autoclaved for 20 min. 50-ml aliquots of bacterial suspension were inoculated in 125-ml Erlenmeyer flasks and incubated at 25 °C. The amoebae were propagated in association with Klebsiella aerogenes and become cohesive by 4 d. The cells do not aggregate and become actinomycosus. For the cell cohesiveness assays (see below), small preparations of cells were obtained by inoculating 10^5 spores in 0.25 ml of bacterial suspension onto SM agar Petri plates (100 x 20 mm). The cells were harvested after 4 d of incubation in the dark.

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4-d surface-grown P. pallidum cells were harvested from pans and washed as described above and then suspended at 10^6 cells/ml in SPS with or without sugars. The cells were swirled at 200 rpm on the G24 Shaker for varying times and then centrifuged at 2,000 rpm for 7 min. Cells were shown to be at least 98% viable by trypan blue exclusion at the time of harvest. A final supernatant which we refer to as conditioned medium was obtained by centrifugation at 20,000 rpm for 30 min. Conditioned medium was concentrated by ultrafiltration on a PM-30 filter (Amicon Corp., Scientific Systems Div., Lexington, MA). It was then boiled for 10 min and dialyzed against NaCl/Pi. Purification of the HAI in the conditioned medium employed the same steps as described above for the AFT extract.

Electrophoresis
Discontinuous SDS electrophoresis was carried out according to Laemmli (19) in 10% slab gels. BSA (66,000), chymotrypsinogen A (25,700), ovalbumin (43,000) and cytochrome c (12,000) were run as molecular weight standards. The gels were stained in 0.2% Coomas Blue (G-250) in methanol/water/acidic acid (50:50:10) for 20 min. The gel was destained in 7.5% acetic acid, 5% methanol. Dilute samples were precipitated with 10% TCA for 30 min (4 °C), after which the precipitates were spun out at 10,000 rpm for 15 min. Before solubilization the pellets were washed three times with absolute ethanol, centrifuging as above after each wash.

Cellulose acetate electrophoresis was performed in a Gelman electrophoresis box using Celllogel 500 (Kallest Scientific Co., Inc., Manhasset NY). Samples were concentrated after dialysis into water by drying under nitrogen and air. 2- to 4- Ag mol were loaded onto strips presoaked for 30 min in the running buffer. The cellulose acetate strips (5 cm x 16.8 cm) were run at 12.5 mAmps for 20 min or 1.5 h in pH 3.5 pyridine-acetic acid buffer. To test other pH's, Pi buffer (pH 7.2), 0.05 M Tris-HCl, and 0.01 M HCl (pH 2.0), and 0.1 M barium acetate buffer (pH 8.6), 0.125 M HCl buffer (pH 3.5, 0.05 ionic strength) were also employed as running buffers. The strips was stained for 15 min in 1% Alcian blue in 2.5% acetic acid and destained in 2.5% acetic acid. In some runs, the strips were treated with periodic acid (20) before staining. In other runs, the strips were stained with Amido black by soaking the strip in a large volume of 0.1% Amido black in 7% acetic acid for 30 min and destaining in 2.5% acetic acid, 40% ethanol. Metabolically labeled material was run for 1 h in pyridine-acetic acid buffer (pH 3.5) and analyzed by cutting the unstained lane into 2-mm strips. Each strip was placed in 1 ml of water in a counting vial, shredded, and shaken for 1 h. Hydrofluoroc scintillation cocktail (10 ml) was added to each vial and the vials were counted.

Cohesiveness Assay
4-d, surface-grown P. pallidum cells were harvested from agar plates and washed in cold distilled water as described above. The amoebae were suspended at 3.2 x 10^6 cells per ml in EDTA/Pi (16.7 mM Na2H-K2HPO4, 10 mM EDTA, pH 6.2) containing 4 mg/ml of bovine serum albumin (BSA). The cell suspension was dispensed into single cells by repeated pipetting with a Manostat (Scientific Products Div., American Hospital Supply Corp., McGaw Park, IL) and was concentrated (0.5 ml) with 0.5 ml of 10 mM 2,4 Dinitrophenol (DNP) in EDTA/Pi to 0.5 ml of EDTA/Pi with or without glycytymic acids in 10-ml Erlenmeyer flasks. The flasks were agitated at 125 rpm on the G-24 shaker at 25°C. 0.5 ml aliquots of the cell suspensions were taken at various times and carefully diluted into 10 ml of EDTA/Pi. The degree of cell aggregation was monitored with a Coulter Electronic Particle Counter (Coulter Electronics, Inc., Hileah, FL). The number of cell aggregates was determined by setting the upper threshold to infinity and the lower threshold to exclude single cells. The change in aggregates (A aggregates) was calculated as the number of aggregates at time (t) minus the number of aggregates at time-zero. We found that maximal aggregation occurred by 30 min.
RESULTS

Purification

As a source of receptor, we started with an acetone powder prepared from a crude membrane fraction of aggregation-competent amoebae. The acetone powder was extracted in NaCl/Pi buffer with boiling to release glycoconjugate receptors that were complexed with pallidin. The soluble extract, obtained after centrifugation, contained a potent inhibitor of the hemagglutination activity (HA) of pallidin. As shown in Table I, 0.8 μg/ml (neutral sugar) of this crude extract inhibited the HA of pallidin by 75%, whereas 5,700 μg/ml (neutral sugar) of lactose was required to inhibit the HA to the same extent. This crude extract also blocked the interaction of 128U/ml-asialofetuin with pallidin (data not shown). Extraction of the AP in NaCl/Pi with detergent (0.5% Empigen BB) resulted in identical HA inhibitory activity, indicating that, within the limits of the assay, detergent did not release any additional inhibitor from the particulate fraction.

Initially, we attempted to purify the inhibitor by conventional affinity chromatography on a column of pallidin covalently coupled to Sepharose. This approach was unsatisfactory, because recovery of activity was only ~10% (data not shown). A more useful technique was lectin affinity precipitation. We found that addition of purified pallidin to the AP extract resulted in the formation of a fine precipitate. With the addition of increasing amounts of pallidin to a fixed amount of extract, the amount of neutral sugar in the precipitate increased concomitantly with the disappearance of hemagglutination inhibitory activity from the supernatant (Fig. 1). The precipitation depended on the carbohydrate-binding activity of pallidin since D-galactose (0.3 M) blocked precipitation formation while D-glucose (0.3 M) did not. After a precipitation that removed 75% of the inhibitory activity, the SDS gel protein patterns of the supernatant (SN) and the starting extract (AP) were indistinguishable except for the augmented pallidin bands in the former (Fig. 2).

The pallidin precipitate was analyzed by solubilizing in boiling guanidine hydrochloride and fractionating on a column of Sepharose 4B. Two major peaks of protein (OD 280), labeled pools 1 and 3 in Fig. 3, and two major peaks of carbohydrate (neutral sugar), labeled pools 2 and 4, were resolved. The same basic profile was observed when the precipitate was solubilized in boiling SDS (not shown). SDS gel analysis established that the two protein peaks (pools 1 and 3) consisted of pallidin, probably denatured to different degrees by the guanidine treatment (Fig. 2, lanes 1 and 3). Almost all of the precipitated HA inhibitory activity was recovered in the first major carbohydrate peak (pool 2), which contained 55% of the applied carbohydrate (Table II). Pools 1, 3 and 4 had little or no activity. The minor carbohydrate peak which comigrated with pallidin at the void volume (pool 1) was probably due to pool 2 material complexed with pallidin. The possible sources of carbohydrate (pool 4) are considered below.

Although pool 2 contained no Coomassie-Blue-staining bands (Fig. 2, lane 2), periodic acid-Schiff staining revealed a diffuse band in the 3% stacking gel of SDS PAGE (data not shown). Cellulose acetate electrophoresis with Alcian blue staining revealed a single diffuse component in pool 2 that migrated slightly towards the anode from the loading line (Fig. 4). Only a single band was seen under a variety of run conditions (between pH 2.0 and 8.6). Short or long electrophoresis

TABLE I

| Inhibitor           | Concentration to reduce HA by 75% (μg neutral sugar eq/ml) |
|---------------------|------------------------------------------------------------|
| Lactose            | 5,700                                                      |
| Asialofetuin       | 0.05                                                       |
| Acetone powder extract | 0.79                                                   |
| Purified HAI       | 0.1                                                        |

Inhibitors were titrated as described in Materials and Methods to determine concentrations corresponding to 1 U of inhibitory activity. Concentrations are given in terms of neutral sugar equivalents. Purified HAI is pool 2 material from a Sepharose 4B fractionation (Fig. 3). The acetone powder extract is the soluble supernatant obtained after boiling the acetone powder of crude membranes in NaCl/Pi and centrifuging.
The P. pallidum amoebae used in our experiments were grown on bacteria in medium that contained complex peptone and yeast extracts. Metabolic labeling was employed to determine whether HAI was a biosynthetic product of the slime mold rather than an undegraded food substance. The amoebae were grown on autoclaved bacteria and then allowed to differentiate without food in an SPS buffer in the presence of $^3$H-acetate. Pallidin precipitation of the labeled AP extract from these cells contained a peak of radioactivity comigrating with the HAI on Sepharose 4B (Fig. 5). Furthermore, on cellulose acetate electrophoresis, a single peak of radioactivity coincided with the Alcian blue band described above (Fig. 6). Hence, during differentiation of the amoebae, the HAI was metabolically labeled. It should be noted that there was relatively little radioactivity associated with pool 4 in this experiment (Fig. 5), suggesting that pool 4 either is derived from an ingested food substance or is poorly labeled under these conditions.

**Metabolic Labeling**

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**Release of HAI into Medium**

As described above, the HAI could be extracted from a particulate fraction of differentiated amoebae. The HAI was also detected in the conditioned medium (CM) of living amoebae. When differentiated amoebae were swirled in SPS buffer at $10^8$ cells/ml, $19$ U/ml of hemagglutination inhibitory activity were released after 5 h. The cells retained full viability during this period. The inhibitory activity was precipitated by the addition of pallidin. Analysis of the precipitate by 4B chromatography and cellulose acetate electrophoresis showed the presence of a component that behaved the same as the HAI. This component, like the particulate-associated HAI, could be metabolically labeled with $^3$H-acetate (data not shown). The specific activity (units of inhibitor per unit neutral sugar) of the conditioned medium was $\sim 15\%$ of that of purified HAI.

If the HAI were associated with the cell surface via an
FIGURE 5 Metabolic labeling of HAI. Amoebae were grown in suspension on autoclaved bacteria and differentiated in the presence of \(^{3}\)H-acetate as described in Materials and Methods. 1 ml of a labeled AP extract (from \(4 \times 10^8\) cells) was precipitated with 2 ml of pallidin (275 \(\mu\)g/ml). The solubilized precipitate (0.3 ml) was fractionated on a 4B column (0.7 cm x 30 cm), and 50 \(\mu\)l of the fractions (0.55 ml) were counted for radioactivity. The total cpm per fraction is given on the y-axis (\(\bullet\)). The peaks of carbohydrate-containing pools 2 and 4 from an unlabeled precipitate run on the same column are indicated by the arrows. The first peak of carbohydrate, corresponding to the inhibitory activity as shown above, was labeled. The ratio of cpm in pool 2 to total cpm in the starting AP extract (0.065) equals the ratio of the mass of protein and carbohydrate in an unlabeled pool 2 to that in its corresponding AP extract (0.066). Since the \(^{3}\)H from the acetate is incorporated into carbohydrates and proteins, the equivalence of the two ratios suggests that the HAI, rather than being a modified food product, is synthesized de novo from the same pool of precursors that go into other slime mold proteins or carbohydrates.

FIGURE 6 Cellulose acetate electrophoresis of labeled HAI. A pool (designated by the bar in Fig. 5) was made corresponding to the first carbohydrate peak in the 4B fractionation. 5 \(\mu\)l of this labeled pool was electrophoresed on cellulose acetate and counted as described in Materials and Methods. The single peak of counts which ran slightly anodally from the loading line corresponded to the Alcian-blue staining band.

interaction with pallidin, then specific saccharides recognized by the lectin should facilitate release of the HAI into the medium. Fig. 7 shows a comparison of inhibitory activity released into the medium in the presence of D-galactose (gal-CM) or D-mannose (man-CM). (The CM's were tested for activity after boiling and dialysis.) The man-CM released no more activity than SPS buffer; however, by 5 h, the gal-CM contained fourfold more activity than the man-CM. The inhibitor released by galactose was precipitated by pallidin and ran the same as HAI on 4B chromatography and cellulose acetate electrophoresis.

The HAI released into gal-CM represented a substantial proportion of the cells' extractable HAI. After 5 h of exposure to galactose, 64 \(\mu\)U/10\(^8\) cells had accumulated in the medium, whereas 27 \(\mu\)U/10\(^8\) cells remained in an AP extract of the cells. In contrast, cells exposed to mannose released only 16 \(\mu\)U/10\(^8\) cells into the medium and retained 94 \(\mu\)U/10\(^8\) cells that could be extracted in an AP extract of the cells. The high yield of activity in the galactose-conditioned medium may make it suitable starting material for the purification of the HAI. Attempts are underway to devise a simplified purification procedure starting with gal-CM.

**Promotion of Cell-Cell Adhesion**

To determine its effect on cell-cell adhesion, the purified HAI was added to cells in a cell cohesiveness assay. In this assay, the number of aggregates that formed in a swirled suspension was measured quantitatively with a Coulter Counter. Under physiological buffer conditions, the HAI produced a small enhancement of cell agglutination relative to untreated controls after 30 min of gyration (data not shown). In the presence of 2,4 DNP, which reduces the endogenous cohesiveness of the amoebae (7), the HAI had significant promoting activity at concentrations as low as 0.1 \(\mu\)g/ml (neutral sugar), whereas control glycoconjugates (heparin, glycogen, or dextran sulfate) were inactive at concentrations up to 25 \(\mu\)g/ml (Fig. 8). Pool 4 was inactive at concentrations up to 10 \(\mu\)g/
which is sulfated and richinglucose. Chosen because their compositions are similar to that of the HAI by the amoebae indicate that the HAI is neither a release of HAI from the cells by galactose and the biosynthesis specific, sugar, releases fourfold less HAI. The HAI released into living amoebae into the medium, whereas mannose, a nonspecific, had no effect at 25 μg/ml. The control substances were chosen because their compositions are similar to that of the HAI which is sulfated and rich in glucose.

ml (neutral sugar). Microscope examination verified that the agglutinates were larger and more numerous in the presence of HAI than in the untreated controls.

**DISCUSSION**

Differentiated *P. pallidum* amoebae contain a high molecular weight, water-soluble glycoconjugate that binds to pallidin and inhibits its HA. This HAI is a biosynthetic product of the cells, is found associated with a particulate fraction of the amoebae, and is also released into the medium by living cells. Extraction of the HAI from the particulate fraction does not require detergent, indicating that its association with membranes is peripheral rather than integral. The HAI is a very abundant component in differentiated amoebae, constituting ~9% of the neutral sugar in a crude particulate fraction and about 2 percent of the cells total neutral sugar. The HAI can be purified using affinity precipitation with pallidin followed by 4B gel chromatography. The HAI is homogeneous by the criterion of cellulose acetate electrophoresis. The precipitation is extremely selective, in that the only components in the solubilized precipitate besides the HAI are pallidin and an inactive carbohydrate fraction. The ability to precipitate with pallidin indicates that the HAI is multivalent.

Our observations strongly suggest that the HAI is held to the surface as a peripheral membrane component via an interaction with pallidin. Galactose, recognized by the sugar binding site of pallidin, markedly facilitates the release of the HAI from living amoebae into the medium, whereas mannose, a nonspecific, sugar, releases fourfold less HAI. The HAI released into the medium by galactose represents a major portion of the particulate-associated material. Our evidence for the facilitated release of HAI from the cells by galactose and the biosynthesis of the HAI by the amoebae indicates that the HAI is neither a fortuitous inhibitor of pallidin without functional interactions nor an absorbed food substance but rather is an extracellular product that has an *in situ* association with pallidin.

Exogenously added HAI has the ability to promote the agglutination of differentiated amoebae. This effect is most clearly seen when the endogenous cohesiveness of the cells is reduced by 2,4 DNP. Presumably, the HAI being a multivalent ligand crosslinks the cells by binding to pallidin molecules on adjacent cells.

Our results are compatible with a model of cell-cell adhesion in which the HAI serves as a multivalent, extracellular receptor with carbohydrate chains that are recognized by pallidin molecules on adjacent cells. There may also be other cell surface receptors for pallidin in addition to the HAI. If pallidin is held to the cell surface by its sugar binding sites, as apparently is the case for the lectin purpurin in *Dictyostelium purpureum* (21), then there would be a class of cell surface receptors responsible for this association. Integral membrane glycoconjugates that bind to the lectin, such as those identified in *D. discoideum* by Breuer and Sui (22), would be candidates for this second class of receptors. In our model the HAI would then be analogous to the large molecular weight, proteoglycan aggregation factors that are involved in species-specific cell-cell adhesion of marine sponges (13, 14). Testing the proposed receptor function for the HAI will require preparing an antibody against this glycoconjugate and determining the effect of F'ab fragments on cell-cell interactions of the amoebae.

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