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Purification and Characterization of the Retina-Specific Cell-Aggregating Factor

(cell recognition/glycoproteins/cell surface/cell ligands)

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ABSTRACT **The tissue-specific, cell-aggregating component of embryonic neural retina cells was purified from the retina cell-aggregating factor and characterized as a glycoprotein. Its molecular weight in solution is in the range of 50,000, and it contains 10–15% carbohydrate. The amino-acid and carbohydrate compositions have been determined. The glycoprotein is produced by embryonic neural retina cells in primary monolayered cultures and is released into the medium. Its tissue-specific, cell-aggregating effect requires integrity of the polypeptide portion, but not of the carbohydrate portion. We suggest that the isolated molecule is a specific determinant of the embryonic retina cell-surface and that it is involved in mediating self-recognition and selective adhesiveness of these cells.**

There is considerable evidence that morphogenetic association and tissue-specific aggregation of embryonic cells are mediated by specific macromolecular constituents of the cell surface which endow cells with self-recognition and selective adhesiveness (1–8). Immunological studies have detected the presence on embryonic cell surfaces of tissue-specific constituents (9), and it has been suggested that some of these function as specific cell-ligands that bind adjacent cells into tissue-forming aggregates (10–12). Earlier work has demonstrated that macromolecules with tissue-specific, cell-aggregating activity are produced and released by certain kinds of embryonic cells in primary monolayer cultures, into the supernatant medium; addition of such "cell-aggregating factors" to suspensions of homologous embryonic cells in rotation cultures enhances their morphogenetic reaggregation. The effect of such factors is tissue-specific (10–14); cell-aggregating factor obtained from embryonic neural retina cells enhances the reaggregation only of neural retina cells, while the factor obtained from cerebrum cells preferentially enhances the reaggregation of cerebrum cells, but not of cells from other brain regions or from other tissues of the same embryo. Studies on the cell-aggregating factor prepared from embryonic neural retina cells have indicated that its effect is consistent with that of the postulated tissue-specific cell-ligands (14). Partial purification of this factor related its activity to a fraction that contained protein-polysaccharide complexes (15). In the present study this factor has been purified and characterized as a further step toward elucidation of its molecular structure and function.

MATERIALS AND METHODS

Preparation of the Retina Cell-Aggregating Factor. Primary monolayer cultures of neural retina cells were prepared from cell suspensions obtained by trypsinization of retina tissue from 10-day chick embryos (details in ref. 14). After 24 hr, the serum-containing medium was removed and the cultures were thoroughly washed and supplied with serum-free medium (Eagle's basal medium with 1% glutamine, and 1% penicillin-streptomycin mixture) (14). At two subsequent 24-hr intervals, the supernatant medium was collected, dialyzed, and fractionated (15). Chromatography of the medium ("crude factor") on Sephadex G-200 (Pharmacia) in a 10 mM NaCl-10 mM Tris·HCl (pH 6.9) buffer yielded three heterogeneous fractions; fraction II, which contained the retina cell-aggregating activity, was separated into three subfractions on a DEAE-cellulose column in a continuous salt gradient [10 mM NaCl-10 mM Tris·HCl (pH 6.9) to 0.5 M NaCl-10 mM Tris·HCl (pH 6.9)]. Subfraction IIB contained the specific cell-aggregating activity and represented the maximal purification previously obtained (15). In the present study, subfraction IIB was further analyzed and the component responsible for its cell-aggregating activity was isolated by isoelectric focusing (16), as described below.

Cell-Aggregating Activity of the preparations was assayed on freshly prepared retina cell suspensions in rotating flask cultures (17), as described (11, 12, 15). Aliquots (3 ml) of retina-cell suspension (2×10^7 cells) were dispensed into 25-ml Erlenmeyer flasks in medium (Eagle's basal medium with 1% glutamine, 25 μg/ml of DNase, and 1% penicillin-streptomycin). The flasks were placed on a gyratory shaker (70 rpm) for 24 hr at 38°. After 24 hr of rotation, control cultures contained cell aggregates of characteristic and consistent size distribution (Fig. 1a), with practically no single cells left; within the aggregates the cells had reconstructed neuroretinal tissue. Under these assay conditions, the size of cell aggregates provides a baseline for assessing enhancement of cell aggregation by the cell-aggregating factor (Fig. 1b).

Density Gradient Centrifugation. The sedimentation coefficient of the cell-aggregating factor was measured according to Martin and Ames (18). Samples containing 0.2–0.3 mg of the cell-aggregating factor and marker proteins in a total volume of 0.1 ml were layered over a 9.9 ml, 5–20% (w/v) sucrose gradient, in a 10 mM phosphate buffer (pH 6.9). Centrifugation was for 19 hr at 38,000 rpm in a Spinco SW 41 rotor at 4°. Tube contents were fractionated and fractions assayed for protein content (19). Cell-aggregating activity was localized by biological assay; catalase, cytochrome *c*, and ferritin markers were identified by enzyme assay and absorbance at 416 nm (18).

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; PAS, periodic acid-Schiff.

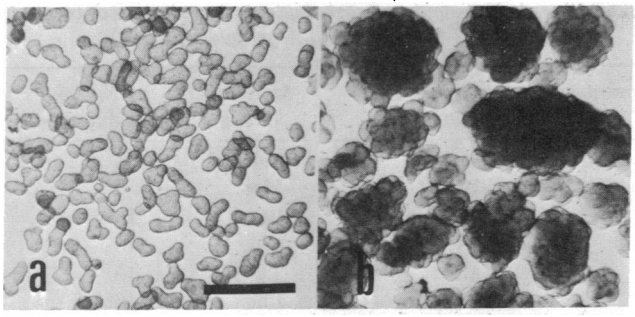

FIG. 1. Twenty-four-hour aggregates of 10-day embryonic neural retina cells obtained in the absence (a) and the presence (b) of 0.5 μg/ml of the retina-specific cell-aggregating glycoprotein (IIB2). Length of bar, 0.5 mm. The cell suspensions were aggregated in rotation flasks as described (14, 15, 17); various concentrations of glycoprotein were added to the culture flasks along with the cell suspension.

Gel Filtration was performed on a 45 × 2.5 cm Sephadex G-200 column in a 40 mM phosphate-5 mM EDTA buffer (pH 8.0), at 4° (20). After equilibration, 1.0 ml of a solution containing 2 mg of ferritin, 5 mg of catalase, 5 mg of cytochrome *c*, and 2.4 mg of retina cell-aggregating factor, all in 20% sucrose, was added to the column. Fractions (1.5 ml) were collected, peak positions in the eluate were determined by absorbance at 280 nm, and the proteins were identified as above.

Amino-Acid and Sugar Composition. The cell-aggregating factor, purified from subfraction IIB by isoelectric focusing, was analyzed for amino-acid and sugar composition by column and gas-liquid chromatography as described in the legend to Table 2.

RESULTS

Purification of the Retina Cell-Aggregating Factor. Examination of the biologically active subfraction (IIB) of the retina cell-aggregating factor (see *Materials and Methods*) by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate ($NaDodSO_4$) resolved it into three to four protein-containing zones. Only one of these stained with the periodic acid-Schiff (PAS) technique as well as with Coomassie blue, indicating the presence of glycosylated proteins (14, 15); this zone was in the region of 50,000 molecular weight, and it contained mostly a single but sometimes a double band (15). Another major protein was localized in the region of 70,000–75,000 molecular weight and gave no reaction with PAS. Since earlier evidence suggested that the retina-specific cell-aggregating activity resided in material localized in the major PAS-staining band (15), we attempted to confirm this by further purifying this material using isoelectric focusing (see legend to Table 1).

Fractionation by isoelectric focusing of IIB (see above) yielded six to seven protein peaks. Only one of these, IIB2, showed cell-aggregating activity. This fraction contained a glycosylated protein from IIB, and its specific cell-aggregating activity was markedly higher than that of IIB (Table 1). On polyacrylamide gel electrophoresis, both with and without $NaDodSO_4$, IIB2 banded in a single zone and stained with both the PAS technique and Coomassie blue (Fig. 2). Its R_F was equivalent to that of a protein of 50,000 ± 5,000. Occasionally, IIB2 produced on $NaDodSO_4$-polyacrylamide gel electrophoresis two closely adjacent bands whose R_F was within the zone of the more common single band. The appearance of this double band was not affected by changes in $NaDodSO_4$ concentration, or by the presence or absence of mercaptoethanol to reduce disulfide bonds. Refractionation of IIB2 by isoelectric focusing or on DEAE-cellulose yielded consistently only a single peak, which nevertheless sometimes yielded the double band on $NaDodSO_4$-polyacrylamide gel electrophoresis. While it is unlikely that

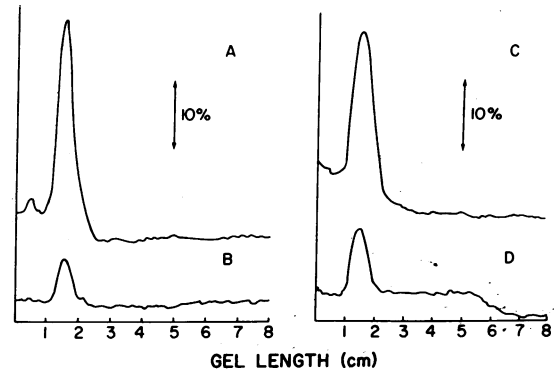

FIG. 2. Absorbance scans of polyacrylamide gels after electrophoresis of the purified retina cell-aggregating glycoprotein (IIB2). (A) Gel without $NaDodSO_4$ stained with Coomassie blue for protein. (B) Gel without $NaDodSO_4$ stained with the PAS technique for polysaccharide. (C) $NaDodSO_4$-polyacrylamide gel electrophoresis with Coomassie blue staining. (D) $NaDodSO_4$-polyacrylamide gel electrophoresis with PAS technique staining. Samples were prepared and gel electrophoresis was done as described (14), according to Weber and Osborn (46). The gels (8 cm, 10% polyacrylamide) were loaded with 30 μg of IIB2. The bar denotes 10% transmission.

TABLE 1. *Cell-aggregating activity of retina cell-aggregating glycoprotein*

Preparation	Protein concentration at which enhancement of cell aggregation is maximal (μg/ml)
Initial "crude factor" preparation	60–70
Sephadex G-200 fraction II	15–20
DEAE-cellulose fraction IIB	3–6
Isoelectric focusing fraction IIB2	0.2–1

Preparation of fraction IIB is described in *Materials and Methods*. For isoelectric focusing, 20–40 mg of IIB were introduced into an LKB 440 electrofocusing column in a 0–50% sucrose gradient with 1% ampholine carrier ampholytes, pH range 3–5. Electrofocusing (16) was conducted at 4° for 3–4 days at 600 V. After equilibration was reached, 1.5-ml fractions were collected and the peaks of protein were determined by absorbance at 280 nm. Fractions within a single peak were pooled, and carrier ampholytes and sucrose were removed by ultrafiltration (15). Preparations of cell-aggregating factor were assayed on retina cell suspensions as described (15). After estimation of protein concentration (19), the factor was tested over a range of 10 concentrations appropriate to the stage of purification. Each concentration range represents more than 50 separate assays.

the occasional presence of the double band indicates a $NaDodSO_4$-resistant dimer, its cause is not yet clear. It is possible that purification may occasionally result in a loss of some terminal residues; or that a minor heterogeneity exists in this material which could not be resolved by isoelectric focusing.

Previous work demonstrated that the production of retina-specific cell-aggregating factor was dependent on macromolecular synthesis, and if synthesized in the presence of radioactive precursors these were incorporated into fraction IIB (14, 15). Fraction IIB2 similarly prepared also contains radioactive amino-acid and sugar residues.

Molecular Weight Determination. Sugar-containing proteins may behave anomalously on $NaDodSO_4$-polyacrylamide gel electrophoresis (21) and, hence, this procedure is inadequate for estimating their molecular weights. We, therefore, redetermined the molecular weight of the purified cell-aggregating factor from its sedimentation coefficient (s), its Stokes radius (a), and its partial specific volume ($\bar{v}$) (18, 20, 22). The Stokes radius was calculated to be 36 ± 1 by determining K_d by gel filtration and plotting $K_d^{1/3}$ of the cell-aggregating factor against marker proteins; s was determined to be 3.3 from density gradient centrifugation; $\bar{v}$ was calculated from the amino-acid composition (see below) to be 0.735. From the relationship $M = 6\pi\eta Nas/1 - \bar{v}\rho$, ($\eta$, viscosity of the medium; N, Avogadro's number; ρ, density of the solvent), the molecular weight of the cell-aggregating factor was calculated to be 50,000 ± 6,000, which is consistent with the earlier value. The frictional ratio, f/f_0, of the cell-aggregating factor was calculated from the above parameters to be 1.3, suggesting an asymmetric molecule with a long and short axis.

Since these data were obtained in the absence of $NaDodSO_4$ and under nondenaturing conditions, they suggest that the retina cell-aggregating factor exists in solution as a 50,000-dalton monomer. This is consistent with the findings that the cell-aggregating activity is not destroyed by treating the factor for 48 hr with 8 M urea, or with 10% $NaDodSO_4$ and 20 mM mercaptoethanol; after removal of urea by dialysis, or of $NaDodSO_4$ by Dowex (23), the factor was found active.

Amino-Acid and Sugar Composition. Analysis of the amino-acid composition of the purified retina cell-aggregating factor (IIB2) demonstrated a relatively large proportion of acidic amino-acid residues (Table 2). Correspondingly, its isoelectric point, pI, determined by isoelectric focusing, was found to be between 3.8 and 4.1. The carbohydrate composition (Table 2) reflects a total polysaccharide content of 10–15%, of which 3–4% is hexosamine. The number of sialic-acid residues detected varied between one and two, depending on the preparation. No glucose was detected by neutral sugar analysis; no galactosamine or uronic acids were found by gas–liquid chromatography. Occasionally, a trace of fucose representing less than one residue per mole of protein was observed; it was not possible to determine whether this trace sugar was actually associated with the protein.

The absence of glucose and the presence of mannose make it unlikely that the retina cell-aggregating factor contains lipid (24); the absence of uronic acids and the relatively low (less than 15%) polysaccharide content classify this molecule as a glycoprotein, as distinguished from glycosaminoglycans (25).

Site of Biological Activity. As previously reported, the cell-aggregating effect of the retina cell-aggregating factor is rapidly destroyed by trypsin (10, 11), but is insensitive to hyaluronidase or neuraminidase (15). The dependence of activity on the polysaccharide moiety was presently further explored. With agarose-bound neuraminidase (Sigma), 95% of the sialic-acid residues were removed (as determined by gas–liquid chromatography) without abolishing the biological activity of the purified factor. Treatment of this desialated material with galactose oxidase (Worthington) also did not destroy its activity. Furthermore, activity was resistant to β-galactosidase (Sigma G-8504) and to treatment for 4 hr with 75 mM periodate.

It, therefore, appears that the activity of the retina cell-aggregating glycoprotein depends on the integrity of the protein moiety, but not on retention of the terminal sialic-acid residues, on the galactose residues, or on those sugar residues that are sensitive to oxidation with periodate under these conditions. It is noteworthy that the cell aggregation factors of mouse teratoma (26) and of marine sponges (27, 28) have a much higher carbohydrate content; the latter show considerable resistance to trypsin but are inactivated by Pronase (29, 30) and periodate (31), and, therefore, were suggested to require the polysaccharide moiety for their characteristic effect (31).

TABLE 2. *Amino-acid and carbohydrate composition of fraction IIB2 of the retina cell-aggregating factor*

Amino acid or sugar	No. of residues/mol		Amino acid or sugar	No. of residues/mol	
Lys	31		Val	34	
His	12		Met	3	
Arg	13		Ile	15	
		56	Leu	40	
Asp	47		Tyr	10	
Glu	55		Phe	10	
		102	Trp	ND	
					112
Thr	23				
Ser	22				
Pro	25				
Gly	24		GlcN	10	
Ala	40		Man	10	
Cys	ND		Gal	10	
		134	Sialic acid	1–2	
					32

Amino acids were analyzed on a Beckman 120C amino-acid analyzer with amino-acid and hexosamine standards. Neutral sugars and amino sugars were analyzed by Dowex column chromatography. Total sugars, including sialic acid, were also analyzed by gas–liquid chromatography. The values obtained by the two techniques were similar; however, neither of the procedures can unequivocally rule out the presence of trace amounts of other sugars. The carbohydrate determinations were done by Drs. J. A. Cifonelli and G. Dawson, and the amino-acid analysis by Dr. R. L. Heinrikson. ND, not done.

DISCUSSION

Our results establish that the retina-specific cell-ligand activity of the retina cell-aggregating factor resides in a glycoprotein, derived from the cells; it has a relatively high content of glutamic- and aspartic-acid residues, and a molecular weight in solution of 50,000 ± 6,000. There is evidence that the cell-aggregating factor specific for embryonic cerebrum is also a glycosylated protein (D. R. McClay, B. B. Garber, and A. A. Moscona, in preparation). The carbohydrate portion

of the retina cell-aggregating glycoprotein represents less than 15% of the total molecular weight; it contains *N*-acetylglucosamine, galactose, mannose, and sialic-acid residues, probably in not more than five side chains (32). The general characteristics of this molecule are those of a membrane protein (21, 33).

We propose that this molecule is a tissue-specific determinant of embryonic retina cell surfaces, and that it is structurally involved in self-recognition and selective ligation of these cells. This suggests analogies with other classes of membrane components that confer surface specificities on cells—such as histocompatibility antigens and immunoglobulins (34)—and raises questions concerning possible relationships between the various molecular mechanisms that mediate different kinds of cell recognition. In fact, linkage through the H-2 locus has been postulated for histocompatibility and immune recognition (35); and the polymorphism of histocompatibility antigens has been explained in terms of linkage of their genes to those that specify molecules for cell recognition (36, 37), such as embryonic cell-ligands. Thus, it is not inconceivable that the various determinants of the different aspects of cell recognition may share certain common features and backgrounds (1–4, 29, 30); this problem is now amenable to examination.

Various hypothetical schemes have been proposed for embryonic cell adhesion, cell recognition, and morphogenetic cell associations (5–8, 31, 38). While most are based on acceptance of the general concept of specific cell ligands (1, 2, 10, 29, 30), i.e., interactions of specific cell-surface components, only few direct attempts have been made to isolate such components from embryonic cells and to characterize them. Hyaluronic acid has been reported to clump cells from certain murine tumor tissue culture lines (39); however, the specificity of this effect and its relevance to the actual mechanisms of histotypic cell associations are not clear. Cells can be clumped and agglutinated by various agents, including polyelectrolytes (40), serum proteins (41), and lectins (42). Some of these reactions are very useful for probing cell surface properties; however, they should not be confused with the mechanism of selective cell adhesion, self-recognition, and morphogenetic interactions of embryonic cells, and hence with tissue-specific cell ligands such as those prepared from retina or cerebrum.

Especially attractive has been the hypothesis that carbohydrates and carbohydrate transferases mediate retina cell adhesion (43); however, since the effect of the retina cell-aggregating glycoprotein appears to be independent of its terminal oligosaccharide residues, the role of carbohydrates in selective adhesion of embryonic retina cells requires further clarification. Our initial results on this subject are consistent with earlier evidence that the retina cell-aggregating glycoprotein does not show retina galactosyltransferase activity, nor is it an acceptor for this enzyme (44).

It remains to be determined if the 50,000 molecular weight established for the retina cell-aggregating glycoprotein in solution represents the actual functional entity, or if a multimeric interaction with homologous or heterologous components is required for the characteristic activity. Our present data do not rule out the existence of a heterogeneity of the kind present in immunoglobulins (34) or glycophorin (45). We have not excluded the possibility that cells from other tissues may possess components with antigenic similarities to the retina cell-ligand glycoprotein.

There is evidence from this (11, 12) and other laboratories (38) that the primary site of action of the retina cell-aggregating factor (and of cell-aggregating factors from other systems) (29, 30) is at the cell surface; however, the possibility of internal effects cannot be ruled out at present. Furthermore, as previously suggested (1, 2, 29, 30), the mechanism of cell-ligand action may involve interactions with still other cell membrane constituents and their detailed patterning on the cell surface. Thus, binding of the cell-aggregating glycoprotein to trypsinized retina cells does not require protein synthesis or temperature-dependent processes (11, 12); however, expression of ligand activity does (14). Diverse preparatory procedures being pursued in this and other laboratories (38) may lead to the isolation of the additional components of the cell-linking mechanism.

Finally, it remains to be determined if the retina cell-aggregating glycoprotein isolated by us is produced by all the cell types present in the embryonic retina during its development and postnatally, and if its binding to the cell surface and its distribution there are similar for all retina cells.

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