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Developmental Regulation of Glycoprotein Biosynthesis in Dictyostelium

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We have examined the glycoprotein-linked oligosaccharides assembled during the life cycle of Dictyostelium discoideum, and found their expression to be dramatically dependent upon the stage of development. During early development mature glycans have a high mannose character, and a substantial proportion acquire a fucose residue that correlates with endo-H resistance. One-third of the glycans also acquire sulfate residues. These glycans diminish in importance during aggregation.

The mature glycans expressed during late development contain fewer mannose residues, from five to ten mannose residues, and are characterized by the absence of sulfate residues and by the presence of fucose residues on endo-H-sensitive glycans. These glycans make their appearance coincident with the construction of tips on tight cell mounds. At this stage glycans characteristic of both early and late stages occur simultaneously.

Developmental regulation of the wide array of protein-linked glycans expressed during the life cycle of Dictyostelium discoideum may be as simple as the controlled transition from a group of structures that are assembled by the vegetative cells to a group of structures that are assembled by the terminally differentiating cells. The potential biological significance of this transition is discussed.

Key words: developmental regulation of glycoprotein, glycoprotein biosynthesis, during life-cycle D discoideum, in D discoideum, assembly of glycoproteins during development, regulation of glycoproteins during development

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The cellular slime mold Dictyostelium discoideum provides a useful model for investigating possible roles of cell surface carbohydrates in the regulation of development. Upon exhaustion of the food supply, Dictyostelium discoideum amoebae undergo a relatively simple developmental process consisting of aggregation of initially free-living amoebae into multicellular masses followed by morphological differentiation of the aggregates. This culminates in the formation of a fruiting body consisting of a spore sac supported by a slender cellular stalk. The initial morphological event, namely, aggregation of individual amoebae into cell mounds, is mediated by pulses of cyclic AMP which acts as a chemotactic agent [reviewed in 1]. Subsequent steps of differentiation include the formation of cell-cell contacts, appearance of a discrete tip region on the cell mounds coincident with a tightening of these cell contacts [2], generation of migratory sluglike pseudoplasmodia, and establishment of a spatial distribution of presumptive stalk and spore cells prior to culmination [reviewed in 3]. Thus the developmental sequence includes a fairly synchronous transition from a noncohesive to a mutually cohesive state and a later reorganization to establish a spatial pattern of two cell types within the aggregate. The regulatory events and mechanisms governing these events are of considerable interest.

Formation of intimate cell contacts is clearly required for development, and there is considerable evidence supporting a role for cell association in the regulation of gene expression [2,4–8]. As in many other systems of cell association, cell surface glycoproteins and their receptors are generally considered to participate in the formation of cell-cell contacts in D discoideum (see Significance section).

Detailed analysis of the participation of cell surface carbohydrates in the regulation of development requires knowledge of the structure of sugar-containing units available for expression at the cell surface and the mechanism that controls their biosynthesis. In the present study we describe the various glycans expressed during development. The observed dependence of glycan structure upon the stage of development has revealed pronounced developmental regulation of the activities of the biosynthetic enzymes.

MATERIALS AND METHODS

Culture Conditions

D discoideum strain A3 was grown at 22°C in shaken suspension (120 rpm) in HL5 broth [9]. Cells were harvested during late exponential growth (5–8 x 10^6/ml) by centrifugation at 500g for 5 min and were washed and resuspended in KPM buffer (M/60 potassium phosphate, pH 6.1, 2 mM MgSO4).

Labeling Conditions

Amebae in KPM suspensions were dispersed onto Nucleopore filters (2 x 10^7 cells per 47 mm diameter filter with 0.2 μ pores) resting on Petri plates containing 2% agar in KPM. For the earliest period of the developmental cycle, the plates were incubated open for about 30 min to allow evaporation of excess liquid prior to labeling. For label incorporation, the filters were transferred to a fresh Petri plate containing a 20μl droplet with 50–100 μCi of tritium-labeled sugar or (^{35}S)-sulfate. For pulse-chase experiments the filters were subsequently

transferred to a Petri dish containing 2% agar in KPM and unlabeled sugar at 2mM. Labeling with galactose, fucose, galactosamine, and sulfate was for 60 min. Labeling with mannose and glucosamine was for 15 min and was followed by a 45-min chase period. Homogeneous populations of migrating slugs were prepared by incubating postaggregation cultures for 2 or 3 in a black box with a pinhole of light. The Petri plates were removed from the slug phototaxis chamber and the population labeled as usual.

Materials

Endo-β-acetylglucosaminidase H (endo-H) was prepared from Streptomyces plicatus according to the method of Tarentino et al [10]. α-Mannosidase type III was obtained from Sigma Chemical Co., Bio-Gel P-4 from Bio-Rad Laboratories, and Pronase CB from Calbiochem. The radiochemicals were from New England Nuclear: D-(2-^{3}H(N))-mannose, 10–20 Ci/mmol; D-(6-^{3}H(N))-glucosamine hydrochloride, 10–30 Ci/mmol; D-(6-^{3}H(N))-galactose, 5–15 Ci/mmol; D-(6^{3}H(N))-galactosamine, 10–30 Ci/mmol; L-(5,6-^{3}H)-fucose, 40.60 Ci/mmol and [^{35}S]-H_2SO_4, 43 Ci/mg.

Isolation of the Lipid-Linked Oligosaccharide

Glycoproteins were obtained by the Folch extraction. The amoebae were washed off the filters in buffer (4 ml) and extracted in five volumes of chloroform/methanol (3:2). The suspension was dispersed and centrifuged. Two phases and a prominent interface formed. The interface and lower phase were washed four times with 8 ml chloroform/methanol/water (1:20:20). Sufficient water and methanol were added to the interface and lower phase to construct a single phase of chloroform/methanol/water (10:10:3). The pellet formed by centrifugation was washed twice more with (10 ml) chloroform/methanol water (10:10:3).

Preparation of the Glycopeptides

The pellets from the chloroform/methanol/water (10:10:3) washes were dried and resuspended in 200 ml of Tris HCl, pH 8.0, 10 mM $CaCl_2$ and incubated with 200 ml aliquots of 1% pronase solution at 50°C with a toluene overlay. Additional 200-ml aliquots of pronase solution were added at 24 and 48, and the total length of the digestion was 72 H.

Glycohydrolase Digestions

Sensitivity to endo-β-N-acetylglucosaminidase H was tested in 0.1 M citrate, pH 5.5, with 90 milliunits of enzyme at 37°C for 4 h under a toluene atmosphere.

Chromatographic Techniques

Gel filtration was performed on Bio-Gel P4 (400 mesh) columns (1.3 x 100 cm) with 50 mM pyridinium acetate, pH 5.5, as eluent. Fractions of 0.45 ml were collected. Descending paper chromatography was performed on Whatman No. 1 paper in the following solvent systems: solvent A, pyridine/ethyl acetate/acetic acid/water (5:5:1:3); solvent B, pyridine/ethyl acetate/water (1:3.6:1.14), upper phase.

RESULTS

The Glycoprotein-Linked Oligosaccharides Assembled at the Onset of Development

The array of mannose-containing oligosaccharides present on mature glycoproteins can be seen in Figure 1 MAN. These oligosaccharides were labeled during a 15-min pulse with radiolabeled (2-^{3}H) mannose, and the glycoproteins were prepared subsequent to a 45-min chase period. The amoebae were extracted with solvent to remove glycolipids, and the glycoprotein residue was exhaustively digested with pronase. The resultant glycopeptides were then digested with endo-β-N-acetylglucosaminidase H (endo-H). This enzyme cleaves between the two core N-acetylglucosamine residues to release high mannose oligosaccharides and peptide fragments that each contain one of the N-acetylglucosamine residues. The endo-H digests were then fractionated on Bio-Gel P-4 columns.

Mannose is incorporated into three classes of oligosaccharide: a large class excluded by the matrix (void volume at fraction 60), a second class eluting between fractions 70 and 90, and a third class eluting between fractions 90 and 110. This last class is derived from the oligosaccharide cleaved by endo-H and is not present prior to this enzymatic digestion. The endo-H-resistant oligosaccharides are the larger oligosaccharides eluting between fractions 70 and 90. The void volume material appears to be high molecular weight polysaccharide resistant to endo-H. At this stage in development, the oligosaccharides present on mature glycoproteins are evenly distributed between endo-H-resistant and endo-H-sensitive glycans.

The endo-H-resistant oligosaccharides eluting between fractions 70 and 90 are also readily labeled with fucose and sulfate (as is material in the void volume) (Fig. 1 FUC and SO_4, respectively). Very little galactosamine is incorporated into the glycopeptides (Fig. 1 GALN). The endo-H-sensitive oligosaccharides are also labeled with glucosamine (Fig. 1 GLN), and with galactose, (Fig. 1 GAL). Strong acid hydrolysis followed by paper chromatography revealed the galactose-derived label to have been converted to glucose prior to its incorporation into the oligosaccharides (unpublished result).

The endo-H-sensitive oligosaccharides have a glucosamine to mannose ratio half that of the endo-H-resistant oligosaccharides (Fig. 1). As half of the glucosamine is lost on endo-H-digestion, the endo-H-resistant and endo-H-sensitive structures have very similar compositions. Indeed, upon mild acid hydrolysis to remove fucose, the endo-H-resistant oligosaccharides become endo-H-sensitive, and upon redigestion with endo-H give rise to the same two oligosaccharides released in the initial endo-H digestion (unpublished result). Whereas large high mannose structures are usually reported not to contain fucose residues, precedent for a fucose residue-blocking endo-H-susceptibility was established by Tarentino and Maley [11]. These workers showed that Man_3-$GlcNAc_2$-ASN was cleaved by endo-H, although slowly, whereas Man_3-GlcNAc- (Fuc)-GlcNAc-ASN was not cleaved at all even after prolonged exposure to high concentrations of enzyme. Thus in Dictyostelium endo-H resistance may be the result of core fucosylation, and there is no evidence of trimming reactions that digest the mannose core.

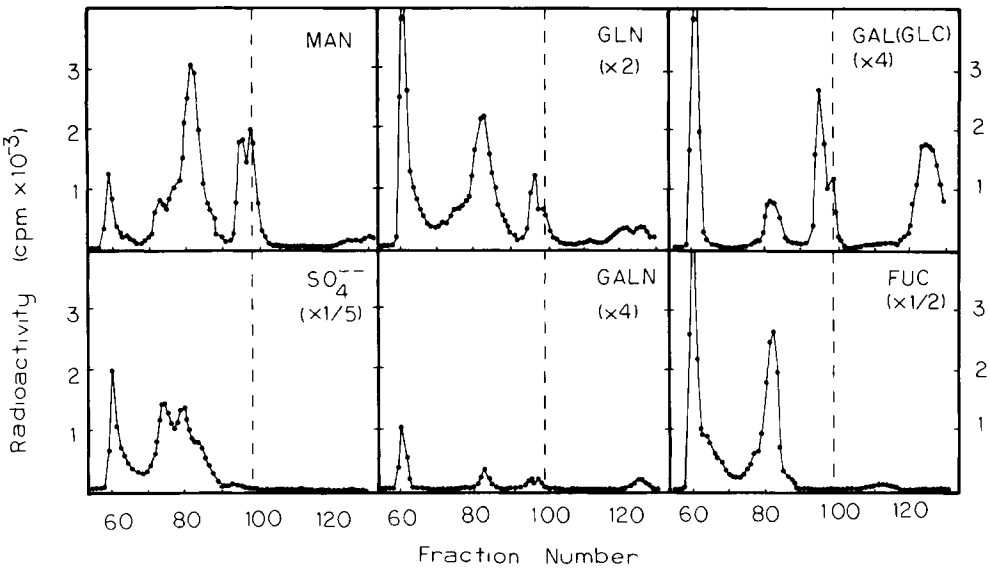

Fig. 1. Glycopeptides from D discoideum labeled during the first hour of starvation. Amoebae were labeled with mannose or glucosamine for 15 min and chase for 45 min with unlabeled sugars, or labeled with galactose, sulfate, galactosamine, or fucose for 60 min. Radiolabeled glycoproteins were prepared from the amoebae by exhaustive solvent extraction (chloroform:methanol:water, 10:10:3 v/v) of the protein residue prepared by the Folch procedure. The glycoproteins were exhaustively digested with pronase and endoglucosaminidase H. The radiolabeled glycopeptides and oligosaccharides were subjected to gel filtration chromatography on Bio-Gel P4 (MAN) (^{3}H)-mannose; (GLN(^{3}H)-glucosamine; (GAL) (^{3}H)-galactose; (GALN) (^{3}H)-galactosamine; (SO4) (^{35}S)-sulfate; (FUC) (^{3}H)-fucose. The scales were adjusted by the factors indicated on the individual panels.

Changes in the Glycoprotein-Linked Oligosaccharides During Development

The glycoprotein-linked oligosaccharide profiles obtained from mature glycoproteins labeled after 4 hours of development are remarkably similar to those shown in Figure 1 for the first hour of development. No new species are observed. The endo-H-sensitive oligosaccharides are increased somewhat relative to the endo-H-resistant oligosaccharides, and fucose and sulfate incorporation into these latter oligosaccharides is increased, by a factor of 2, on a per-cell basis. The patterns remain similar when amoebae are labeled during aggregation between 8 and 9 hours of development. Again there are no new species present, and the trend toward an increase in endo-H sensitivity increases. Within the endo-H-sensitive oligosaccharides there is an increase in the material that has lost two hexoses (dashed line) relative to the material that has lost just one hexose.

The first significant changes in the profiles are observed when labeling is performed at the completion of aggregation when tight mound form a distinguishable tip (Fig. 2). The incorporation of fucose and sulfate into endo-H-re-

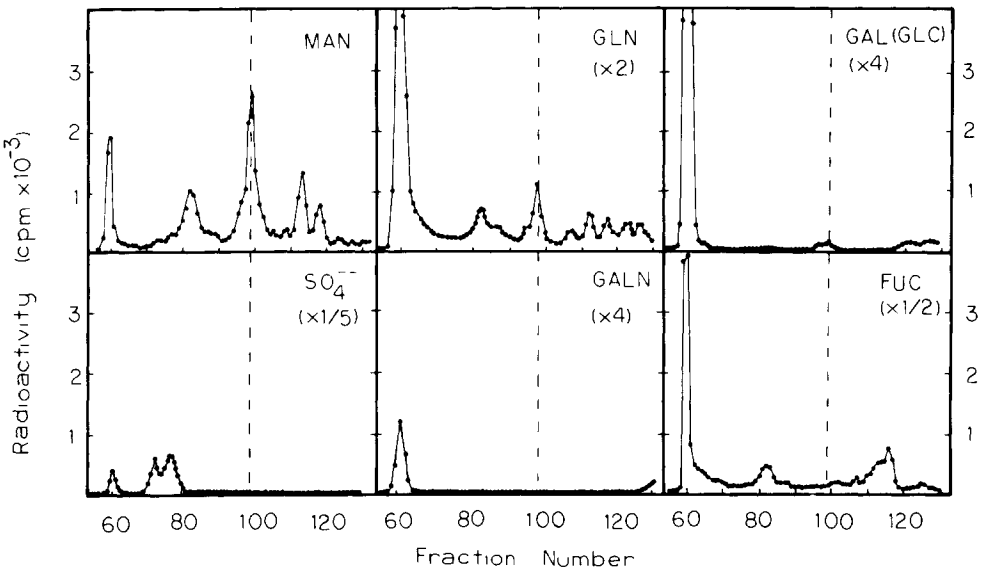

Fig. 2. Glycopeptides from D discoideum labeled after 13 hours of starvation (TIP). Legend to Figure 1 for details.

sistant structures is severely depressed. Very little glucose survives processing to appear in mature glycoproteins. New species are also present that are released by endo-H and elute at fractions 113 and 118. These oligosaccharides contain mannose and glucosamine, and they are also fucosylated. In these cases the fucosylation is on peripheral portions of the oligosaccharides and provides protection against subsequent α-mannosidase digestions.

The trends observed at the tip stage continue when labeling is performed after 16 hours of starvation when the aggregates were migratory slugs (not shown). The prominence of this migratory phase is dependent upon the environmental conditions and, in the present study, it was achieved by culturing the aggregates in the dark except for a point source of light. This produced a synchronous population of migrating slugs that were aligned toward the light source. The glycopeptide patterns show that the sulfated glycopeptides and the endo-H-resistant fucosylated glycopeptide at elution position fraction 83 have almost completely disappeared. The fucosylated oligosaccharides at elution positions fraction 113 and 118 are more prominent, and new species (positions 102 and 107) containing mannose, glucosamine, and fucose are observed.

Figure 3 shows the gel filtration profiles of the glycopeptides labeled with the various metabolites after 20 hours of starvation when the aggregates are beginning to form fruiting bodies. In spite of the cellulose matrix and the less extensive contact with the supporting material (filter) the amoebae at this stage of development very actively incorporate the metabolites into protein-linked oligosaccharides. Sulfated and endo-H-resistant fucosylated species remain severely depressed. The bulk of the mannose label is now in endo-H-sensitive species that

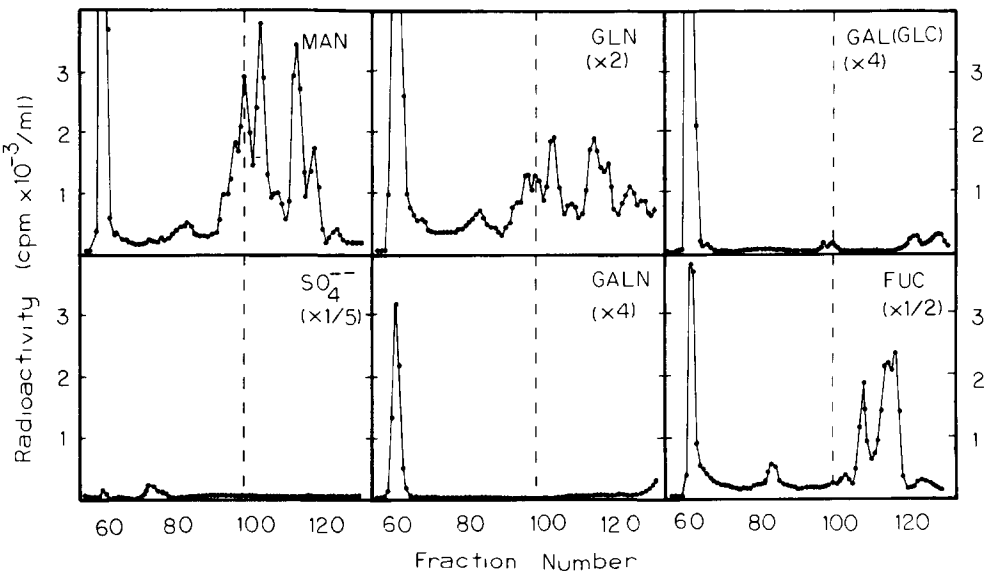

Fig. 3. Glycopeptides from D discoideum labeled after 20 hours of starvation (CULM). Legend to Figure 1 for details.

elute to the right of the dashed line (fraction 99), whereas prior to tip construction there was no material that eluted to the right of this line. There is now a spectrum of oligosaccharides with peaks at fractions 93, 96, 99, 103, 107, 117, and 123. All of these oligosaccharides contain N-acetylglucosamine and some contain fucose (fractions 102, 107, 113, and 116).

The Processing Pathways for the Protein-Linked Oligosaccharides

The data of Figures 1–3 suggest that two alternative sequences for processing the precursor oligosaccharide after transfer to protein occur during development. These pathways occur either independently as at early (Fig. 1) and late (Fig. 3) stages or simultaneously as at the tight mound stage (Fig. 2). The postulated pathways are summarized in Scheme 1. The early pathway (A–E) appears to be the incomplete removal of glucose followed by a fucosylation that correlates with resistance to endo-H. There are also sulfated oligosaccharides that appear to contain either one of two sulfate residues and are probably derivatives of the fucosylated species. Fully 40% of the oligosaccharides on mature glycoproteins have lost only a single hexose residue, and another 40% of the oligosaccharides have lost only two hexose residues. The mannose cores of the entire array of oligosaccharides remain intact.

The late pathway (A, B, F–H) is also shown in Scheme 1 and appears to be the sequential removal of the glucose residues and several of the mannose residues, so that a nearly complete spectrum of the intermediately trimmed oligosaccharides is present. No sulfate incorporation occurs, and the fucosylation of the oligosaccharides occurs on endo-H-sensitive glycans.

Enzymes of Oligosaccharide Biosynthesis

By analysis of the oligosaccharide products a number of conclusions can be drawn about in vitro activities of the biosynthetic enzymes during development. Three different classes of enzymatic behavior are identified and are summarized in Scheme 1. First, the enzymes responsible for synthesis of the lipid-linked precursor oligosaccharide (A) and its transfer to polypeptides (B) persist from the vegetative phase throughout development. Second, the activity of the fucosyl transferase (D) that correlates with endo-H resistance is greatly reduced during development. Coordinately, incorporation of sulfate (E) is depressed. This could indicate a loss of the sulfate transferase. Alternatively, these transferases may require prior fucosylation of their substrates, and the apparent loss of activity could reflect reduced availability of the appropriate acceptor substrate.

Third, several activities obviously increase during development. These most probably are an elevation in levels of active glucohydrolase(s) (C, F) since loss of glucose is more extensive and since the substrate is always present. Transfer of fucose to peripheral regions of the oligosaccharides (H) also appears in late development, and this could be due to elevation of enzyme levels or to a new availability of the acceptor substrate.

Finally, the data suggest an elevation of mannohydrolase activity (G). This is provocative in light of the previous studies of Loomis [12] who showed that the major isozyme of α-mannosidase increases in activity early in development reaching a maximum in late culmination. This enzyme has an acidic pH optimum, is probably lysosomal and, as such, would not be expected to participate in biosynthetic processing. Later, Free and Loomis [13] isolated mutants that lacked this enzyme and revealed a minor, apparently membrane-bound α-mannosidase with a higher pH optimum which was expressed late in development. This enzyme may be responsible for the biosynthetic processing reported here.

Developmental Regulation of Polysaccharide Synthesis

Incorporation of precursors into polysaccharide material (Fig. 4) undergoes transitions of type similar to those seen with glycoproteins. Galactose (Fig. 4C and D) incorporation increases in the first four hours and then diminishes during aggregation, although considerable galactose incorporation persists through late development. As tips and, more dramatically, as slugs form, incorporation of mannose (Fig. 4A) and glucosamine (Fig. 4B) rises markedly. The high level of fucose incorporation at early stages drops significantly at aggregation but then rises sharply in later development (Fig. 4F). Galactosamine labeling (Fig. 4E) is quite low at early stages and rises slightly after tip formation.

SIGNIFICANCE

There is considerable evidence supporting a role for cell surface carbohydrates in the cell-cell interactions required for development. Gerisch and his coworkers have identified a cell surface glycoprotein with a molecular weight of 80,000 daltons [14] called contact site A. Monovalent Fabs against contact site A block cell aggregation [15]. A set of closely related lectins called discoidins, also appear on cell surfaces coincidental with acquisition of cohesiveness by the amoe-

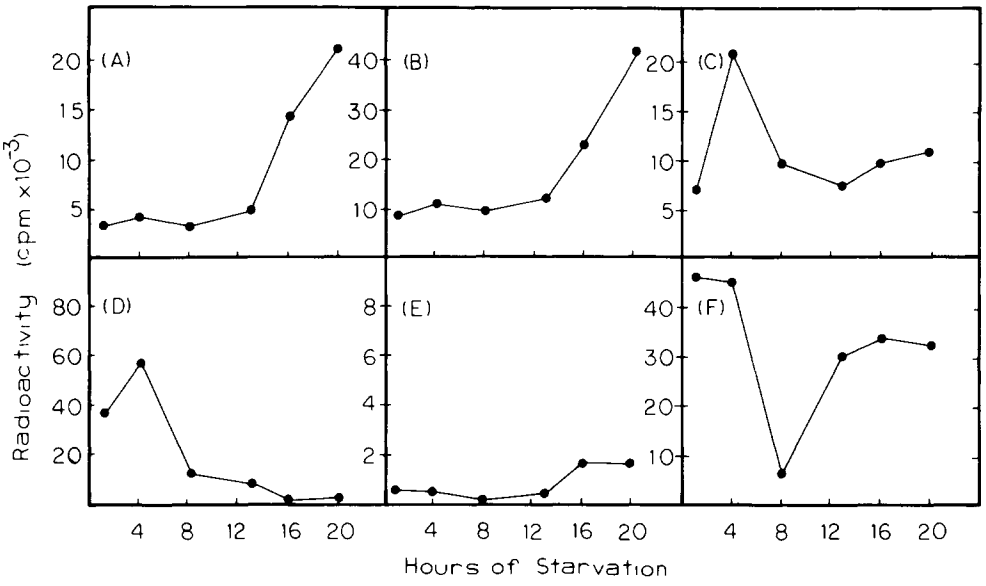

Fig. 4. Time course for the developmental regulation of the enzymatic activities involved in polysaccharide biosynthesis. A) Mannose incorporation; B) glucosamine incorporation; C) galactose incorporation; D) sulfate incorporation; E) galactosamine incorporation; F) fucose incorporation. For each time point 2 x 10^7 amoebae were plated and at the appropriate time labeled as described in Materials and Methods. Very little cell division occurs subsequent to the initiation of development (ie, the onset of starvation), and therefore similar cell numbers were labeled at each time point.

bae [16, 17]. A mutant strain producing inactive discoidin fails to aggregate [18]. The glycoprotein antigen described by Huesgen and Gerisch [14] is distinct from and does not interact with discoidin. More recently, it has been reported that monovalent antibodies to a 150,000-dalton cell surface, concanavalin A-binding glycoprotein will block reagglutination of mechanically dispersed cell aggregates [19]. A possible cell surface receptor for this glycoprotein remains to be identified.

The present study provides a description of oligosaccharide structures available for surface recognition. The appearance of fucosylated oligosaccharides over the same short period that the aggregates acquire their tight cohesion may be directly related to this increased intercellular recognition. The developmentally regulated lectins, discoidins, which are usually considered to be galactose-binding proteins, are equally competently inhibited by fucose [20]. We have been unable to find galactosylated glycopeptides. Therefore, if such an oligosaccharide exists, it must be of very minor abundance. Other alternatives therefore are that the ligand for discoidin-like activity is either the galactose-containing polysaccharide excluded from this matrix or the fucosylated-oligosaccharides that are attached to proteins. Although the fucose binding protein from Lotus tetragonolobus does not agglutinate D discoideum amebae [21, 22], it could easily differ from discoidin in ligand specificity.

The developmental regulation of the wide array of protein-linked oligosaccharides displayed during the developmental cycle of D discoideum is here postulated to be the result of a carefully modulated transition between two alternative

processing pathways (Scheme 1). Like yeast, another unicellular eukaryote, vegetative amoebae do not extensively degrade the precursor oligosaccharide. In contrast, the multicellular form of D discoideum trims the precursor oligosaccharide fairly extensively, as is the case in higher eukaryotes. The complex processing pathways of higher eukaryotes where the high-mannose type of oligosaccharides are degraded to form the simple pentasaccharide core of complex oligosaccharides have seemed overelaborate. However, the gradual addition of more extensive cleavage and processing reactions to the basic pathway of unicellular eukaryotes is a simpler evolution than the de novo appearance of an alternative glycosylatation route for the complex oligosaccharides that contain very small mannose cores. The acquisition of a more extensive processing pathway by the multicellular phase of D discoideum may therefore recapitulate the evolution of the processing pathways of higher eukaryotes.

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