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# Cell surface oligosaccharides participate in cohesion during aggregation of *Dictyostelium discoideum*

(glycoproteins/development)

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ABSTRACT **Plasma membrane glycoproteins from *Dictyostelium discoideum* amoebae at three stages of early development were digested with Pronase and endoglycosidase H and fractionated by gel filtration. This gave three classes of glycans (polysaccharides, endoglycosidase H-resistant glycopeptides, and endoglycosidase H-released oligosaccharides), which were tested for their ability to block agglutination of amoebae from vegetative, aggregation (8-hr), and late-aggregation (13-hr) stages of development. The endoglycosidase H-resistant glycopeptides from 8-hr cells inhibited agglutination of disaggregated 8-hr cells but not vegetative or 13-hr cells. The 8-hr polysaccharide and endo H-sensitive oligosaccharides did not inhibit. The glycopeptides from 8-hr cells were resolved into five species by electrophoresis in borate-containing buffer. Two of these had agglutination-inhibiting activity, and three did not. None of the glycan fractions from vegetative or 13-hr cells inhibited agglutination of vegetative, 8-, or 13-hr cells. These data implicate specific cell surface glycans in aggregation-stage intercellular cohesion and suggest that both these glycans and receptors for them are developmentally regulated.**

*Dictyostelium discoideum* has been a useful model for study of intercellular cohesion by virtue of its orderly and sequential expression of stage-specific cohesion systems during development (reviewed in refs. 1 and 2). When vegetatively growing amoebae are starved, within a few hours they begin to aggregate into multicellular masses. At the completion of aggregation, after about 13 hr of starvation, the multicellular mound begins reorganization to produce a migratory, slug-like pseudoplasmodium composed of the two precursor cell types (prestalk and prespore) that will generate the eventual fruiting body.

Cohesion between vegetative amoebae is blocked by EDTA, while cohesion between developing amoebae is EDTA resistant (3). Serological studies have further refined this analysis. Antibodies that block cohesion between vegetative cells are not effective on aggregating amoebae and vice versa (4). At least one additional cohesion system appears during postaggregation development; antibodies that block aggregation-stage cohesion are not effective at later developmental stages, and antibodies that block cohesion in postaggregation amoebae are not effective for aggregation- or vegetative-stage amoebae (5–7).

Isolation of the major antigens recognized by cohesion-blocking antibodies has allowed identification of stage-specific glycoproteins, which are thus strongly implicated in intercellular cohesion (5, 8–10). Since all are glycoproteins and since periodate treatment inactivates some of the antigens (11, 12), one model for cohesion is that the sugar-containing units of these glycoproteins bind receptors on adjacent cells.

If this model is correct, an exogenous supply of such glycans should compete for receptors and inhibit intercellular cohesion. We have tested this hypothesis and find that two specific glycopeptides from aggregation-stage cell plasma membrane glycoproteins inhibit cohesion of cells at that stage but not other stages. No glycans from other stages showed cohesion inhibition at any stage.

## MATERIALS AND METHODS

**Cell Culture and Metabolic Labeling.** *D. discoideum* strain AX-3 amoebae were grown at 22°C in shaken suspensions (120 rpm) in HL5 broth (13) or TGC broth (as for HL5 except with trypticase rather than thiotone peptone and supplemented with 0.45 g of cysteine and 0.4 g of glycine per 650 ml). Cells were harvested during exponential growth (5–8 × $10^6$ cells per ml) by centrifugation at 500 × *g* for 5 min at room temperature and were washed in cold KPM buffer (16.7 mM potassium phosphate/2 mM $MgSO_4$, pH 6.1). Amoebae dispersed on Nuclepore filters were metabolically labeled for 60 min with 100 μCi of $^3$H-labeled sugars (1 Ci = 37 GBq) at the appropriate developmental stage. All the above procedures were as previously described (14, 15).

Cells harvested or labeled immediately after plating (plus 30 min of incubation to allow evaporation of excess liquid) are referred to in the text as vegetative (0-hr) cells. Cells harvested or labeled between 7.5 and 8.5 hr of starvation were actively aggregating at the time of harvest and are referred to in the text as aggregation-stage (8-hr) cells. Cells harvested or labeled between 13 and 14 hr of starvation had completed aggregation and initiated tip formation. These are referred to as late-aggregation (13-hr) cells.

**Plasma Membrane Isolation.** Cell lysis and plasma membrane isolation were as described (16), using large numbers of cells (70–80 plates of $10^8$ cells each). Usually, only 20 plates of cells were metabolically labeled. The three plasma membrane bands in the center of the gradient were collected by lateral puncture of the tube and diluted 3- to 4-fold with KPM, and the membranes were harvested by centrifugation for 90 min at 12,000 × *g*, at 2°C.

**Glycoprotein Extraction and Enzymatic Digestions.** These procedures were as described (15). Briefly, membrane pellets were resuspended in 4 ml of KPM, and delipidated proteins were prepared by a modified Folch procedure and digested exhaustively with Pronase and endo-β-*N*-acetylglucosaminidase H (endo H).

**Column Chromatography.** Lyophilized Pronase and endo H digests were dissolved in 50 mM pyridinium acetate, pH 5.5, and chromatography was performed on 1.3 × 100 cm

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Abbreviation: endo H, endo-β-*N*-acetylglucosaminidase H.

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columns of Bio-Gel P4 (−400 mesh) or (200–400 mesh) in 50 mM pyridinium acetate, pH 5.5/0.02% $NaN_3$; 0.47-ml fractions were collected.

**Electrophoresis and Fluorography.** These were as described by Prem Das and Henderson (15). Briefly, the method uses a 10% polyacrylamide slab preequilibrated with 2,5-diphenyloxazole, then reequilibrated and run in 100 mM Tris borate, pH 8.3. The sample buffer concentration is 10 mM. Borate binds oligosaccharides with suitably positioned hydroxyl groups such that otherwise neutral glycans can be fractionated. After electrophoresis, the gel is immediately dried and fluorography is performed. Elution of bands from the gel was as described (15).

**Agglutination Assays.** Cells that had been harvested from various developmental stages were suspended in 1 ml of KPM buffer per $10^8$ cells and recovered by centrifugation for 30 sec in a Beckman Microfuge B. The cells were resuspended in 1 ml of KPM containing 10 mM EDTA (pH 6.0) using repeated Vortex agitation at intervals for 10 min. Cell dissociation was usually complete. If not, mechanical dissociation using a needle and syringe was employed. These methods generated a suspension of single cells with little or no cell lysis. The cells were then sedimented again (30 sec in the Microfuge), the supernatant was quickly discarded, and the cells were resuspended in 1 ml of KPM, with or without EDTA or a competitor glycan, and the suspension was agitated for 5 sec. The suspension was then quickly added to 9 ml of KPM, with or without EDTA, in a 25-ml Erlenmeyer flask. After a "zero-time" sample had been taken, the flask was placed on a Gyrotory shaker [New Brunswick G10, with 2-inch (50-mm) displacement, 120 rpm, 22°C], and aliquots were removed for counting (by hemocytometer) at 10-min intervals.

Percent particles remaining is the number of singlet and doublet cells at each time point, divided by those at the zero time and multiplied by 100. Thus, 60% particles remaining means that 60% of the cells were still as singlets and doublets and 40% were in agglutinates, which usually contained many cells.

**Cell Equivalent Units.** We do not know the cellular pools of precursor sugars, and, since they probably change during early development, radioactivity cannot be used as a comparative measure of numbers of glycan molecules. Therefore, the "unit" of glycans to be tested for inhibition of agglutination was based on cell equivalents. One cell equivalent is defined as that amount of a glycan type from the plasma membrane fraction of $1 \times 10^8$ starting cells. Thus, 5 cell equivalents is the amount of oligosaccharide obtained

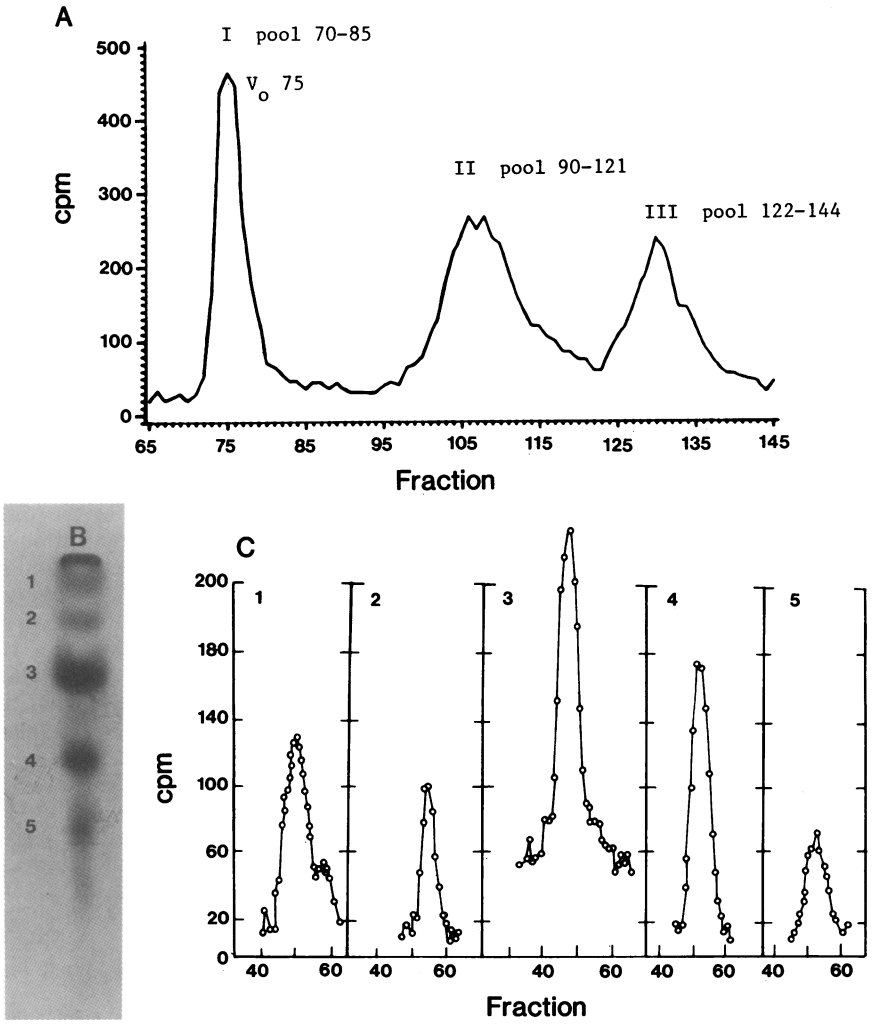


FIG. 1. Purification of metabolically labeled glycans. Amoebae were plated, starved for 7.5 hr, and labeled for 60 min with 100 μCi of [$^3$H]mannose (*A*) or [$^3$H]glucosamine (*B*, *C*). Plasma membranes were isolated and subjected to Folch extraction, and the glycoproteins were digested with Pronase and endo H. (*A*) Bio-Gel P4 (−400) elution profile of 100,000 cpm of mannose-labeled glycans; radioactivity was measured in 0.05 ml of each fraction. $V_0$, void volume. (*B*) Fluorogram of polyacrylamide gel loaded with 130,000 cpm of pooled glucosamine-labeled column fractions 90–121 and exposed 5 days. On some gels, as is the case here, some material does not enter the gel. The reason for this is not known. (*C*) Bands 1–5 in *B* were eluted and chromatographed on a Bio-Gel P4 (200–400) column, measuring radioactivity in 0.015 ml of each fraction. Panels 1–5 correspond to gel bands 1–5, respectively.

from the isolated plasma membranes of $5 \times 10^8$ cells, not corrected for losses during the purification. There are $10^8$ cells (1 real cell equivalent) in 10 ml in the standard agglutination assay.

Five cell equivalents of 8-hr plasma membrane endo H-resistant glycopeptides in a 10-ml assay mixture corresponds to at least nanomolar levels. This is based on the following: (*i*) [$^3$H]glucosamine, which is a very efficient label (compared to mannose) for *Dictyostelium*, so that the intracellular pool must be relatively small; (*ii*) the extreme assumption of no intracellular pool; and (*iii*) the amount of radioactivity recovered in the glycans. The true concentration will be increased above nanomolar by the actual size of the metabolic pool.

**Materials.** Pronase CB was from Calbiochem. The endo H (from *Streptomyces plicatus*) was from the New York State Department of Health. The radiochemicals were from New England Nuclear or ICN: D-[2-$^3$H(N)]mannose, 10–20 Ci/mmol; D-[6-$^3$H(N)]glucosamine hydrochloride, 10–30 Ci/mmol.

## RESULTS

**Preparation of Plasma Membrane Protein Glycans.** Cells were metabolically pulse labeled with tritiated sugars for 60 min after 0.5, 7.5, and 12.5 hr of starvation. After the labeling, plasma membranes were isolated, and glycoproteins extracted by the Folch procedure were digested exhaustively with Pronase and then with endo H. The Bio-Gel P4 (−400) column profile of the digest obtained from aggregation stage cells (Fig. 1*A*) shows three prominent peaks. Fraction I is "polysaccharide" material eluting in the void volume. Fraction II contains high-mannose glycopeptides resistant to digestion by endo H, which cleaves between the two core *N*-acetylglucosamine residues unless one is substituted, in this case by fucose (see *Discussion*). Fraction III contains oligosaccharides released from peptide carriers by endo H; without this digestion, they elute with fraction II. No material smaller than fraction III is observed with mannose labeling.

The plasma membrane glycan profiles are very similar for all three developmental stages tested (not shown). In all, the endo H-resistant and endo H-sensitive glycans are present in approximately equal amounts. The mannose label also indicates a large amount of polysaccharide material, in contrast to the relatively small amount of polysaccharide seen with this label in whole cell samples (14, 15). Whole cell profiles also show a peak between fractions II and III which consists of polyanionic glycans (15) and is absent from these purified plasma membranes.

**Effects of Exogenous Glycans on Cellular Cohesion.** If intercellular cohesion is based on recognition by cell surface receptors of glycans on neighboring cell glycoproteins, an exogenous supply of the glycans should compete with the endogenous ligands and prevent establishment of stable contacts between cells. The assay is based on the ability of cohesive cells to agglutinate in gently shaken suspensions. For vegetative cells, endogenous cohesion is inhibited by EDTA, whereas cohesion of aggregation-stage cells is resistant (3). Therefore, agglutination of vegetative cells was scored in the absence of EDTA and agglutination of later stages was scored in the presence of 10 mM EDTA, though this reduces the rate and extent of agglutination.

Fig. 2 *A–C* shows the effects of 5 cell equivalents (see *Materials and Methods*) of exogenous glycan fractions from vegetative (0-hr), aggregation (8-hr), and late-aggregation (13-hr) stage cells on agglutination of cells from the same stages. The 8-hr endo H-resistant glycopeptides strongly inhibited the agglutination of 8-hr cells (Fig. 2*B*). The results of three separate experiments with such glycopeptides are shown to emphasize that inhibition is always observed under this condition. At 40 min, 60% of the control cells had agglutinated, but only 0–15% of cells had agglutinated in the presence of these glycopeptides.

In contrast, none of the glycans from 0-hr cells inhibited agglutination of 0-hr cells (Fig. 2*A*), and the same was true for combinations of 13-hr cells and 13-hr glycan fractions (Fig. 2*C*). With 8-hr cells, there was no inhibition by the endo H-sensitive oligosaccharides or the polysaccharide

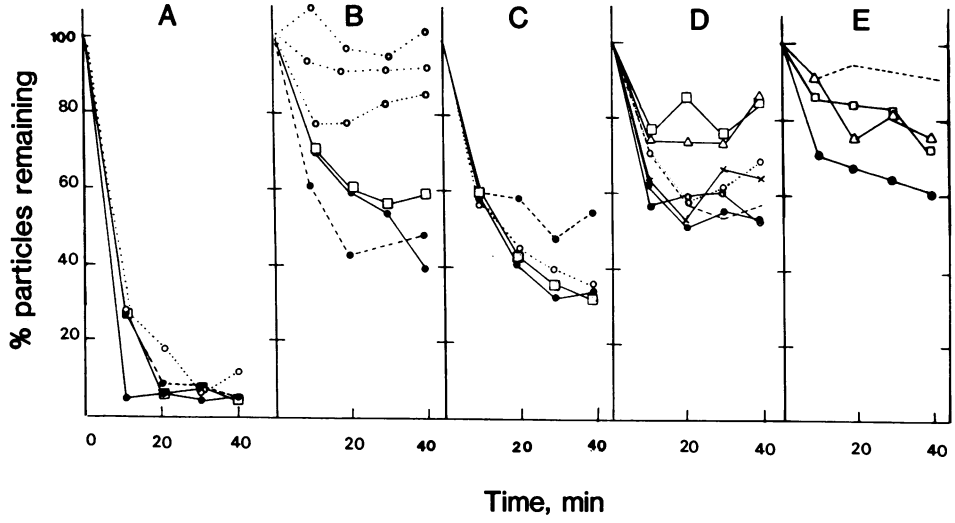


FIG. 2. Effects of pooled glycan fractions on cohesion. In *A*, *B*, and *C*, respectively, $10^8$ cells from 0, 8, or 13 hr were tested with 5 cell equivalents of glycans from the same stage. All assay mixtures except those in *A* contained 10 mM EDTA. In *B*, three results for endo H-resistant glycopeptides are shown. These were obtained with three different sets of 8-hr starved cells, and a different 8-hr plasma membrane preparation was used as a source of glycans in each case; agglutination of control cells without added glycans was the same in the three experiments. The symbols for *A–C* are ●—●, control; ●---●, polysaccharide; ○·····○, endo H-resistant glycopeptides; □—□, endo H-released oligosaccharides. In *D* and *E*, 1.25 cell equivalents of each purified gel band glycopeptide was added to 5 ml (*D*) or 10 ml (*E*) of cells at $10^7$ per ml. In *D*, one control contained an amount of Tris borate equivalent to that eluted from a gel band. For E, the eluted bands were purified by gel filtration, lyophilized, and redissolved in buffer prior to use. The symbols for *D* are ●—●, control; ----, control + Tris borate; ×—×, band 1; ○·····○, band 2; △—△, band 3; □—□, band 4; ○—○, band 5. The symbols for *E* are ●—●, control; △—△, band 3; □—□, band 4; ----, bands 3 + 4.

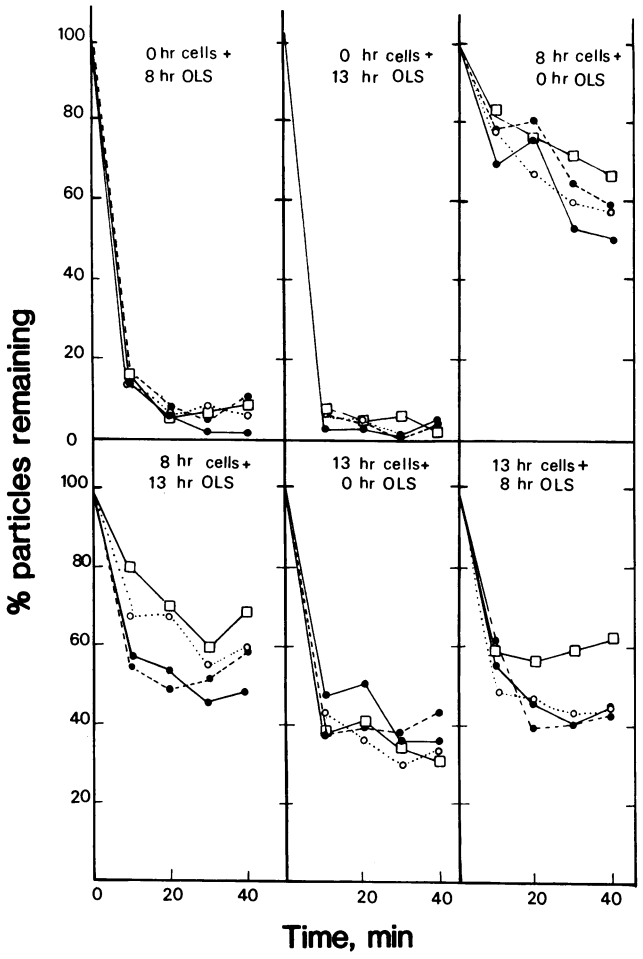


FIG. 3. Developmental stage specificity. In these experiments, 5 cell equivalents of glycan pools (OLS, oligosaccharides) isolated from plasma membranes of cells at one stage of development was tested in 10 ml with $10^8$ cells from other stages. The specific combinations are shown in the individual panels. All assay mixtures contained 10 mM EDTA except those with 0-hr cells. The symbols in each panel are ●—●, control; ●---●, polysaccharide; ○·····○, endo H-resistant glycopeptides; □—□, endo H-sensitive oligosaccharides.

fraction. Only the endo H-resistant glycopeptides were inhibitory with 8-hr cells (Fig. 2*B*).

The data in Fig. 2 suggest small stimulatory (*B*) or inhibitory (*C*) effects of the polysaccharide. These effects were not reproducible. That is, the small effect was suggested only once in a minimum of three different experiments. By contrast, the inhibition of agglutination of 8-hr cells by 8-hr plasma membrane endo H-resistant glycopeptides has been observed in every case in a large number of experiments using 5 cell equivalents of glycopeptides. One cell equivalent of glycans did not inhibit in any case (not shown).

**Effects of Individual Glycopeptides.** The endo H-resistant glycopeptide fraction from 8-hr cells was resolved into 5 bands by electrophoresis in Tris borate buffer (Fig. 1*B*). Each band was eluted separately from the gel and tested for inhibition. The results are shown in Fig. 2*D*. Bands 3 and 4 caused inhibition, whereas the other bands did not. In this experiment, the cell number and assay volume were reduced by a factor of 2 and assayed with 1.25 cell equivalent of glycopeptide. Thus, the equivalent ratio of glycopeptides to cells was 2.5 compared to 5 in Fig. 2 *A–C*.

Chromatography of the eluted gel bands on Bio-Gel P4 columns gave very sharp peaks for bands 2, 3, and 4 (Fig. 1*C*), as opposed to the broad peak for the mixture (Fig. 1*A*), suggesting they may be homogeneous species (Fig. 1*C*), and also removed any contaminating Tris borate from the gel. Gel-filtered bands 3 and 4 were tested separately and together for agglutination inhibition (Fig. 2*E*). In this experiment, the amount of added glycopeptide was further reduced by a factor of 2, so that the equivalent ratio of glycopeptides to cells was 1.25. Bands 3 and 4 gave partial inhibition when added separately and almost complete inhibition when added together (where the equivalent ratio of glycopeptides to cells was 2.5). Bands 1, 2, and 5 did not inhibit when added at the same concentration (based on counts of eluted gel bands) as band 4 in this experiment (not shown).

**Developmental Stage Specificity.** The glycan fractions from each of the three developmental stages were tested against cells from the other two stages (Fig. 3). None of these combinations showed the inhibition seen with 8-hr glycopeptides and 8-hr cells. Partial effects suggested in some cases in Fig. 3 were not reproducible observations; that is, they were obtained only once among several experiments. The only consistently reproduced result has been the inhibition of agglutination of 8-hr cells by 8-hr endo H-resistant glycopeptides.

## DISCUSSION

Serological evidence indicates that the molecular elements of *D. discoideum* intercellular cohesion are distinct in vegetative, aggregation, and postaggregation stages. Antibodies capable of blocking these distinct cohesion systems have identified several glycoproteins that may be the mediators at different stages (5, 8–10), and some of the antigens are periodate sensitive, suggesting that carbohydrates may be involved. Further, inhibition of N-linked glycosylation by tunicamycin (17–19) inhibits both aggregation and appearance of EDTA-resistant cohesivity. Such observations suggest a role of protein-linked carbohydrate, but such a role could be indirect. For example, the sugars could be required to maintain an active conformation of the carrier protein.

The simplest interpretation of our results is that there is direct participation of N-linked oligosaccharides in recognition and binding between cells at the aggregation stage of development, presumably by interaction with one or more receptors. Those receptors could be part of the cohesion system itself or could regulate the activity or exposure of cohesion components. The receptors have structural specificity, as inhibition of agglutination was not observed with polysaccharide material or with endo H-sensitive oligosaccharides, and only two of the five endo H-resistant glycopeptides were inhibitory. Since no glycan fraction from 0- or 13-hr cells inhibited 8-hr cells, the expression of the inhibitory glycans is developmentally regulated. Further, since no glycan fraction, including the 8-hr endo H-resistant glycopeptides, was able to inhibit agglutination of 0- and 13-hr cells, it is unlikely that cohesion is based on a single type of receptor common to all stages. Therefore, the implication is that the receptors as well as the glycans are developmentally regulated.

A remarkable feature of the inhibition by 8-hr glycopeptides is the low dosage required to inhibit cell–cell association. Reproducible inhibition was observed with individual glycopeptide species recovered from only 1.25-fold more starting cells than cells in the assay. Further, purified plasma membranes are obtained in only 30–50% yield, a small portion of each column fraction is consumed in scintillation counting, and recovery from gels is 75–80%. Thus, the added glycopeptide is considerably less than the true 1.25 cell equivalent amount, and the assay is under dilute conditions compared to aggregation. These features suggest a very high affinity of the glycopeptide for putative receptors.

We do not know which structural features of the glycans are critical for their inhibition. All of the endo H-resistant

N-linked oligosaccharides in plasma membranes of early developing cells are high-mannose glycans. None of the five gel bands is phosphorylated (data not shown), but all five contain fucose (J. A. Boose and E.J.H., unpublished data). Mild acid hydrolysis under conditions highly selective for fucose removal renders these plasma membrane glycans sensitive to endo H (ref. 14; O. Prem Das and E.J.H., unpublished data). Therefore, the fucose is likely to be on core *N*-acetylglucosamine residues and is not likely to participate directly in cohesion, since noninhibitory glycopeptides are also fucosylated. The size shift after endo H digestion of the defucosylated glycans confirms the expectation, from the conditions of the Pronase digestion, that the peptide portion of the inhibitory glycopeptides is a few amino acids at most. Since the mass of the intact glycopeptides is only about 2500 daltons, inhibition through nonspecific steric hindrance is also very unlikely.

If core regions of the glycopeptides are not involved, substitutions of outer mannose residues must be critical. Peripheral substituents such as glucose (O. Prem Das and E.J.H., unpublished data), *N*-acetylglucosamine (20), or sulfate (21) would alter borate binding and electrophoretic mobility, thus explaining the generation of multiple gel bands from a pool of high-mannose structures. A key role for mannose would also be consistent with the reports that amoebae have a mannose receptor (22) and that digestion with $\alpha$-mannosidase, alone of several glycosidases tested, reduced mutual cohesion between the amoebae (23). Full structural analysis of the inhibitory glycopeptides, along with tests of effects of glycosidase digestions on their inhibitory capacity, is the next step in these studies.

We currently do not know what glycoproteins carry these glycans or might serve as receptors for them. Antibodies that block aggregation-stage cohesion have implicated two glycoproteins, a gp69-72 (10) and gp80 (8). The latter has been most fully characterized. There are two types of glycans on gp80: N-linked oligosaccharides, at least some of which are sulfated, and a glycan that is either O-linked or in an unusual N-linkage (21), which is eliminated in *modB* mutants (24). The latter glycan is immunodominant and no antibodies specific for the N-linked sulfated glycans have been reported. Antibodies to the *modB*-dependent glycan inhibit EDTA-resistant agglutination (12), and mutants lacking this glycan are severely impaired in EDTA-resistant agglutinability (24, 25). However, such mutants do aggregate and produce apparently normal fruiting bodies. Similarly, a mutant lacking any gp80 peptide determinants has impaired agglutinability but develops normally (26).

There are several possible interpretations of these results. One is that critical N-linked glycans are usually carried not only on gp80 but also on other cell surface glycoproteins. In this case, the loss of gp80 could make cohesion fragile under shear conditions in agglutination assays, but when cells aggregate on a two-dimensional unstirred surface the remaining glycans are sufficient. According to this model, gp80 is a carrier of these glycans rather than a receptor for them. Further, according to such a model, cohesion is not a single-component system but has multiple components, which reinforce each other. An alternative model is that gp80 is the receptor, and this is compatible with the report that a monoclonal antibody apparently against a peptide of gp80 can inhibit cohesion (7). The glycoproteins that carry and bind these oligosaccharides have not yet been identified.

The major assay routinely used to monitor cohesion is based on agglutination of cells in shaken suspensions, and the degree of shear will obviously effect this measure. Therefore, glycans that had no inhibitory effect may play a role in cohesion but one not observed in this assay. The assay is also limited in its ability to determine a precise role for implicated molecules. Cells in shaking suspension are presumably symmetrical and lack any substratum-induced cytoskeletal organization that could control the distribution and activity of membrane proteins. In real aggregation streams the EDTA-resistant contacts provide end-to-end (head-to-tail) cohesion, and the EDTA-sensitive sites provide lateral association of cells (27).

To further analyze the role of these glycans in true aggregation, we have selected mutants with temperature-sensitive defects in oligosaccharide processing (28). A number of these mutants are temperature sensitive for both aggregation and expression of EDTA-resistant agglutinability. Analyses of such mutants should help to clarify the exact contribution of glycans to aggregation-stage cohesion.

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