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The Turnover of a Tissue Specific Cell Surface Ligand Which Inhibits Lectin Induced Capping

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Ten-day-old embryonic chick neural retina release into the environment glycoprotein ligands which bind to homologous cells, inhibiting the lectin-induced redistribution of cell surface receptors. Material with identical activity is released from trypsin-dissociated neural retina cells that are allowed to repair in culture for 2 h and are then transferred to fresh medium. Release of ligand is inhibited by cytosine arabinoside, hydroxyurea, UDP, and EDTA, and is potentiated by $MnCl_2$. These data suggest that a glycosyltransferase reaction plays a critical role in the turnover of the cell surface ligand. Reactivation of enzymatically deglycosylated ligand solutions by intact cells provides further support for this hypothesis.

Release of ligand is also accompanied by a loss of the agglutinability of the cells by a tissue-specific component which accumulates in monolayer conditioned medium. Conditions which inhibit release maintain maximal agglutinability suggesting similar mechanisms mediate both processes.

Key words: capping of surface receptors, adhesive ligand, glycosyltransferase

Cell surface proteins accumulate in cell and tissue culture media, presumably due to the continual "shedding" or turnover of the cell surface (1). Our laboratory has been studying such components released from embryonic chick neural retina and cerebral lobe cell and tissue cultures. Our observations strongly suggest that among the components released are tissue specific ligands which can participate in the formation of intercellular adhesions (2, 3) and which inhibit the redistribution of a variety of cell surface receptors into "caps" on single cells freshly prepared by trypsinization (4).

While there has been a great deal of effort by our own laboratory and others (5, 6) at purification and characterization of the role of such "shed" components in intercellular adhesion, little or no information is available on the mechanism mediating release into the environment. To examine this process we have used trypsin-prepared single cells which were allowed to repair in culture; such cells have lost the ability to cap Concanavalin A

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(Con A) receptors (7). Capping ability is regained following transfer of the cells to fresh medium containing cycloheximide; furthermore, activity which inhibits capping on freshly dissociated cells is concomitantly released into the medium. Quantitative evaluation of capping ability of cells and the amount of released ligand together with the effects of various chemicals suggest that release is accomplished by addition of a terminal sugar to the ligand via a glycosyltransferase reaction at the cell surface.

Repair of neural retina cells in culture is also accompaned by an increase in their agglutinability by a tissue-type specific component which accumulates in serum-free monolayer cultures (3). The same conditions which prevent release of ligand and the regaining of capping ability also prevent loss of agglutinability. Therefore, either the same component mediates both processes, or the various components are turned over by similar mechanisms.

MATERIALS AND METHODS

Cell Preparation and Repair

Trypsin dissociated 10-day-old chick neural retina cells were prepared as described previously (7). 2×10^7 cells were allowed to repair in still culture at 37°C for 2 h in 60-mm Falcon bacteriological dishes in 4 ml of Eagle's basal medium containing an additional 2 mg/ml glucose, 2% nonessential amino acids, 1 mM glutamine, and 50 μg/ml gentamycin (Schering Diagnostics, Union, New Jersey) under an atmosphere of 10% CO_2 in air.

Assay for Redistribution of Con A Receptors

Cells in suspension were assayed by methods previously described (4, 7) with the following modifications: 0.1 ml of cells (2.5×10^7 cells/ml) suspended in HBSG-CH (Hepes-buffered saline, pH 7.4, with 1 mg/ml glucose and 5 μg/ml cycloheximide) was added to 0.05 ml of FITC-Con A (fluorescent labeled Con A, Miles Laboratories, 100 μg/ml in HBSG) and 0.4 ml of HBSG containing various additives. Following 10 min at 0°C, the samples were incubated for 60 min at 37°C with intermittent shaking, pelleted for 5 min at 200 × g at 5°C, and resuspended in 0.4 ml of 2% glutaraldehyde in 0.02 M Na_3PO_4 (pH 7.4) at 5°C. The proportion of the cell populations exhibiting cap fluorescence was determined using a Zeiss Universal microscope modified for epifluorescence.

Assay for Activity of Released Material

To collect the released material (RM) 10^8 cells repaired as above were resuspended in 1 ml of HBSG-CH and incubated for 10 min at 37°C (release period). The cells were then pelleted at 200 × g at 5°C for 10 min. The supernatant was collected and recentrifuged for 30 min at 10,000 × g. The high speed supernatant was then dialyzed overnight at 5°C against 0.15 M NaCl–0.01 M Na_3PO_4 (pH 7.4). Protein concentration in the dialyzed RM was determined by the method of Lowry et al. (8) using crystalline bovine serum albumin as a standard. Capping inhibition activity was determined by incubating various concentrations of RM in 0.5 ml of HBSG-CH containing freshly dissociated neural retina cells (5×10^6 cells/ml) and FITC-Con A (5 μg/ml). Incubation conditions were as described above. Released material was considered active if it inhibited capping of Con A receptors by greater than 50% at a concentration of less than 30 μg/ml. Retina tissue culture supernatants inhibit capping by greater than 50% at a concentration of 5 μg/ml and maximally inhibit capping (80% inhibition) at a concentration of 25 μg/ml (see McDonough and Lilien, Ref. 4).

Preparation of Ligand and Deglycosylated Ligand

Ligand which inhibits the induced redistribution of Con A receptors was collected from tissue culture medium as previously described (4). Ligand-containing solutions were terminally deglycosylated by incubating 7 ml of the culture supernatant solution with 3 ml of 0.15 M sodium acetate (pH 4.2) containing 0.033 Units/ml of purified β-N-acetyl-hexosaminidase (Miles Laboratories, Turbo cornutus) for 30 min at 30°C, boiling for 10 min, and dialyzing overnight at 5°C against 0.01 M Na_3PO_4–0.15 M NaCl (pH 7.4). Boiled control preparations lacking enzyme were carried through the same procedure and retained greater than 95% of initial activity.

Fixed Cell Agglutination Assay (see Ref. 3)

Cells incubated at reduced temperature or in the presence of drugs during the release period were fixed in 2% glutaraldehyde (Ladd, EM grade) in 10 mM Na_3PO_4-buffered saline (0.12 M) (pH 7.4) for 30 min at 4°C. The cell suspension was then pelleted at 1,000 × g for 5 min at 4°C, resuspended in 0.2 M glycine in 10 mM Na_3PO_4 (pH 7.4), and incubated for 10 min at 22°C. Following 4 washes with 0.15 M NaCl – 0.01 M Na_3PO_4 (pH 7.4), aliquots of 10^6 cells/3 ml/35-mm Falcon dish were incubated at 70 rpm for 24 h in serum-free medium conditioned by monolayers of 10-day-old neural retina cells. Agglutination was scored by Coulter Counter determination of the number of single cells remaining.

Monolayers were prepared by aliquoting 2×10^7 freshly dissociated cells to 35-mm Falcon plastic tissue culture dishes in 4.0 ml of Eagle's basal medium containing an additional 2 mg/ml glucose, 2% nonessential amino acids, 1 mM glutamine, and 50 μg/ml gentamycin. After 24 h the medium was collected, centrifuged at 10,000 rpm for 30 min and made 2 mM with phenyl methyl sulfonyl fluoride in 2-propanol. This additive prevents the rapid loss of agglutination mediating activity (3). It should be stressed that this activity is distinct from the tissue-specific ligand which accumulates in organ culture conditioned medium previously described by our laboratory (11).

RESULTS

I. Characterization of the Release Process

Embryonic chick neural retina cells prepared by trypsinization are able to redistribute Con A receptors into caps (7). With increased time of repair in culture such cells progressively lose capping ability and by 2 h are unable to cap Con A receptors (7). When such cells are transferred to fresh medium containing cycloheximide (5 μg/ml) for as little as 10 min they regain completely their initial capacity to cap Con A receptors. That this reacquisition of capping ability is due to the release of surface associated macromolecules is demonstrated in 2 ways: 1) culture medium from the 10-min incubation period is active in inhibiting capping of Con A receptors on freshly dissociated neural retina cells, while medium collected from freshly trypsinized cells subjected to a similar 10-min incubation is inactive as is medium from repaired cells subjected to a second 10-min incubation period. 2) Treatments which prevent the reacquisition of capping ability also prevent the accumulation of capping inhibitory activity in the supernatant medium (see below).

We have established that there exists an intercellular pool of the capping inhibitory ligand which is mobilized to the cell surface in the presence of cycloheximide (8). In addition, release of surface-bound ligand does not occur until the entire pool has been

mobilized to the surface (8). Thus the amount of surface ligand is not affected by the presence of cycloheximide during the 2-h repair period. The presence of cycloheximide during the 10-min release period prevents further synthesis of surface ligands. Such synthesis reduces the capacity of the cells to cap Con A receptors but allows release of capping inhibitory material, altering the one to one relationship between capping ability and activity of the released material.

To further characterize the release process, various drugs, cations, and altered temperature were assayed for their effect during the 10-min release period following a 2-h repair period in the presence of cycloheximide. Table I summarizes these results. The data are normalized relative to the extent of capping recorded following a release period of 10 min at 37°C in medium containing only HBSG-CH. Low temperatures, cytosine arabinoside, and hydroxyurea all inhibit release while treatment with either cytochalasin B or puromycin has no effect. The effect seen with cholchicine is not due to inhibition of release, since colchicine inhibits capping and its effect is partially irreversible (7). Cells incubated with colchicine either during the repair period only or during the release period have a similar degree of inhibition of capping following the release period incubation. In all cases where release is inhibited no capping inhibitory activity is found in the supernatant medium.

These data suggest that a process which is inhibited by low temperatures, cytosine arabinoside, and hydroxyurea mediates release of the cell surface ligand. It has been shown that both cytosine arabinoside and hydroxyurea can inhibit the activity of glycosyltransferases (10). Experiments were therefore undertaken to test the hypothesis that glycosyltransferase activity is required for release of the cell surface ligand.

As above, cells repaired for 2 h in medium containing cycloheximide were harvested, washed, and incubated for 5 or 10 min at 37°C in HBSG containing various additives. After washing, the cells were assayed for the extent of capping. Since release appears to be maximal in 10 min it was necessary to reduce the release period to 5 min in order to test the effects of cations, as certain of them might be expected to accelerate the rate of a glycosyltransferase reaction. As shown in Fig. 1, neither calcium nor magnesium when present during release has any effect on the extent of capping after the release period. However, manganese dramatically increases the extent of capping, its effect being maximal at a concentration of approximately 5 mM.

Other compounds which might be expected to inhibit glycosyl transferase activity were tested during 10-min release periods. Ethylenediaminetetraacetic acid (EDTA, 0.005%) completely prevents release. Manganese ion at concentrations above 10 mM can overcome the inhibition caused by EDTA and stimulate activity while calcium and magnesium ions restore activity to the basal level.

Since nucleotides have been reported to affect both soluble (11) and membrane-bound (12) glycosyltransferases they were also tested for their effect on release. GDP, GMP, UTP, UDP, UMP, ADP, CMP, cyclic AMP, and dibutyryl cyclic AMP all inhibit release (Table II). However, UDP is approximately 1,000 times more effective in inhibiting the release process than GDP and ADP and approximately 10,000 times more effective than the other nucleotides tested.

These data support the contention that glycosyltransferase activity catalyzes the release of cell surface ligands into the surrounding medium. If release is mediated through glycosylation at the cell surface, repaired cells that have been stripped of ligand should retain the transferase at their surface, where it should be directly demonstrable. While ligand released from repaired cells inhibits capping of freshly prepared cells, it has little

TABLE I. Effects of Additives on Release of the Cell Surface Ligand

Treatment during release period[a]	% Caps following release period
Cycloheximide (5 μg/ml, CH)	100
CH + colchicine (20 μg/ml)	65 ± 10
CH + cytochalasin B (40 μg/ml)	107 ± 9
CH + NaN_3 (2.5 mM)	80 ± 6
CH, 22°C	75
CH, 4°C	0
CH + cytosine arabinoside (1 mM)	4 ± 8
CH + cytosine arabinoside (0.25 mM)	79
CH + hydroxyurea (1.3 mM)	7 ± 5
CH + hydroxyurea (0.65 mM)	32
Puromycin (10 μg/ml)	93

[a]The release period incubation was carried out for 10 min at 37°C except as indicated. Following the release period the cells were washed with ice-cold HBSG-CH and assayed for their extent of capping at 37°C as described in Methods.

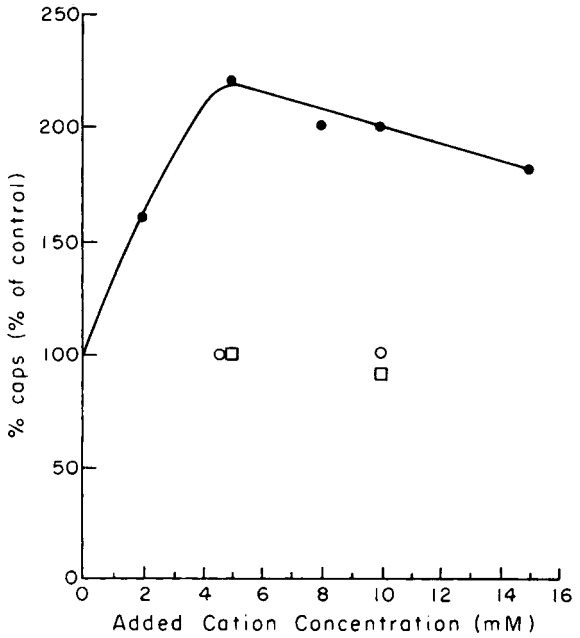

Fig. 1. Effects of different cations on release of cell surface material from 10-day-old chick neural retina cells. Cells were cultured for 2 h at 37°C in the presence of cycloheximide, harvested, washed with ice-cold HBSG/CH, then incubated for 5 min in HBSG-CH at 37°C containing various additions (10^8 cells/ml). The cells were then pelleted, washed with HBSG-CH, and assayed for their extent of capping of Con A receptors. Values shown are means from duplicates from 2 experiments. The control level of capping was that for a sample incubated with no additional ions. •) Mn^{2+}; ○) Ca^{2+}, □) Mg^{2+}.

TABLE II. Effects of Exogenously Added Nucleotides on Release of the Cell Surface Ligand

Nucleotide added	Effective concentration[a] (μM)
UDP	10^{-1}
UTP	10^{3}
UMP	10^{3}
GDP	10^{2}
GMP	10^{3}
CMP	$> 10^{3}$
ADP	10^{2}
Cyclic AMP	10^{3}
Dibutyryl cyclic AMP	10^{3}

[a]Effective concentration is considered to be the lowest concentration of nucleotide which inhibits by greater than 90% the release of cell surface material that restricts capping of Con A receptors. Nucleotides were added only to the release period incubation medium. Following the release period the cells were washed with ice-cold HBSG-CH and assayed for their extent of capping at 37°C. Results are expressed as the means of duplicate samples from 2–3 separate experiments.

effect on the repaired stripped cells. Conversely, ligand solutions treated with β-N-acetylhexosaminidase inhibit capping on repaired, stripped cells but not on freshly prepared cells (McDonough and Lilien, unpublished). Therefore, if there is a glycosyltransferase active during release of ligand, then repaired, stripped cells should catalyze reglycosylation of hexosaminidase-treated ligand, reactivating it with respect to freshly prepared cells and inactivating its ability to inhibit capping of repaired, stripped cells.

The results of these experiments are shown in Table III. In the absence of any added deglycosylated ligand the cells release into the medium material which can only inhibit capping minimally. Similarly when cells are incubated with deglycosylated ligand, $MnCl_2$, and 1 μM UDP (which inhibits release and therefore should inhibit the transferase) a small amount of activity is seen. However, when the cells are incubated with $MnCl_2$ and deglycosylated ligand alone the resultant medium contains capping inhibition activity for freshly trypsinized cells while that for 2-h cultured cells is much reduced. The presence of capping inhibition activity in the medium requires an interaction between deglycosylated ligand and some cell surface component(s). The supernatant medium from cells was unable to reactivate deglycosylated ligand in a 10-min incubation at 37°C. Moreover, treatment of the reactivated ligand with purified β-N-acetylhexosaminidase reduces capping inhibition activity to the initial minimal level (Table III). In addition, reactivated deglycosylated ligand solutions do not inhibit capping on freshly dissociated 10-day-old chick cerebral lobe cells. Thus the specificity of the reactivated ligand mimics that previously reported for tissue culture conditioned medium (4).

The observation that 2-h repaired and stripped cells will reactivate deglycosylated ligand prompted us to determine whether prior to the repair period such activity was also present. The ability of fresh, trypsin-prepared single cells to reactivate deglycosylated ligand was compared to 2-h repaired, stripped cells. The results are illustrated in Fig. 2; freshly prepared single cells lack the ability to reactivate deglycosylated ligand. Similarly, 2-h repaired, stripped cells subjected to a brief trypsinization also lack activity. Furthermore, such trypsin-treated repaired cells are no longer subject to inhibition of cap formation by deglycosylated ligand.

TABLE III. Reactivation of Deglycosylated Ligand*

Activity of ligand preparations prior to incubation with cells	% Capping inhibition	
	Freshly trypsinized cells	2 h cultured cells
Glycosylated ligand	68 ± 4	10
Deglycosylated ligand	30 ± 6	59
Reactivation of deglycosylated ligand		
Cells alone	26 ± 5	14
Cells + deglycosylated ligand (75% vol/vol) + 1 μM UDP	25 ± 15	–
Cells + deglycosylated ligand (75% vol/vol)	62 ± 7	23
Supernatant from cells following 10 min at 37°C + deglycosylated ligand (75% vol/vol) incubated 10 min at 37°C	30	–
Cells + deglycosylated ligand (75% vol/vol) 10 min at 37°C followed by treatment of supernatant with β-N-acetylhexosaminidase[a]	30 ± 8	55 ± 5

*Cells were incubated for 2 h at 37°C in medium containing cycloheximide (5 μg/ml), harvested, incubated for 10 min at 37°C in HBSG-CH and washed with cold HBSG. Cells were then incubated in HBSG-CH, 5 mM $MnCl_2$ containing the indicated additives in a total volume of 1 ml (10^8 cells/ml). After 10 min at 37°C the cells were pelleted at 5°C for 10 min at 200 × g and the supernate collected and assayed (at 80% vol/vol) for its Con A induced capping inhibition activity on 10-day-old neural retina cells either immediately following dissociation or after incubation for 2 h in medium containing cycloheximide followed by a release period. Values shown are mean ± standard deviation from 3–5 separate experiments.

[a]The release period medium was treated for 30 min at 30°C with purified β-N-acetylhexosaminidase (Miles Laboratories, Turbo cornutus), boiled for 10 min, and dialyzed overnight against 0.01 M Na_3PO_4-0.5 M NaCl (pH 7.4).

II. Agglutinability of Fixed Cells

During the 2-h repair period, cells coordinately lose the ability to cap lectin receptors and gain agglutinability (3). To characterize this relationship trypsin dispensed single cells were allowed to repair for 2 h in the presence of cycloheximide. The cells were then transferred to HBSG-CH containing the various affectors of release. Following the 10-min release period cells were fixed and tested for agglutinability by the tissue specific component of monolayer conditioned medium. Table IV shows that agglutinability is lost following a 10-min release period. Agglutinability is maintained under conditions which inhibit release and, therefore, prevent acquisition of capping ability.

DISCUSSION

Embryonic chick neural retina cells release into the environment a cell surface ligand which restricts the capping of lectin receptors. The data are consistend with the notion that release is mediated by the enzymatic addition of a terminal sugar to the ligand.

The ion specificity of release and inhibition of release by cytosine arabinoside, hydroxyurea, and UDP, are consistent with known properties of glycosyltransferases. However, it is the restoration by intact cells of biological activity of the enzymatically deglycosylated ligand which is most convincing. This interpretation of the mechanism of release is further strengthened by the result that after digestion with purified β-N-acetyl-

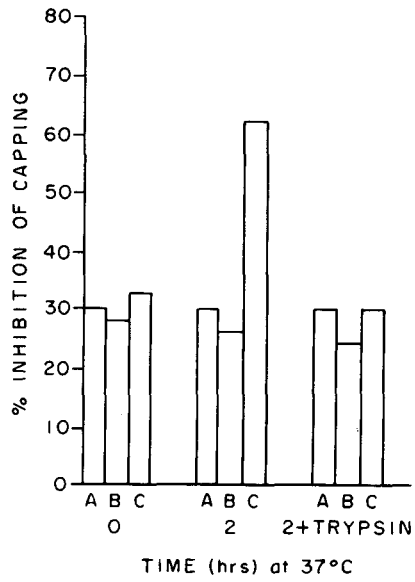

Fig. 2. Reactivation of deglycosylated ligand on interaction with cells. Freshly dissociated cells (0 time), and cells incubated for 2 h in the presence of cycloheximide (5 μg/ml) with or without subsequent trypsin treatment (4,000 National Formulatory Units/10^8 cells/ml, 3x crystallized trypsin, Miles Laboratories) were harvested, incubated in a total volume of 1 ml (10^8 cells/ml) of HBSG containing 5 mM $MnCl_2$ with or without deglycosylated ligand (75% vol/vol). After 10 min at 37°C the cells were pelleted at 5°C for 10 min at 200 × g and the supernate was collected and assayed (80% vol/vol) for its ability to inhibit Con A induced capping on freshly dissociated 10-day-old chick neural retina cells [1]. Results shown are the mean of duplicates from 2 experiments. A) DL solution alone; B) supernate from cells + 5 mM $MnCl_2$; C) supernate from cells + 5 mM $MnCl_2$ + DL.

TABLE IV. Agglutinability of Fixed Cells Following Release

Treatment during the 10-min release period	% of maximal[a] agglutination
No release	100
Cycloheximide (5 μg/ml, CH)	15
CH + NaN_3 (2.5 mM)	27
CH, 22°C	65
CH, 4°C	82
CH + cytosine arabinoside (2 mM)	70
CH + hydroxyurea (1.3 mM)	79
CH + UDP (1 μM)	71
CH + GDP (1 μM)	26

[a]Experimental variation is within 10% of the expressed values.

hexosaminidase the biologically reactivated ligand loses capping inhibition activity. Thus, with respect to its sensitivity to β-N-acetylhexosaminidase and its tissue specificity, the reactivated ligand is identical to the ligand originally released from the cell surface.

That UDP is the most effective nucleotide in inhibiting release of the retina ligand is consistent with the donor requirements for transferases catalyzing the addition of N-acetylgalactosamine, the previously identified terminal sugar of the retina ligand (4, 14). It is interesting that UDP is effective in inhibiting the glycosylation reaction at 0.1 μM, a concentration notably less than that observed for partially isolated or solubilized transferases (13). This extreme sensitivity may be due to the fact that the enzyme is in its native configuration at the cell surface. As far as we are aware the effect of nucleotides in other intact cell systems has not been examined so that no comparisons can be made.

In similar studies being conducted on cerebral lobe cells (Hermolin and Lilien, unpublished data), we have found that GDP is the most effective nucleotide in inhibiting release of the cerebral lobe ligand. The nucleotide specificity in this system is consistent with the donor requirements for mannosyltransferases and thus is also in agreement with our previous report that binding and inhibition of capping by the cerebral lobe ligand are α-mannosidase labile (14).

An obvious implication of these studies is that glycosyltransferase acts as a cell surface receptor for deglycosylated ligand, and that upon binding of deglycosylated ligand the ability of cells to cap lectin receptors is lost. This is consistent with release of ligand only under conditions which support catalysis and with reactivation of deglycosylated ligand by repaired, stripped cells. Further support for this hypothesis derives from the experiments showing that trypsinization of repaired, stripped cells destroys not only the capacity to reglycosylate but also sensitivity to inhibition of capping by deglycosylated ligand. However, fully glycosylated ligand both binds to fresh, trypsin-dispersed cells and inhibits capping among them. This observation suggests that there is a separate receptor for this form of ligand or that the receptor for deglycosylated ligand (transferase) has a trypsin-stable portion which recognizes fully glycosylated ligand.

In addition to ligand which inhibits lectin-induced cap formation, tissue culture conditioned media contains a component essential for the formation of intercellular adhesions (2, 3). That the 2 activities reside in the same molecule is suggested by the sensitivity of both capping inhibitory activity and agglutinability enhancing activity to β-N-acetylhexosaminidase. Furthermore, single cells acquire agglutination-mediating activity and lose capping ability concomitantly during repair (3). In addition our preliminary data indicate that both activities copurify. The data presented here are also indicative of an identity between the macromolecular species which mediate both processes. Agglutinability is lost following release and is retained under conditions which inhibit release.

By considering the data presented in the context of cell adhesion it is possible to integrate the notion that glycosyltransferases are involved in intercellular adhesion (15, 16) with the model previously proposed by our laboratory (2, 3). The model suggests that intercellular adhesions are mediated by 3 components: a cell surface receptor which interacts with the oligosaccharide moiety of the ligand and a third component which interacts with the polypeptide moiety of ligands on opposing cells to establish an adhesion. The data presented here suggest that the ligand receptor is a glycosyltransferase. While this model is substantially different from that proposed by Roseman (15) and Roth et al. (16) it does incorporate the virtue of known, highly specific enzyme-substrate interactions as partial determinants of adhesive specificity.

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