Hydration and the Lamellar to Hexagonal II Phase Transition of Phosphatidylethanolamine†

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ABSTRACT: The effects of chaotropic agents on the lamellar to hexagonal II phase transition of soy phosphatidylethanolamine were examined. Guanidine hydrochloride, urea, and NaSCN were used as chaotropic agents. In each case, the lamellar phase was stabilized by the presence of the chaotropic agent. In the case of NaSCN, the temperature of the lamellar to hexagonal phase transition of soy phosphatidylethanolamine was increased by more than 60 °C. Guanidine hydrochloride was capable of substantially reducing the aggregation of phosphatidylethanolamine vesicles. These data lead to a thermodynamic understanding of the lamellar to hexagonal phase transition.

Phospholipids such as phosphatidylcholine spontaneously form lamellar phases in aqueous media. The bilayers of pure phospholipid lamellar phases are similar to the dominant bilayer structure found in biological membranes. Not all phospholipids found in cellular membranes spontaneously form lamellar phases, however. Notable among such phospholipids is phosphatidylethanolamine. For aqueous dispersions of pure phosphatidylethanolamine or of mixtures of lipids enriched in phosphatidylethanolamine, the lamellar phase is frequently not stable. Instead, the phospholipids can adopt the hexagonal II phase, which consists of phospholipids arranged in a tube surrounding an aqueous cavity. The phospholipid head groups face the inside of the tubes. The characteristics of the lamellar to hexagonal phase transition have been reviewed (Cullis & de Kruijff, 1979).

In an earlier paper (Sen et al., 1982), it was stated that the organization adopted by any given lipid, or lipid mixture, in aqueous dispersion is determined by the balance of forces existing between the molecules of the system. These can be divided into those arising from lipid–lipid, lipid–water, and water–water interactions.

An important question that arises is the following: Why the inclusion in biological membranes of hexagonal phase forming lipids? Since the hexagonal phase is not likely to be formed in cellular membranes, is the role of these lipids contained in their tendency to form the hexagonal phase or in some other property? A related question concerns the driving force for the formation of the hexagonal phase. It has been suggested that hexagonal phase formation is a property of the molecular shapes of the molecules involved (Israelachvilli et al., 1980).

The purpose of this study is to examine factors involved in the formation of the hexagonal phase by phosphatidylethanolamine. In the process, clues to the role of lipids like phosphatidylethanolamine in membranes have been obtained.

Materials and Methods

All phospholipids, including transphosphatidylated (from egg phosphatidylcholine) phosphatidylethanolamine and soy phosphatidylethanolamine, were obtained in pure form from Avanti Polar Lipids, Birmingham, AL. Octyl glucoside was obtained from Calbiochem. Phosphate was assayed by the method of Bartlett (1959), as modified (Litman, 1973). Guanidine hydrochloride was obtained from Fisher Scientific.

Preparation of Vesicles. Lipids were dissolved in chloroform to ensure complete mixing. Chloroform was evaporated under a stream of nitrogen gas and then under high vacuum. The dry lipid film was then hydrated and vortexed to suspend the lipid. These preparations were used for the NMR measurements, X-ray measurements, and freeze–fracture electron microscopy. Large unilamellar vesicles were formed by octyl glucoside dialysis at 4 °C as described previously (Yeagle et al., 1982). The large vesicles were further purified by centrifugation in a 50 rotor using a Beckman L5-50 ultracentrifuge. These vesicles were centrifuged at 45000 rpm for 15 min, the supernatant was completely removed and finally the pellet was resuspended in the same buffer. These preparations were used for the vesicle aggregation measurements.

X-ray Diffraction. X-ray diffraction studies were performed by using a Jarrel-Ash fine-focus X-ray generator and a Frank's type camera. Lipid dispersions were placed in thin-walled glass capillaries, and the sample temperature was controlled by a thermoelectric device. Diffraction patterns were recorded on Kodak X-ray film.

^{31}P Nuclear Magnetic Resonance (^{31}P NMR). ^{31}P NMR spectra at 109 MHz were obtained in 10-mm tubes with a JEOL FX270 Fourier transform spectrometer. A fully phase cycled Hahn echo sequence was used to eliminate spectral distortions, as previously described (Rance & Byrd, 1983). Data were collected prior to refocusing of the echo and transformed from the point of focusing of the echo. No first-order phase corrections were employed. The decoupler was gated on only during acquisition to prevent sample heating; 50-kHz spectra were obtained. Repetition rates of 1 s were used and 2048 data points collected. About 2000 free induction decays were collected for each spectrum. The samples

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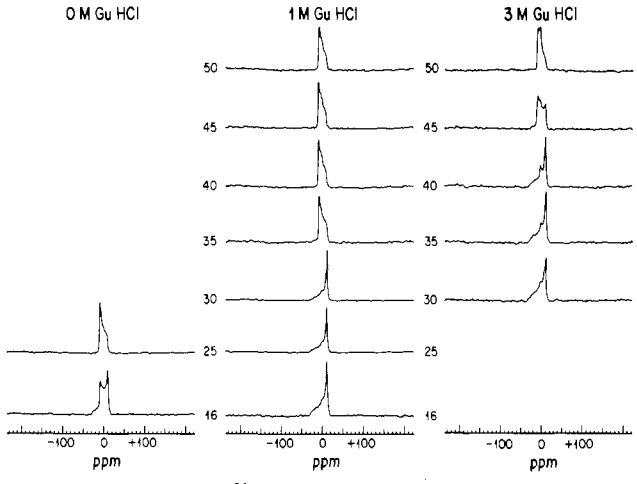

FIGURE 1: 109-MHz ^{31}P NMR spectra of soy phosphatidylethanolamine unsonicated aqueous dispersions in 10 mM histidine, 100 mM NaCl, and 1 mM EDTA, pH 7, as a function of temperature and guanidine hydrochloride (GuHCl) concentrations.

contained 50 mg of phospholipid in each case.

^{13}C Nuclear Magnetic Resonance (^{13}C NMR). ^{13}C NMR spectra were obtained at 67 MHz in 10-mm tubes with a JEOL FX270 Fourier transform (FT) NMR spectrometer; 4096 data points were collected over 15 kHz with a 2-s repetition rate. No more than 1-Hz line broadening was used in the FT calculations.

RESULTS

A number of different forms of phosphatidylethanolamine exhibit the transition from the lamellar phase to the hexagonal phase when the temperature of the dispersion is increased. Generally, the more unsaturated the fatty acyl chains, the more unstable is that phosphatidylethanolamine in the lamellar form.

^{31}P NMR provides an excellent means to monitor the phase structure of aqueous phospholipid dispersions. The lamellar to hexagonal II phase transition is manifest in the ^{31}P NMR spectra as a reduction (by half) of the expressed chemical shift anisotropy and an apparent reversal of the powder pattern. The spectral shape change arises from diffusion of the lipids around the hexagonal tubes. As seen in the left side of Figure 1, soy phosphatidylethanolamine undergoes the lamellar to hexagonal II phase transition at relatively low temperatures. This has been observed previously (Cullis & deKruijff, 1979).

The effects of guanidine hydrochloride, a chaotropic agent, on the lamellar to hexagonal II phase transition were examined. This experiment was tried because we hypothesized that the interaction between the aqueous phase and the membrane surface (and its hydration) might be important in the formation of the hexagonal II phase. Figure 1 shows ^{31}P NMR spectra which reflect the phase behavior of soy phosphatidylethanolamine as a function of temperature and guanidine hydrochloride concentration. The effect of guanidine hydrochloride is to increase the temperature at which the phase transition from the lamellar to the hexagonal II phase takes place. Furthermore, the greater the concentration of the chaotropic agent, the higher the temperature of the phase transition. Another way to state the observation is that the chaotropic agent in the aqueous phase stabilizes the lamellar phase structure for phosphatidylethanolamine.

The effect of guanidine hydrochloride on the aggregation of phosphatidylethanolamine vesicles was examined. Phosphatidylethanolamine (transphosphatidylated from egg phosphatidylcholine, and stable in the lamellar phase at 21 °C) vesicles were made by octyl glucoside dialysis as described under Materials and Methods in 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, (EDTA), and 10 mM histidine, pH 7, buffer. These vesicles proved to be aggregated in clumps at 21 °C as seen in the phase-contrast light microscope. These vesicles phase separated macroscopically in the test tube into a compactly packed lipid phase and largely lipid-free phase. Addition of 5 M guanidine hydrochloride to these vesicles eliminated all evidence for aggregation. Under the light microscope, only individual vesicles were seen, with no evidence for the aggregated clumps seen in the absence of guanidine hydrochloride. Likewise, macroscopically no phase separation was observed. Intermediate concentrations of guanidine hydrochloride, including 1, 2, 3, and 4 M, exhibited intermediate behavior, directly dependent upon the guanidine hydrochloride concentration.

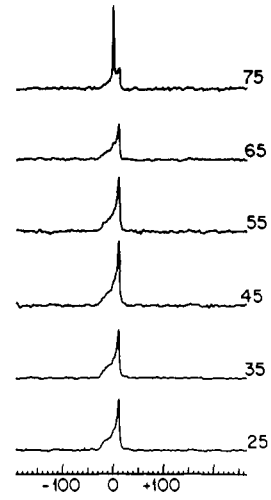

FIGURE 2: 109-MHz ^{31}P NMR spectra of soy phosphatidylethanolamine unsonicated aqueous dispersions in 10 mM histidine, 100 mM NaCl, and 1 mM EDTA, pH 7, as a function of temperature and the presence of 1 M NaSCN.

Table I: Low-Angle X-ray Diffraction Spacings of Soy Phosphatidylethanolamine at 35 °C in the Presence and Absence of 1 M NaSCN

	d (nm)	
diffraction	−NaSCN	+NaSCN
1	6.73	5.24
2	3.89	2.62
3	3.36	1.75
4	2.53	

If the stabilization of the lamellar phase of phosphatidylethanolamine is truly due to the chaotropic properties of guanidine hydrochloride, then chaotropic agents of different chemical structure and charge should have the same effect. This was tested.

Figure 2 shows the effect of another chaotropic agent, NaSCN, on the phase behavior of soy phosphatidylethanolamine (the same lot number as that used in Figure 1). NaSCN stabilizes the lamellar phase even more effectively than guanidine hydrochloride. Note that with NaSCN, there is no hint of nonlamellar phase up to 65 °C, even though in the absence of NaSCN, a pure hexagonal II phase would exist as low as 25 °C. Similar results were obtained with urea (data not shown).

X-ray diffraction studies were conducted on soy phosphatidylethanolamine in the presence and absence of 1 M NaSCN to determine the phase of the lipid dispersions at 35 °C. The low-angle "*d*" spacings are given in Table I. In the absence of NaSCN, the reflections index on a hexagonal lattice. The diffraction pattern obtained from a dispersion of soy phos-

phatidylethanolamine with 1 M NaSCN is that of a lamellar phase lipid with a repeat distance of 5.24 nm. The X-ray data are thus in complete agreement with the ^{31}P NMR results. Measurements were not obtained at higher temperatures because of sample deterioration in the X-ray beam.

Freeze–fracture electron microscopy was used to verify the phase structure deduced from ^{31}P NMR and X-ray at selected points. The freeze–fracture electron micrographs confirmed the ^{31}P NMR and X-ray results (data not shown).

As a control, the behavior of each of the chaotropic agents was examined in the presence and absence of phosphatidylethanolamine bilayers at 25 °C. ^{13}C NMR was used, and the line width and resonance intensity were measured. All chaotropic agents were used at 1 M, and the phospholipid was at 60 mM, as in the case of the other measurements reported here.

If these chaotropic agents bound to the phospholipid surface, the binding would likely be characterized by low-affinity sites with substantial off rate constants. When a small molecule binds to the membrane surface, it experiences a dramatic increase in rotational correlation time. In the case of rapid isotropic motion and carbon–proton dipolar relaxation, the line width is directly proportional to the rotational correlation time:

$$\Delta\nu_{1/2} \propto K\tau_R$$

where $\Delta\nu_{1/2}$ is the half-height line width, τ_R is the rotational correlation time, and K is a set of constants describing the nuclei and internuclear distances. While this equation is not precise for the bound species, it nevertheless illustrates that a large increase in rotational correlation time will lead to a large increase in line width.

In the case of rapid exchange of the chaotropic agents between bound and unbound "sites", the observed line width would be a weighted average of the bound and unbound line widths:

$$\Delta\nu_{1/2,obsd} = f\Delta\nu_{1/2,b} + (1 - f)\Delta\nu_{1/2,u}$$

where f is the fraction bound and b and u refer to bound and unbound, respectively. This equation assumes no significant change in chemical shift, though qualitatively similar behavior obtains if there is a chemical shift change.

The ^{13}C NMR measurements of all of the chaotropic agents show no detectable change in line width due to the presence of phosphatidylethanolamine bilayers (data not shown). Furthermore, there is no change in resonance intensity which might occur in the unlikely event of tight binding. Therefore, the ^{13}C NMR data provide no evidence for appreciable binding of the chaotropic agents to the bilayer surface.

One of the reasons for using the three chaotropic agents was the difference in charge on the active species. If the positively charged species was attracted to the surface of the membrane, then the negatively charged species would likely not be attracted in the same manner. However, all three agents stabilized the lamellar phase.

^{31}P NMR powder patterns from phospholipid head groups in membranes are sensitive to the conformation of the head group because changes in conformation lead to changes in orientation of the chemical shift tensor with respect to the director for rotational diffusion (Thayer & Kohler, 1981). Binding of cations such as Ca^{2+} and La^{3+} to zwitterionic phospholipids, while weak, perturbs the chemical shift tensor as revealed in a change in the isotropic chemical shift (Hutton et al., 1977). ^{31}P NMR spectra of the phosphatidylethanolamine bilayers show normal axially symmetric powder patterns with and without the chaotropic agents. Therefore, the ^{31}P NMR data provide no evidence for binding of the chaotropic agents to the membrane surface.

Therefore, the effects reported here are likely different than those observed on protein stability and solubility by SCN^- in which the effects were attributed to binding of the ion to the protein (Arakawa & Timasheff, 1982).

One more control was performed. Triethanolamine would be expected to have an effect opposite to that of the chaotropic agents. Therefore, the effect of 1 M triethanolamine, pH 7, on the stability of the lamellar phase of phosphatidylethanolamine was tested. The effect was to reduce by about 5 °C the lamellar to hexagonal II phase transition temperature for phosphatidylethanolamine transphosphatidylated from egg phosphatidylcholine (data not shown).

Discussion

For some time, phosphatidylcholine and phosphatidylethanolamine have been known to have substantially different properties in aqueous suspension. Phase transitions from the gel state to the liquid-crystal state are generally about 20 °C higher for the ethanolamine derivative than for the choline derivative having the same hydrocarbon chains. More recently, considerable interest has been generated by the observation that phosphatidylethanolamine is unstable, under many conditions, in the lamellar phase and instead adopts the morphology of the hexagonal II phase. In contrast, phosphatidylcholine is stable under most conditions in the lamellar phase, and addition of a modest amount of phosphatidylcholine to a phosphatidylethanolamine membrane will stabilize that membrane in the lamellar structure (Cullis & de Kruijff, 1979).

What is the driving force for the formation of the hexagonal II phase by phosphatidylethanolamine? The difference in chemical structure of phosphatidylcholine and phosphatidylethanolamine is limited to the three methyl groups on the nitrogen in phosphatidylcholine, in place of the three hydrogens in phosphatidylethanolamine. Previously, it had been suggested that the head-group size of the phosphatidylethanolamine was significantly smaller than the head-group size of the phosphatidylcholine and that this encouraged packing of the ethanolamine head groups on the inside surface of the cylinders characterizing the hexagonal II phase (Israelachvilli et al., 1976, 1980). In the present report, the lamellar phase is stabilized simply by the addition of a chaotropic agent to the aqueous phase. This operation does not change the chemical structure (and therefore size) of the phospholipid head groups. Furthermore, it has recently been shown that increasing the size of the ethanolamine by addition of methyl or ethyl substituents to the C_2 of the ethanolamine destabilizes the lamellar sphase (Brown et al., 1986).

The observation that guanidine hydrochloride affects the aggregation of phosphatidylethanolamine vesicles in the lamellar phase provides an interesting clue. The data reported here on phosphatidylethanolamine suggest that the surface of a phosphatidylethanolamine bilayer is "hydrophobic" relative to the surface of a phosphatidylcholine bilayer. The hydrophobic nature is manifest in the aggregation of the surfaces of the phosphatidylethanolamine bilayers that excludes some of the water. In the presence of guanidine hydrochloride, this aggregation is disrupted. It is known that guanidine hydrochloride and urea act as disaggregants in systems where hydrophobic interaction is believed to be the main driving force for association (Franks, 1975). The effects on phosphatidylethanolamine phase behavior are analogous to the denaturation of proteins by guanidine hydrochloride, in which changes in the nature of the aqueous phase (by the chaotropic agent) make the hydrophobic portions of the protein more compatible

with the aqueous phase, negating the driving force of the hydrophobic effect stabilizing the native structure of the protein. The consequence is that the protein unfolds in the presence of guanidine hydrochloride (Tanford, 1968).

Why is the surface of phosphatidylethanolamine apparently hydrophobic (relative to phosphatidylcholine)? Consider the interaction of water with the surface of phospholipid bilayers. Phosphatidylethanolamine head groups bind significantly fewer water molecules than do phosphatidylcholine head groups (Sen et al., 1986a). Therefore, when a phosphatidylethanolamine bilayer surface interacts with the aqueous phase, a smaller percentage of the water molecules that must be ordered by the surface of the membrane are bound to the lipids. The ordering of the water molecules at the membrane surface contributes an unfavorable ΔS to the interaction of the surface with the aqueous phase. This may be partially compensated by a favorable ΔH associated with the binding of water molecules to the phospholipid head groups. In the case of phosphatidylethanolamine, such a ΔH compensation in the overall ΔG of the interaction of the membrane surface with the aqueous phase is less effective than in the case of phosphatidylcholine. Furthermore, the head groups of phosphatidylethanolamine are hydrogen bonded intermolecularly (Sen et al., 1986b). This will reduce the polarity of the ethanolamine head groups, much like hydrogen bond formation in α helices of proteins allows the otherwise polar amide bonds of those helices to exist in the hydrophobic interior of the protein or the hydrophobic interior of a membrane. The hydrogen bonding likely further contributes to the entropic problems arising from the interaction of the phosphatidylethanolamine surface with the aqueous phase.

A relatively hydrophobic surface of phosphatidylethanolamine bilayers offers an explanation for the aggregation of phosphatidylethanolamine bilayers since aggregation leads to an exclusion of some of the aqueous phase from the vicinity of the membrane surface. In addition, the data reported here provide important clues to the formation of the hexagonal II phase. Since the interaction of the phosphatidylethanolamine head groups with the aqueous phase is relatively unfavorable, the structure of the system will organize so as to minimize the contact of the head groups with the aqueous phase.

If the phospholipids are in the hexagonal II phase, the exposure of the head groups to the aqueous phase is reduced, compared to their exposure in the surface of a bilayer, because of close packing of the head groups on the inside of the hexagonal II tubes. Therefore, the formation of the hexagonal II phase can be explained on thermodynamic grounds, within the context of the hydrophobic effect (Tanford, 1980).

The data from the experiments with the chaotropic agents provide a good test of this thermodynamic analysis. If there are entropic problems associated with the interactions of the aqueous phase with a phosphatidylethanolamine surface, then changing the nature of the aqueous phase should change the stability of the lamellar phase. Chaotropic agents tend to disrupt water structure. Such a disruption would reduce the unfavorable entropic contributions to the ΔG of interaction of a phospholipid bilayer surface with the aqueous phase. The concepts discussed above would then predict that chaotropic agents should stabilize the lamellar phase. A dramatic stabilization of the lamellar phase by three different chaotropic agents is just what is observed.

Gruner (1985) has provided a model for the relationship between the lamellar and hexagonal phases based on the intrinsic radius of curvature (R_0) associated with a particular lipid and on packing constraints. The radius of curvature is small and positive for the hexagonal II phase and is very large in the nearly flat lamellar phases. Differences in R_0 in this model contribute to the differences in phase behavior of the lipids. When this nomenclature is used, the present data suggest that differences in R_0 arise from differences in the thermodynamics of the interaction with the aqueous phase of the surfaces created by the phospholipids.

It is interesting to reexamine the transmembrane distribution of phosphatidylethanolamine in small sonicated vesicles. It was found that phosphatidylethanolamine was distributed preferentially toward the inside surface of the vesicle (Litman, 1974). Because of the small radius of curvature of the sonicated vesicle, this distribution may result from the need to minimize the contact of the phosphatidylethanolamine head groups with the aqueous phase. Measurements have shown that the head groups are more tightly packed on the inside surface of the vesicle than on the outside surface of the vesicle (Yeagle & Martin, 1976; Schmidt et al., 1977) and the head groups are therefore less exposed to the aqueous phase on the inside. The aqueous phase itself is quite limited on the inside of the vesicle due to the small intravesicular volume involved.

More recently, we have studied the distribution of cholesterol between membranes of different lipid composition (Yeagle & Young, 1986). The results indicated that cholesterol preferentially partitions into a phosphatidylcholine membrane relative to a phosphatidylethanolamine membrane. This preferential partitioning can be completely defeated by measuring the partitioning in guanidine hydrochloride. Since cholesterol binds even fewer water molecules than does phosphatidylethanolamine (Sen et al., 1986a), inclusion of cholesterol in the phosphatidylethanolamine membrane would lead to a membrane surface even more poorly structured for interaction with the aqueous phase than the pure phosphatidylethanolamine membrane surface. Thus, the model presented here also explains these cholesterol partitioning data.

A transition from a lamellar phase to a hexagonal phase would presumably be highly disadvantageous to the functioning of most cellular membranes. One of the primary responsibilities of a biological membrane is to create a semipermeable barrier, passage through which is carefully controlled by the properties of the membrane. Formation of a hexagonal phase by a membrane would destroy the permeability barrier, which in many cases would be lethal to the system. In fact, available evidence indicates that biological membranes exist primarily in the bilayer form.

Therefore, a role for phosphatidylethanolamine in cell membranes may be in the control of the nature of the hydration of the membrane surface. This may be important in the close approach of two membrane surfaces as in fusion. It may also be important to the interaction between membrane proteins (both integral and peripheral) and the bilayer surface.

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Membrane Biogenesis in Embryonal Carcinomas: Glycoproteins Destined for the Cell Surface Are Delayed in a Pre-Golgi Compartment†

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ABSTRACT: Embryonal carcinoma and early embryonic cells assemble a family of unusually large and complex carbohydrates. These glycans are highly branched, repeating copolymers of the sugars galactose and *N*-acetylglucosamine, referred to as polylactosamines, and are frequently decorated with fucose, sulfate, and sialic acid. We have previously shown that in teratocarcinoma cells these glycans are part of a large spectrum of glycans assembled on mannose cores derived from a common precursor glycan. Metabolic studies revealed a large excess of high-mannose glycans at a time when complex-type glycans cease to accumulate. The present studies demonstrate that these high-Man glycans are not degraded internally or secreted directly but are on glycoproteins destined for the cell surface. These unprocessed glycoproteins replace material lost during the extensive membrane turnover that occurs in these cells. Their export to the cell surface is delayed in a pre-Golgi compartment.

Murine teratocarcinoma stem cell lines resemble the cells of the inner cell mass of the preimplantation mouse embryo in their biological (Kleinsmith & Pierce, 1964; Pierce et al., 1964; Brinster, 1973; Mintz & Illmensee, 1975; Papaioannou et al., 1975), serological (Artzt et al., 1973), and biochemical (Bernstine et al., 1973) properties. A class of unusually large and complex carbohydrates has been identified on teratocarcinoma stem cells and on the early embryo (Szulman, 1964; Muramatsu et al., 1978, 1980). These carbohydrates are progressively lost during development (Szulman, 1964; Muramatsu et al., 1978, 1980). These glycans have been examined (Fukuda et al., 1985; Muramatsu et al., 1983) and appear to have structural characteristics in common with erythroglycan—a polylactosamine-containing glycan on the surface of red blood cells (Finne et al., 1978; Jarnefelt et al., 1978; Fukuda et al., 1979).

Several observations have implicated these developmentally programmed carbohydrates as an adhesive system for teratocarcinoma stem cells. In particular, Grabel et al. (1979, 1981, 1983) have demonstrated a cell surface carbohydrate-binding component from teratocarcinoma stem cells that has apparent specificity for fucose-rich glycans, Oppenheimer and Humphreys (1971, 1975) have identified an adhesive factor that requires terminal galactose, and Shur (1982, 1983) has demonstrated a cell surface galactosyltransferase that will bind polylactosaminoglycans that are either on other cells or immobilized on a substrate. Our own studies demonstrated that embryonal carcinomas can recognize a subset of their own carbohydrates—specifically branched, sulfated polylactosamines—and have implicated these glycans in cellular adhesiveness.

We investigated the assembly of glycoproteins in embryonal carcinomas, paying particular attention to the biosynthesis of the polyactosamine-containing glycoconjugates described above. Complex-type glycans assembled on mannose cores are the products of a long and complex biosynthetic pathway (Elbein, 1979; Parodi & Leloir, 1979; Spiro & Spiro, 1979; Kornfeld & Kornfeld, 1980; Struck & Lennarz, 1980; Hubbard & Ivatt, 1981). These glycan products are the result of competition between rival glycosylation pathways (Paulson et al., 1978; Beyer et al., 1981; Ivatt, 1981) for a common acceptor glycan derived by extensive trimming from the lipid-linked precursor glycan. We identified a lipid-linked precursor glycan, identical in properties to the one described for fi-

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