A NOVEL FUCOSYLATION PATHWAY IN THE CYTOSOL OF *DICTYOSTELIUM DISCOIDEUM*

By

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A mis padres
(To my parents)

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LIST OF ABBREVIATIONS

APA	Asparagus Pea Agglutinin
ATP	Adenosine-5'-triphosphate
BSA	Bovine Serum Albumin
^{14}C	Radioactive Carbon
cDNA	Complementary Deoxyribonucleic Acid
cm	Centimeter(s)
Con A	Concanavalin Agglutinin
cpm	Counts per Minute
CHO	Chinese Hamster Ovary
dH_2O	Distilled Water
dpm	Disintegrations per Minute
EDTA	Ethylenediaminetetraacetic Acid
EtOH	Ethanol
GlcNAc	N-acetyl Glucosamine
GDP	Guanosine-5'-diphosphate
h	Hour(s)
^{3}H	Tritiated
HMG	High Mobility Group
HPLC	High Performance Liquid Chromatography
kD	Kilodaltons
K_m	Michaelis Constant
l	Liter(s)

M	Molar Concentration
mCi	Millicurie(s)
mAb	Monoclonal Antibody
MES	2-(N-Morpholino)ethanesulfonic Acid
mg	Milligram(s)
ml	Milliliter(s)
min	Minute(s)
mm	Millimeter(s)
mM	Millimolar
MW	Molecular Weight
μCi	Microcurie(s)
μM	Micromolar
nm	Nanometers
p	probability
PAGE	Polyacrylamide Gel Electrophoresis
pmol	Picomole(s)
PMSF	Phenylmethylsulfonyl Fluoride
PNGase F	Peptide N-glycosidase F
Rev	Relative Elution Coefficient
RNAse B	Ribonuclease B
SDS	Sodium Dodecyl Sulphate
TCA	Trichloroacetic Acid
Tris	Tris(hydroxymethyl)aminomethane
U	Unit(s)
UEA-I	*Ulex europaeus* Agglutinin
Ve	Elution Volume

Vi	Inclusion Volume
V_{max}	Maximal Velocity
Vo	Void volume
v/v	Volume per Volume
WGA	Wheat Germ Agglutinin
w/v	Weight per Volume

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A NOVEL FUCOSYLATION PATHWAY IN THE CYTOSOL OF *DICTYOSTELIUM DISCOIDEUM*

By

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Chairman: Dr. Christopher M. West
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The existence of a fucosylation pathway in the cytosol of *Dictyostelium discoideum* was investigated in the glycosylation mutant strain, HL250, and the normal parental strain, Ax3. HL250 was characterized as a conditional mutant that cannot convert GDP(guanosine-5'-diphosphate)-mannose to GDP-fucose, resulting in a lack of macromolecular fucosylation unless grown in the presence of extracellular fucose. HL250 or Ax3 cells were metabolically labelled with [^{3}H]fucose, filter-lysed, and fractionated by high speed centrifugation into sedimentable (P100) and soluble (S100) fractions. The fractions exhibited unique profiles of fucoconjugates as analyzed by gel electrophoresis. The major acceptor in the S100 was a 21 kilodalton molecular weight protein, FP21. Analysis of FP21 oligosaccharide by mild alkaline hydrolysis and gel filtration chromatography

revealed that fucose was incorporated into an O-linked oligosaccharide with an average size of 4.8 glucose units. FP21 appeared to be endogenous to the cytosol, based on the failure to release FP21 from P100 vesicles by sonication, and the absence of FP21-like glycopeptides derived by pronase digestion of the P100.

To determine if FP21 was fucosylated in the cytosol, S100 and P100 fractions from HL250 were assayed for fucosyltransferase activity, measured as ability to transfer [^{14}C] from GDP-[^{14}C]fucose to endogenous acceptors. Fucosylation of FP21 by the S100 was time- and protein concentration-dependent. The cytosolic activity was distinguished from the bulk P100 activity by its absolute divalent cation dependence, alkaline pH sensitivity, and very low apparent K_m for GDP-fucose. Fucosyltransferase activity was not detectable in Ax3 cytosol. However, activity was reconstituted by addition of purified mutant FP21, suggesting FP21 was already fucosylated in living cells, and Ax3 possessed a cytosolic fucosyltransferase equivalent to the HL250 enzyme. S100 fractions from Ax3 and HL250 were able to fucosylate an α1,4fucosyltransferase-specific acceptor glycolipid analog, but not analogs capable of being modified by α1,2 and α1,3 fucosyltransferases. Since fucosylation of the analog was reduced by addition of purified FP21, the same enzyme appears to be responsible for fucosylation of both molecules. Thus it is proposed that

FP21 is synthesized and fucosylated in the cytosol by an α1,4fucosyltransferase.

CHAPTER I
HISTORICAL REVIEW AND BACKGROUND

Introduction

Glycoprotein synthesis and localization have been subjects of intense study in the last few decades. Much has been learned about glycosylation and some excellent reviews are available (Kornfeld and Kornfeld, 1985; Hirschberg and Snider, 1987). Carbohydrate moieties are usually categorized as N and/or O-linked depending on whether the carbohydrate moiety is attached to an asparagine by an amide glycosidic linkage or to a serine or threonine, respectively. Glycosylation is generally considered to be a modification restricted to macromolecules that pass through the secretory pathway, starting in the rough endoplasmic reticulum where N-linked glycosylation is initiated (Kornfeld and Kornfeld, 1985), but in some instances O-linked glycosylation also takes occurs (Spielman et al., 1988). Glycosylation then continues in the Golgi apparatus where further processing of N-linked oligosaccharides may occur and O-linked glycosylation takes place (Abeijon and Hirschberg, 1987). In the case of fucosylation, L-fucose has been shown to be added as a terminal modification to either N-linked or O-linked oligosaccharides in the Golgi

apparatus utilizing GDP-fucose as the sugar nucleotide donor (Bennett et al., 1974; Hirschberg and Snider, 1987). However, there are a few exceptions to these remarks, since fucose has been found to be attached directly to serine and threonine, although the site for this modification has not been identified (Hallgreen et al., 1975), and to be present in homopolymers, as in fucoidans (Flowers, 1981). It has been suggested that fucosylation also occurs in the endoplasmic reticulum as well as in the Golgi apparatus in thyrotrophs under different physiological conditions (Magner et al., 1986). There are two pathways for the biosynthesis of GDP-fucose. The main source of GDP-fucose is the conversion pathway of GDP-mannose to GDP-fucose (Yurchenco et al., 1978; Flowers, 1985). Alternatively, synthesis of GDP-fucose may occur by the fucose salvage pathway in the presence of extracellular L-fucose (Yurchenco et al., 1978; Flowers, 1985; Ripka and Stanley, 1986; Reitman et al., 1980).

Glycosylation is carried out by diverse specific glycosyltransferases which have been located in the endoplasmic reticulum and Golgi apparatus. One underlying assumption is that the acceptor macromolecule must colocalize with the transferase enzyme in order to be glycosylated. However, there have been occasional reports of glycoproteins in compartments topologically discontinuous with the lumen of the rough endoplasmic reticulum and Golgi

apparatus. These findings contradict the dogma that glycosylation is strictly an endoplasmic reticulum- and Golgi apparatus-dependent event. Two models could account for the existence of glycoproteins outside the realms of the secretory pathway; one postulates that lumenal glycosylated proteins translocate across the membrane back to the cytosolic space, the other that glycosyltransferases are localized outside of the lumen of the endoplasmic reticulum or Golgi apparatus. Evidence has been accumulating in the past decade that supports the latter model. In this chapter I shall be concerned with the presence of fucosylated proteins in compartments topologically discontinuous with the lumen of the endoplasmic reticulum and Golgi apparatus. Subsequently, I shall analyze some of the studies that suggest the presence of fucosyltransferases that would co-compartmentalize with such fucoproteins. In light of the results presented in this dissertation, a review focussing on the presence of fucoproteins and fucosyltransferases outside the secretory pathway will be useful. An excellent review is available that examines nuclear and cytosolic glycosylation in general (Hart et al., 1989a).

Fucosylated Macromolecules

The presence of fucosylated proteins in compartments discontinuous with the secretory pathway has been documented since the 1970's. Various techniques have been employed in

these reports, including the direct analysis of carbohydrate content, use of lectins, use of radiolabelled fucose and/or a combination of biochemical and morphological techniques.

Nuclear Fucosylated Macromolecules

In contrast to the prevailing dogma, fucosylated macromolecules have been detected in the nucleus. Such studies demonstrated lectin binding to nuclear membranes, chromatin, nuclear proteins, and the nuclear matrix. These studies generally involved localization by binding of labelled lectins (fluorescent, radiolabelled, ferritin-conjugated, etc.) to isolated nuclei. Binding specificity was assessed by competition of labelling with hapten inhibitors. Occasionally, these studies were supplemented by metabolic labelling experiments with radioactive fucose which is primarily incorporated into fucoproteins with little metabolism into other molecular species in eukaryotic cells (Yurchenko et al., 1978).

Nuclear membranes. One early report suggested the presence of fucose-containing structures on the cytoplasmic face of isolated bovine nuclei (Nicolson et al., 1972). Nicolson et al. (1972) found that purified nuclei were agglutinated by the L-fucose-specific lectin UEA-I from *Ulex europaeus* (Lis and Sharon, 1986), and agglutination was inhibited by incubation in the presence of L-fucose,

suggesting that there were fucose-containing membrane-bound oligosaccharides on the outer nuclear membrane. Similar results were obtained with the mannose- and glucose-specific lectin concanavalin A (Con A), which was also found to agglutinate purified nuclei (Nicolson et al., 1972). However, subsequent work by another group revealed that, as evidenced by electron microscopic examination, ferritin-Con A appeared to stain only damaged nuclei (Virtanen and Wartiovaara, 1976) raising some concerns about the studies by Nicolson et al. (1972). Likewise, in the aforementioned studies (Nicolson et al., 1972), integrity of the isolated nuclei was not determined, allowing for the possibility that the lectin was interacting with lumenal fucoproteins that escaped organelles during nuclei isolation or with contaminating fucoprotein-containing membranes, such as plasma membrane.

Chromatin. The existence of chromatin associated fucose-containing proteins has been suggested by several laboratories. Early on, Stein et al. (1975) reported the presence of [^{3}H] labelled glycoconjugates in purified chromatin from HeLa cells grown in the presence of [^{3}H]fucose. Although the label was not examined to corroborate its presence as fucose, in the case of radiolabelling macromolecules with radioactive sugar precursors, fucose is an excellent candidate because it has

been shown to be incorporated as such, with minimal metabolizing of the label (Yurchenko et al., 1978). In these studies the authors examined a very pure chromatin preparation, with essentially no contamination from nuclear or plasma membranes. The authors argued against plasma membrane contamination based on mixing experiments, in which radiolabeled cell-surface trypsinates were combined with unlabeled nuclear preparations prior to chromatin isolation. The fucosylated chromatin-associated macromolecules were deemed to be fucoproteins based on their sensitivity to pronase digestion. Unfortunately, these studies have not been pursued further, and many questions remain unanswered with respect to their structure and biosynthesis.

Chromatin-associated fucoproteins were also detected in normal rat liver and Novikoff hepatoma ascites cells (Goldberg et al., 1978) using the L-fucose-specific lectin asparagus pea agglutinin (APA). The authors reported a strongly basic fucoprotein, that was sensitive to pronase digestion. Based on the reactivity with the lectin, they calculated that the protein was three times more concentrated in tumor chromatin than in normal liver cells. However, the method for chromatin purification did not eliminate adequately nuclear inner membrane contamination, and since binding to the lectin was assayed by affinoelectrophoresis a molecular weight for the protein was not determined (Goldberg et al., 1978).

In a more recent study on duodenal columnar cells, Kan and Pinto da Silva (1986) used UEA-I conjugated to colloidal gold in freeze-fracture electron microscopy of cross-fractured nuclei. They compared binding of the conjugated lectin to euchromatin, heterochromatin, and nucleolus; compartments which can be differentiated ultrastructurally. Binding of UEA-I showed that colloidal gold particles were almost exclusively confined to cross-fractured areas where euchromatin was exposed. Labelling was abolished by pretreatment and incubation in the presence of L-fucose, as expected for a specific label. Pre-digestion of the fractions with trypsin also abolished labelling, suggesting the receptors for UEA-I binding were glycoproteins. The preferential binding to euchromatin may be of importance, because replication and transcription take place at euchromatin regions. Although the results reported are intriguing, the authors did not identify the type of fucose-containing proteins detected. DNA-associated proteins can be classified as either histone or non-histone proteins, and as summarized below, both types of proteins appear to be fucosylated.

Histones. The histones are the most abundant proteins associated with DNA. Histones are very basic proteins and are found in all nuclei (Darnell et al., 1986). Based on the specific binding to UEA-I, Levy-Wilson (1983) presented

evidence that histones isolated from the macronucleus of *Tetrahymena thermophila* appear to contain fucose. These results were strengthened by metabolic incorporation of [^{3}H]fucose into histones, which showed that all five *Tetrahymena* histones, H1, H2A, H2B, H3, and H4, appear to contain fucose, with H2A incorporating the highest amount (Levy-Wilson, 1983). In these studies macronuclei were isolated to high purity, and highly pure histone preparations were obtained after extensive washing in high salt to remove nonhistone proteins. Using Con A, the author showed specific binding to histones, which was interpreted as evidence for the presence of mannose residues. However, Con A has previously been shown to also recognize D-glucose residues. Based on the extent of fucose incorporation and its specific radioactivity, the author estimated, as the lowest estimate, that one in a thousand nucleosomes contained a fucosylated H2A molecule. To date, the glycosylation pathway of histones and the oligosaccharide structure(s) present in histones remain unknown.

Nonhistone proteins. High mobility group (HMG) proteins are fairly abundant nonhistone chromosomal proteins classified according to their relative electrophoretic mobilities (Darnell et al., 1986). HMG proteins undergo a variety of posttranslational modifications, including glycosylation. Since they appear to be preferentially

associated with actively transcribed DNA, it has been speculated that glycosylation may influence gene activity. Highly purified HMG14 and HMG17 from mouse Friend erythroleukemic cells were found to contain fucose, among other sugars, by direct composition analysis (Reeves et al., 1981). These proteins bound UEA-I specifically and could be metabolically labelled with [^{3}H]fucose. The oligosaccharides were largely insensitive to ß-elimination, suggesting an N-linkage to protein (Reeves et al., 1981). When purified HMG14 and HMG17 were digested with mixed glycosidases, the binding of HMG14 and HMG17 to the nuclear matrix was abolished. Even though they did not employ a fucosidase, making it difficult to ascertain the role of fucose in binding, it was evident that glycosylation influenced binding to the nuclear matrix (Reeves and Chang, 1983). These studies presented convincing evidence that HMG 14 and 17 are fucosylated, although they did not explore the site of modification or the composition of the oligosaccharide(s).

Nuclear fucoproteins and transcriptional activity. Since some glycoproteins have been found associated with DNA there has been speculation about a possible correlation between the state of nuclear glycosylation and transcriptional activity (Hart et al., 1989a). There are examples in the literature of glycosylated transcription

factors and, at least in one case, glycosylation may have influenced transcriptional activity (Lichtsteiner and Schibler, 1989; Jackson and Tjian, 1989). As mentioned earlier, in the case of fucosylated macromolecules, it was found that in Novikoff hepatoma cells chromatin had three times the amount of a fucoprotein of normal liver cells based on APA binding (Goldberg et al., 1978). However, the identity, size, or number of such proteins were not well documented. Putative fucoproteins were found preferentially associated with euchromatin (Kan and Pinto da Silva, 1986). Fucosylated histones were found in the macronucleus of *Tetrahymena*, where transcriptionally active chromatin is compartmentalized (Levy-Wilson, 1983). Nevertheless, histones from non-active chromatin were not studied, so no comparisons can be made with heterochromatin. Levy-Wilson (1983) suggested that the reason why other investigators have failed to detect fucosylation in mammalian histones is due to the low proportion of transcriptionally active genome which would imply a low concentration of fucosylated histones.

Although the data gathered in these reports are interesting, they are the result of isolated studies from diverse organisms and it is difficult to draw generalized conclusions. In addition, virtually nothing is known about the structure or biosynthesis of the fucose-containing moieties. Even though the fractions in all these reports

appeared to have almost no contamination, structural studies showing a different glycoconjugate from those found in other organelles would convincingly argue against contamination. Nevertheless, these provocative studies are encouraging and deserve to be pursued further.

Cytosolic Fucoconjugate

The existence of glycoproteins in the cytosol has been documented in the past and reviewed recently (Hart et al., 1989a). Studies suggesting the existence of glycoproteins in the cytosol were based on determinations of lectin binding sites or biochemical compositional analyses (Hart et al., 1989a). Some of the negative results reported by those studies, that relied on lectin binding as confirmation for the presence of a sugar residue, may be misleading since lack of binding may reflect a poor choice of lectins to probe with and not necessarily the absence of a fucose residue. While there are several fucose-binding lectins available that serve as useful biochemical tools, they do not recognize every possible fucose-containing structure. Lectin binding is dependent on a specific array of sugar residues, and does not depend solely on the presence of fucose (Lis and Sharon, 1986). However, as will be described below, there is one biochemical study that reports the existence of a cytosolic fucoconjugate. In many cell fractionation experiments, the cytosolic compartment is

defined by the lack of sedimentation during high-speed centrifugation. However, this criterion alone is not sufficient, since cytosolic glycans might arise from contamination by other organelles. The most convincing evidence describes fucoconjugates that appear to be preferentially enriched in the cytosol in relation to other compartments.

In studies in rat brain, a soluble proteoglycan that contains novel O-linked mannose-containing oligosaccharides was recovered in the cytosolic fraction (Margolis et al., 1976; Finne et al., 1979). The proteoglycan was characterized as a soluble chondroitin sulfate proteoglycan, that contained neutral oligosaccharides releasable by mild alkaline borohydride treatment. The oligosaccharides contained mannose at their proximal ends, and one oligosaccharide was proposed to be composed of mannose, GlcNAc, fucose, and galactose (Finne et al., 1979). However, the possibility remains that the oligosaccharides are not integral components of the proteoglycan, but were associated with other co-purified material. Nevertheless, the oligosaccharides appeared to be endogenous to the cytosol and not the result of contamination from other fractions, since they were present in only trace amounts in the microsomal or synaptosomal membrane fractions (Finne et al., 1979). Unfortunately, the function and biosynthetic pathway of these oligosaccharides remain unknown.

Fucose-Binding Proteins

The presence of fucoproteins in the nucleus and cytosol prompted the idea that there might be specific proteins inside the cell that bind, and/or modify these fucoproteins, as is the case with the fucoproteins in the secretory pathway. This would include fucose-binding proteins, fucosyltransferases responsible for fucosylation, and fucosidases responsible for fucose removal. There are some studies suggesting the existence of fucose-binding lectins and fucosyltransferases. However, there is no evidence for a nuclear or cytosolic fucosidase.

Endogenous Lectins

In light of evidence for glycoproteins in the cytosolic and nuclear compartments, investigators sought the existence of carbohydrate-binding proteins that would colocalize with such glycoproteins. Endogenous lectins have been detected in preparations of nucleoplasmic and/or cytosolic fractions of a variety of cells (Hart et al., 1989a).

Aided by fluorescein-labeled neoglycoproteins, Sève et al. (1986) have postulated the presence of endogenous fucose-specific lectins. Baby hamster kidney cell nuclei were isolated by two different procedures, cell lysis and enucleation in Ficoll, to argue against contamination by cytoplasmic or membrane-derived components. Using fluorescein-labelled BSA conjugated to fucose in the order

of 20±5 sugar units per molecule, fluorescence microscopy experiments suggested that the majority of the binding appeared to be associated with nucleoli and nucleoplasmic ribonucleoprotein elements (Sève et al., 1986). Interestingly, it was shown by quantitative flow microfluorometry that nuclei from exponentially growing cells bound one order of magnitude more fucose-BSA than nuclei from contact-inhibited cells. In spite of these results, the authors acknowledge that it is impossible based on the data to ascribe biological roles to the nuclear fucose-binding sites or to conclude that they influence cellular physiology (Sève et al., 1986).

In *Dictyostelium discoideum* a family of lectins, the discoidins, has been identified, and discoidin I has been the most extensively studied isoform. In erythrocyte agglutination assays, agglutination by discoidin may be inhibited by galactose, modified galactose residues, L-fucose, D-fucose, and other sugars, suggesting the existence of cell-surface sugar-dependent epitopes on erythrocytes recognized by Discoidin (Barondes and Haywood, 1979). Although discoidin I was originally thought to be a cell surface protein involved in cell-cell adhesion, it has since been established that it is primarily present in the cytosol (Erdos and Whitaker, 1983). Although galactose and modified galactose residues are the ligands bound by discoidin I with highest affinity, it is possible that a fucose-containing

macromolecule may serve as a ligand for it or that another lectin, possibly from the discoidin family, will be present in the cytosol with fucose-binding capability.

Fucosyltransferases

Intracellular fucosyltransferases identified to date, are localized in the lumen of the Golgi or possibly, endoplasmic reticulum. Thus the presence of fucoproteins in the nucleus and cytosolic compartments poses questions pertaining to the mode of synthesis and/or intracellular transport of such glycoproteins. Early on, Kawasaki and Yamashina (1972) theorized, based on metabolic labelling, that nuclear membrane glycoproteins were not synthesized in and transported from microsomes, but were made in the nuclear membrane or its vicinity. A few reports have suggested the presence of glycosyltransferases in the nucleus, nuclear membranes, or cytosol that may be involved in the modification of several glycoproteins; however, none of these glycoproteins was reported to contain fucose (Richard et al., 1975; Galland et al., 1988, Haltiwanger et al., 1990).

Though there is evidence for nuclear and cytosolic fucosylated macromolecules, to my knowledge there are no reports of nuclear or cytosolic fucosyltransferases. Louisot and collaborators reported the purification and separation of two soluble fucosyltransferase activities from

rat small intestinal mucosa (Martin et al., 1987). They report the isolation of α1,2 and α1,3/1,4fucosyltransferases that could be candidates for cytosolic fucosyltransferases based on their inability to sediment after homogenization of cells in 0.25 M sucrose, followed by 90 min, 200k x g centrifugation (Martin et al., 1987). They compare the α1,2fucosyltransferase with the α2,6sialyltransferase, which is normally a Golgi enzyme converted to a soluble form by cleavage of the amino terminal signal anchor to allow for secretion (Weinstein et al., 1987). In the case of the fucosyltransferases, the authors did not report using protease inhibitors during isolation, nor did they document the partitioning of cellular markers in the different fractions (Martin et al., 1987). The reasons the enzymes localize to the high speed supernatant, which would usually be considered as the cytosolic fraction, may include the breakage of the microsomal vesicles or the fact that they were initially present extracellularly in the body fluids of the mucosa. In another report these investigators tested for the presence of glycosyltransferases in the nucleus. In highly purified rat liver nuclei there was a total absence of fucosyltransferase activity when endogenous macromolecules or asialofetuin were used as acceptors (Richard et al., 1975).

One explanation for the lack of evidence of nuclear and/or cytosolic fucosyltransferases activity in any

organism may be that there indeed are no fucosyltransferases. Alternatively, if there are no cytosolic fucosyltransferases, the presence of fucoconjugates in the cytosol or nucleus may be explained by membrane-associated enzymes that face the cytosolic compartment, as has been reported for other glycosyltransferases (Haltiwanger et al., 1990). In addition, the lack of adequate acceptors, unfavorable conditions for in vitro activity, and the scarcity of sustained interest in the field, could account for the lack of evidence for such fucosyltransferase(s) that, just as is the case with other glycosyltransferases, are not of lumenal origin.

Evidence for fucosylation, and glycosylation in general, has been accumulating in the past decades. Until recently, only sporadic reports about fucoproteins appeared in the literature. With the identification of newly reported glycoconjugates (Hart et al, 1989b), interest has been revived in this area of research and currently there appears to be much interest in the field. However, a careful examination and sustained interest will be necessary in order to elucidate the structures, and modes of biosynthesis and functions of nuclear and cytosolic fucoconjugates.

CHAPTER II
CHARACTERIZATION OF A FUCOSYLATION MUTANT

Introduction

Since fucosylation comprises numerous steps which are potential sites for mutations, many fucosylation mutants have been obtained. Fucosylation consists of the synthesis of the sugar nucleotide donor GDP-fucose and the transfer of fucose from the GDP-fucose to an acceptor (Kornfeld and Kornfeld, 1985). The main source of GDP-fucose is the conversion pathway of GDP-mannose to GDP-fucose (Yurchenco et al., 1978; Flowers, 1985). Alternatively, synthesis of GDP-fucose by the fucose salvage pathway can occur in the presence of extracellular L-fucose, allowing cells defective in the conversion pathway to phenotypically revert (Ripka and Stanley, 1986; Reitman et al., 1980). Lesions may affect the formation of GDP-fucose, the transport of GDP-fucose to the fucosylation compartment, the transferases responsible for fucosylation and/or the biosynthesis or transport of the acceptor species to the fucosylation compartment. Two mutants deficient in protein-associated fucose were shown to be defective in the formation of GDP-fucose (Reitman et al., 1980; Ripka et al., 1986). A chinese hamster ovary (CHO) cell line that showed a marked

reduction of incorporation of fucose into macromolecules was unable to synthesize complex-type N-linked oligosaccharides, resulting in a deficiency of acceptors (Hirschberg et al., 1982). In another case, two glycosylation mutants were shown to express a fucosyltransferase activity absent in the parental cell line with the concomitant expression of a novel linkage (Campbell and Stanley, 1984).

There are several putative glycosylation mutants. One of these mutants, HL250, was selected after mutagenesis of the parental normal strain Ax3 for the inability to bind anti-SP96 antiserum (Loomis, 1987). This mutant also failed to express a fucose-dependent epitope recognized by the monoclonal antibody 83.5 (West et al., 1986). The focus of my initial investigation was to characterize the mutation in HL250 with the help of biochemical and morphological techniques. We determined that HL250 is a fucosylation mutant that lacked cellular fucose when grown in the absence of fucose and that the defect is probably a result of a lesion detectable in vitro in the conversion pathway that forms GDP-fucose from GDP-mannose.

Materials and Methods

Materials

GDP-[1-^{3}H]mannose (9.1 Ci/mmol) was purchased from New England Nuclear and L-[(5,6)-^{3}H]-fucose (60 Ci/mmol) from American Radiochemical Corporation. KCl and $MgCl_2$ were

obtained from Mallinckrodt; formic acid from Fisher; ATP (disodium salt, catalog number A-5394), niacinamide, NAD^+, NADPH, Trizma base, phenylmethylsulfonyl fluoride, Dowex-1 (1x8, minus 400, chloride form), hexokinase, GDP-mannose, and Amberlite MB-3 were obtained from Sigma. ATP was stored frozen at a concentration of 500 mM in 1 mM Tris-HCl (pH 7.4), resulting in a final pH of approximately 5.5. Dowex-1 formate form was made as follows: 1) The column was washed with 1 M HCl until pH of eluate was below 2 (as determined by pH paper). 2) The column was washed with water until pH was higher than 4.5. 3) Subsequently, the column was washed with 1 M NaOH until pH was higher than 13. 4) The column was washed with water as described in step 2. 5) The column was washed with 3 volumes of 1 M formic acid until pH of eluate was below 2. 6) Lastly, the column was washed with water as described in step 2. This procedure was also followed for regeneration of column.

Strains and Conditions of Growth and Development

Dictyostelium discoideum amoebae were grown on HL-5, a medium that contains glucose, yeast extract, and proteose peptone (Loomis, 1971). The axenic strains Ax3 (from F. Rothman) and HL250 (from W.F. Loomis) were maintained by passage during logarithmic growth phase. Ax3 is the normal strain and HL250 is a mutant obtained from Ax3 by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis. For metabolic

labelling experiments, cells were grown for 4-6 doublings in 8-20 μCi/ml (0.10-0.26 μM) of [^{3}H]fucose in FM medium, a minimal defined medium that lacks fucose (Franke and Kessin, 1977). When appropriate, the medium was supplemented with L-fucose (Sigma). For development, cells were plated in PDF buffer (20 mM KCl, 45 mM sodium phosphate, 6 mM $MgSO_4$, pH 5.8) on filters as previously described (West and Erdos, 1988).

Cell Lysis and Fractionation

Logarithmically growing amoebas were harvested and washed in 50 mM Tris-HCl (pH 7.5) and resuspended to a concentration of $2x10^8$ cells/ml in the lysis buffer consisting of 0.25 M sucrose, 50 mM Tris-HCl buffer (pH 7.5) supplemented with 1 mM PMSF. All operations were carried out at 0-4°C. Cells were immediately lysed by forced passage through a 5 μm nuclepore polycarbonate filter, with pore diameter slightly smaller than the diameter of the cells (Das and Henderson, 1986). This method routinely yields more than 99% cell breakage. The lysate was centrifuged at 100k xg for 1 hour. The supernatant (S100) was made 2.5% (v/v) in glycerol by addition of 100% glycerol and either used immediately or saved at -80° without significant loss of activity for four weeks.

Localization by Immunofluorescence

Prespore and spore cells were examined as described previously (West and Loomis, 1985; González-Yanes et al., 1989). The monoclonal antibodies utilized have been described previously elsewhere (West et al., 1986; González-Yanes et al., 1989).

Determination of Fucose Content and Specific Activity

The method is a modification of the protocol developed by Yurchenco and Atkinson (1975) for HeLa cells. Spores were harvested from sori not more than a day old and resuspended in water without washing, since it has been determined that a significant amount of spore coat protein may be lost after washing spores in water (West and Erdos, 1990). Vegetative cells were grown in FM (in the presence or absence of extracellular L-fucose) for determination of sugar content. For determination of specific activity, cells were grown in FM media supplemented with [^{3}H]fucose. After harvesting, cells were washed twice in PDF followed by EtOH precipitation. The ethanol supernatant was reextracted with EtOH and the resulting pellet pooled with the previous precipitate. EtOH was evaporated under a stream of air. Alternate methods such as TCA precipitation of the ethanol supernatant, did not significantly increase the yield. Samples were then hydrolyzed in a reacti-vial (Pierce) in 0.1 N HCl for 45 min at 100° on a heating block.

Macromolecules were EtOH precipitated and the remaining supernatant dried down, resuspended in water, desalted on an Amberlite MB-3 column and dried by vacuum centrifugation. The samples were redissolved in water and analyzed using a Dionex Bio-LC ion chromatograph by the method of Hardy et al. (1988). Standards and modifications to the chromatography procedure have been published elsewhere (González-Yanes et al., 1989). Specific activity, when applicable, was determined by counting elution fractions and was expressed as radioactivity present in the fucose peak divided by the amount of fucose detected by the pulsed amperometric detector coupled to the HPLC, with reference to previously established calibration curves for L-fucose (Hardy et al., 1988). Greater than 95% of the eluted radioactivity was recovered from the column eluate at the elution position of fucose.

Assay for Conversion of GDP-mannose to GDP-fucose

The conversion assay was carried out essentially as in Ripka et al. (1986). In short, the standard assay mixture contained in a final volume of 1 ml 600-750 μg S100 protein, 10 mM niacinamide, 5 mM ATP, 0.2 mM NAD^+, 0.2 mM NADPH, 7.5 μM GDP-[^{3}H]mannose (approximately 10^5 cpm) in 50 mM Tris-HCl, pH 7.5. After incubation at 37° the reaction was stopped with 50 μl of 2 N HCl, the reaction mixture boiled for 20 min and subsequently neutralized with 55 μl of 2 N

NaOH. Quantitation of the conversion of GDP-mannose to GDP-fucose was achieved by determining the amount of fucose present after acid hydrolysis. Free mannose, released from GDP-mannose, was phosphorylated by adding 4 units of hexokinase in the presence of 5 mM ATP and 5 mM $MgCl_2$ at 37° for 1 hour. Hexokinase catalyzes the transfer of one phosphate to C-6 of any acceptor hexose. Since fucose lacks a hydroxyl group at position C-6, it cannot be phosphorylated. Parallel controls with no enzyme were used to correct for losses. The mixture was passed over a Dowex-1 (formate) column (0.6 x 5 cm) and eluted with water. 1 ml fractions were collected an aliquot of 100 μl from the eluate was counted using ScintiVerse LC (Fisher); fucose-associated radioactivity usually eluted by the first 2 ml. Calculation of K_m and V_{max} was done by the Lineweaver-Burk double reciprocal plot (1/v vs. 1/[S]) as discussed by Henderson (1985).

Results

Phenotypic Description of a Fucosylation Mutant

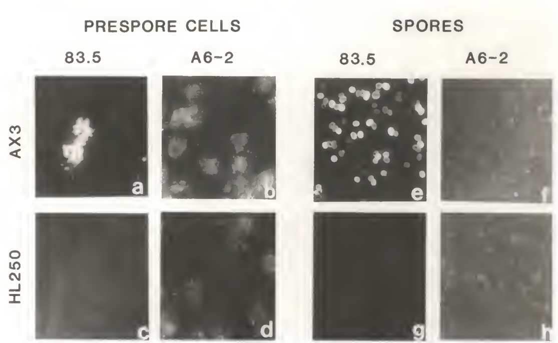
The normal strain Ax3 was mutagenized with nitrosoguanidine and surviving clones were screened with anti-SP96 antiserum (Loomis, 1987). One of the clones, HL250, was selected due to its inability to bind anti-SP96 antiserum which recognizes carbohydrate and peptide epitopes. HL250 has been found to have a more permeable

spore coat, lower germination efficiency in older spores, and a longer doubling time when compared to the parental strain Ax3 (González-Yanes et al., 1989; West et al., manuscript in preparation).

Absence of a fucose-dependent epitope. The failure of HL250 to react with anti-SP96 antiserum suggested that the mutant might be deficient in a form of protein glycosylation. This hypothesis was confirmed by finding that HL250 did not react at appreciable levels with the fucose-dependent monoclonal antibody (mAb) 83.5 (West et al., 1986; González-Yanes et al., 1989). Spores from mutant and the parental normal strains were subjected to SDS-PAGE and Western blotting and probed with mAbs 83.5 and A6.2 (West et al., 1986; González-Yanes et al., 1989). The latter monoclonal is specific for SP96. Consistent with the supposition that glycosylation is affected in HL250, SP96 was reduced in apparent molecular weight compared to SP96 from Ax3 spores (West et al., 1986). The absence of the fucose-dependent epitope was further examined in developing cells and spores. Cells were plated for development and slugs dissociated by shearing in the presence of EDTA. Cells and spores were then processed for indirect immunofluorescence. Figure 2-1 shows the localization of SP96 in spores and prespore cells. Ax3 exhibits peripheral labelling of spores and a punctate labelling from prespore

Figure 2-1. Localization of SP96 in prespore and spore cells.

Slugs cells were dissociated, placed onto polylysine-coated glass slides, fixed, permeabilized, and processed for indirect immunofluorescence using mAbs 83.5 or A6.2.


PRESPORE CELLS
SPORES
83.5
A6-2
83.5
A6-2
AX3
HL250
a
b
e
f
c
d
g
h

vesicles using both mAb. However, HL250 shows the same pattern only when A6.2 is used, in agreement with the results observed by Western blotting. The fact that labelling of spores with A6.2 is similar in both strains indicates that spore coat localization of SP96 is not affected by the mutation.

Fucose content of normal and mutant strains. Epitope recognition by mAb 83.5 was inhibited by L-fucose (West et al., 1986), so the possibility of a defect in fucosylation in strain HL250 was investigated. Initially, the macromolecular fucose content of vegetative cells grown in fucose-free media, and of spores was investigated. Ethanol insoluble macromolecules were acid hydrolyzed, ethanol precipitated, and the supernatant deionized and chromatographed on an alkaline anion-exchange column equipped with a pulsed amperometric detector. When authentic [^{3}H]fucose was fractionated in this manner, more than 95% of the radioactivity eluted at the fucose position (not shown). Fucose content was found to be negligible in the mutant, HL250, both in spores and vegetative cells when compared to the normal strain, Ax3 (table 2-1). However, when HL250 cells were grown in FM supplemented with 1 mM L-fucose, they possessed detectable amounts of fucose. Previous investigators have found by autoradiography that [^{3}H]fucose incorporation is highest in prespore cells

Table 2-1. Fucose content of normal and mutant spores and vegetative cells.

strain	cell type	conditions of growth	fucose	glucose	mannose
Ax3	spores		581±18	n.d.	n.d.
HL250	spores		0.44	n.d.	n.d.
Ax3	amoebae	FM	11.0±1.3	83.6±5.0	30.0±0.7
		FM + 1mM L-fucose	6.5±0.2	86.8±1.4	17.1±1.4
HL250	amoebae	FM	0.075	116.3±3.8	39.5±1.3
		FM + 1mM L-fucose	2.5±0.0	69.0±1.3	40.3±1.4

Spores were collected from fresh fruiting bodies from cells that were plated after growing in HL-5. Vegetative cells were grown in fucose-free media (FM) or FM supplemented with fucose. Fucose content was determined as described in Materials and Methods. Results expressed as nmoles of sugar/ mg of protein as the mean of three determinations ± s.e.m.; n.d., not determined.

compared to prestalk and vegetative cells, so higher levels of fucose would be expected in spores (Lam and Siu, 1981; Gregg and Karp, 1978). The levels of glucose and mannose were measured for comparison to determine if there was a difference in the amounts of other sugars in the mutant. None of the contents of the other sugars were reduced in the mutant when grown in FM. Thus it seems that the lesion in HL250 is selectively affecting fucose metabolism, relative to that of other sugars. This is consistent with the fact that fucosylation is usually a terminal modification of oligosaccharides, so its addition is not a prerequisite for the addition of other sugars (Kornfeld and Kornfeld, 1985).

Characterization of the Mutant Lesion

Earlier investigations have described mutants with a fucose minus phenotype that are the result of a defect in GDP-fucose biosynthesis. The main source of GDP-fucose is the conversion pathway of GDP-mannose to GDP-fucose (Yurchenco et al., 1978; Flowers, 1985). It consists of the reactions presented in figure 2-2. Alternatively, synthesis of GDP-fucose by the fucose salvage pathway can occur in the presence of extracellular L-fucose (figure 2-2), allowing cells defective in the conversion pathway to phenotypically revert (Ripka and Stanley, 1986; Reitman et al., 1980). HL250 cells grown and developed in the presence of 1 mM L-fucose reexpressed the fucose epitope (González-Yanes et

Figure 2-2. Biosynthesis of GDP-fucose.

Diagrams of the conversion pathway elucidated in bacteria and mammalian cells (adapted from Flowers, 1981) and the salvage pathway as described in HeLa cells (adapted from Yurchenko et al., 1978).

A CONVERSION PATHWAY

B. SALVAGE PATHWAY

al., 1989), and vegetative cells grown in 1 mM L-fucose had detectable amounts of macromolecular-associated fucose (table 2-2). These results suggested that HL250 had a normal salvage pathway, but a defect in the GDP-mannose to GDP-fucose conversion pathway.

In vitro conversion of GDP-mannose to GDP-fucose. Conversion of GDP-mannose to GDP-fucose has been reported in bacteria, in higher plant cells, and mammalian cells (Kornfeld and Ginsburg, 1966; Liao and Barber, 1971; Ripka et al., 1986). It was assumed that *Dictyostelium* would share this ability with other species, so I assayed if cell extracts in vitro were able to convert GDP-mannose to GDP-fucose. High speed supernatants from Ax3 and HL250 were assayed in vitro for their ability to convert GDP-[^{3}H]mannose into GDP-[^{3}H]fucose. Cells were homogenized and a 100k x g soluble fraction (S100) assayed as described by Ripka et al. (1986) and the effects of time, varying protein and GDP-mannose concentrations were examined. Table 2-2 shows that the conversion activity in Ax3 was proportional to the time of incubation. In contrast, mutant extracts showed negligible activity. Ax3 and HL250 cytosols were mixed to determine if a soluble inhibitor of GDP-mannose to GDP-fucose conversion activity was present. When equal amounts of extracts were mixed, activity was commensurate with the Ax3 contribution (table 2-2) indicating HL250 does

Table 2-2. Effect of time on conversion of GDP-mannose to GDP-fucose.

	nmol fucose/mg protein		
time (min)	Ax3	HL250	Ax3+HL250 (0.5:0.5)
7.5	0.57	0	0.21
15	2.5	0	1.3
30	2.9	0.15	2.2
90	9.2	0	4.3

Protein (600 μg total) from a 100,000 xg supernatant of a vegetative cell-free extract was assayed for ability to convert GDP-[^{14}C]mannose (7.5 μM initial concentration) to GDP-[^{14}C]fucose; data are the result of the average of two determinations

not contain an inhibitor for the activity. Conversion was linear with respect to protein through 800 μg (figure 2-3, panel A). In agreement with the results from the time dependence experiment, HL250 expressed less than 1% of the activity possessed by Ax3 at all concentrations of protein assayed (figure 2-3, panel A). Conversion by Ax3 S100 was also dependent on the amount of GDP-mannose present while the activity present in HL250 was insignificant (figure 2-3, panel B). The Ax3 GDP-mannose to GDP-fucose conversion activity showed an apparent K_m of 14.1 μM and V_{max} of 18.3 nmol fucose/mg protein/30 min (fig. 2-3, panel C). Previous reported values for the apparent K_m of the conversion pathway range from 2 μM in CHO cells (Ripka et al., 1986) to 160 μM in the higher plant *Phaseolus vulgaris* (Liao and Barber, 1971).

The conversion activity is absent in mutant extracts in vitro, if absent in vivo, this would explain the lack of fucose in living cells and the correction by exogenous fucose. Taken together, all these results point to the conversion pathway as the site of the lesion in HL250. Furthermore, the defect seems to be in the first step of the conversion of GDP-mannose to GDP-fucose because no radioactivity was recovered after hexokinase treatment. If the 4,6-dehydratase was active, the product would have eluted from the ion-exchange column after hydrolysis and phosphorylation by hexokinase (see figure 2-2).

Figure 2-3. Conversion of GDP-mannose to GDP-fucose by normal and mutant strains. Closed circles, Ax3; open circles, HL250. Results are the average of two determinations.

Panel A. Effect of protein concentration on conversion. 30 min. assay, 7.5 μM GDP-[^{3}H]mannose.

Panel B. Effect of GDP-mannose concentration on conversion. 30 min assay, 750 μg protein for each strain.

Panel C. Apparent Michaelis constant for GDP-[^{3}H]mannose, determined for Ax3 conversion. Apparent K_m was determined to be 14.1 μM and apparent V_{max} 18.3 nmol/mg protein/30 min by the Lineweaver-Burk double reciprocal plot method.

A

nmol fucose/30 min

0 1 2 3 4 5

0 200 400 600 800

µg protein

B

nmol fucose/mg protein/30 min

0 5 10 15

0 5 10 15 20 25

GDP-mannose (µM)

C

1 / v

0.0 0.1 0.2 0.3

-0.09 -0.00 0.09 0.18 0.27

1/[S]

Specific activity of fucose pools. A lesion in the GDP-mannose to GDP-fucose conversion pathway would render the mutant defective in macromolecular fucosylation when grown in the absence of fucose, as was shown earlier. Such a scenario would require that the GDP-fucose inside the cell be derived from the salvage pathway fed exclusively by fucose from the extracellular media. In the case of the Ax3, however, there would be a contribution of GDP-fucose derived from the conversion pathway. If the mutation in HL250 was indeed in the GDP-mannose to GDP-fucose conversion, macromolecular fucose of cells grown in the presence of L-[^{3}H]fucose would have the same specific activity as the fucose present in the media. To test this hypothesis, mutant and normal cells were grown in fucose-free defined media supplemented with [^{3}H]fucose. Whole cell preparations were then assayed for fucose content as described above and the specific activity expressed as the radioactivity present in the fucose peak divided by the amount of fucose detected. As expected, the macromolecular pool of the mutant had essentially the same specific activity as the fucose in the medium. In contrast, the specific activity in Ax3 was diluted approximately 400-fold compared to the medium (table 2-3). These results confirmed that HL250 derived its intracellular fucose from the salvage pathway. Meanwhile it appears that in Ax3 the contribution

Table 2-3. Specific activities of fucose.

strain	fucose concentration	cpm/nmol fucose medium	macromolecular
HL250	50 μM	$1.1x10^5$	$9.5x10^4$
Ax3	0.1 μM	$1.8x10^7$	$4.7x10^4$

Cells were grown for 3 days in FM media in the presence of $6x10^6$ dpm/ml of [^{3}H]fucose supplemented with non-radioactive fucose to yield the noted concentration of fucose in the media. Specific activity was determined as described in Materials and Methods.

of fucose by the salvage pathway is minimal, 1/400 of the total content. These studies agree with earlier reports on fucose metabolism, where the majority of fucose in mammalian cells is derived from the conversion of GDP-mannose to GDP-fucose (Yurchenko et al., 1978).

Discussion

HL250 failed to express a fucose-dependent epitope recognized by the mAb 83.5. However, it expressed SP96, one of the polypeptides that bears the carbohydrate epitope recognized by 83.5. These results were reproduced by Western blot (González-Yanes et al., 1989) and indirect immunofluorescence. Interestingly, the immunofluorescence microscopy studies showed that the mutant is not defective in its ability to package SP96 in vesicles or in the targeting of the glycoprotein to the spore coat. Similar results were reported for other proteins in another *Dictyostelium* glycosylation mutant (Aparicio et al., 1990; West and Loomis, 1985). Measurements of fucose content of cells and spores demonstrated that the mutant contained almost undetectable amounts of fucose, in contrast to Ax3 which contained macromolecular-associated fucose in both cell types.

Once HL250 was identified as having a mutation that resulted in decreased macromolecular fucose, I tried to identify the nature of the lesion. It was speculated that

the mutant HL250 may have a defect in (1) fucosyltransferase activities, (2) endogenous acceptors for fucosyltransferases, (3) transport of GDP-fucose into microsomal vesicles, and/or (4) synthesis of GDP-fucose. In vitro microsomal extracts of normal and mutant cells were active in the transfer of [^{14}C]fucose from GDP-[^{14}C]fucose to endogenous acceptors and the activity was latent (see Chapter IV), so it was reasoned that fucosyltransferases may be normal and probably the uptake of GDP-fucose by vesicles was not impaired, so the lesion might be at another point in the fucosylation pathway. Since fucose is normally added as a terminal modification, the fucose minus phenotype could be the result of a lack of formation of acceptors for the fucosyltransferases (Stanley, 1984; Hirschberg et al., 1982). HL250 expresses levels comparable with Ax3 of other carbohydrate epitopes and has normal neutral monosaccharide composition (González-Yanes et al., 1989; West et al., 1986), for these reasons it was speculated that the defect was not in an earlier step of glycosylation but it involved the fucosylation pathway directly.

The glycosylation defect in HL250 appears to result from an inability to produce GDP-fucose. I have found that the GDP-mannose to GDP-fucose conversion activity in vitro is reduced to undetectable levels in mutant cell extracts. The fact that there is partial rescue when the cells are grown in the presence of fucose, suggests that the cells are

producing GDP-fucose via the salvage pathway and that the rest of the fucosylation machinery is probably normal. Earlier studies have reported the phenotypic reversion of mammalian mutants with a defective GDP-mannose to GDP-fucose conversion pathway when the cells were supplied with extracellular fucose (Ripka and Stanley, 1986; Reitman et al., 1980). However, Ripka and Stanley (1986) used the recovery of lectin sensitivity as a marker for phenotypic reversion, but did not report measuring the fucose content of the cells or show the data for lectin binding compared to the parental strain. Reitman et al. (1980) showed that a mouse lymphoma cell line which has a defect in the conversion of GDP-mannose to GDP-fucose was defective in pea lectin binding compared to the parental cell line. The ability to bind pea lectin was restored to wild type parental cell line levels after culturing in 10 mM fucose. Fucose is an important determinant in the carbohydrate-binding specificity of pea lectin (Kornfeld et al., 1981). It is important to note that the mutant mouse lymphoma cell line had approximately one fifth the amount of fucose and one third the number of high affinity lectin-binding sites as the parental line, indicating that mutant cells were salvaging fucose from the medium, or that the mutation was only partial. The evaluation for phenotypic reversion of HL250 is more rigorous since it demands the expression of a

carbohydrate epitope and measures total levels of fucose from a cell that was grown in fucose-free media.

The specific activity of the medium was compared to the specific activity of the intracellular macromolecular fucose. Consistent with a lesion in the GDP-mannose to GDP-fucose conversion pathway, the mutant cells relied on extracellular fucose as their only fucose source. In contrast, extracellular fucose only contributed to a small fraction of the total Ax3 fucose pool. This is useful because it means that radioactivity from cells grown in [^{3}H]fucose can be used as a direct measure of fucosylation in HL250.

In conclusion, even though HL250 has a severe glycosylation lesion which renders it unable to carry out fucosylation when grown in the absence of fucose, the strain is able to grow, develop, and form spores. To my knowledge, there are no previous reports in the literature of eukaryotic cells defective in the GDP-mannose to GDP-fucose conversion pathway that can survive in fucose-free media. The fucosylation mutants reported are cell lines that have a functional salvage pathway maintained in culture in the presence of animal serum, so they can synthesize GDP-fucose from the fucose present in the cell culture media (Ripka and Stanley, 1986; Reitman et al., 1980). For this reason, these investigators were unable to totally deprive the mutants of fucose, as I am able to do with *Dictyostelium*.

Other lower eukaryotes, such as yeast, do not carry out fucosylation (Kukuruzinska et al., 1987) so the existence of a *Dictyostelium* fucosylation mutant could be very important to study fucosylation. In any event, HL250 has already served as a very useful tool in which to study fucosylation events, as will be evident in the following chapters.

CHAPTER III
IDENTIFICATION OF A CYTOSOLIC FUCOPROTEIN

Introduction

There is some evidence that fucoconjugates are present in the nucleus and cytosol (Hart et al., 1989a; Chapter I). However, virtually nothing is known about the structure or biosynthesis of these fucosylated macromolecules. Most studies have limited themselves to reporting the existence of evidence for nuclear or cytoplasmic glycoproteins, but have not gone further to characterize the sugar-peptide linkage or compare it with material derived from the secretory pathway. As discussed in Chapter II, there is a conditional fucosylation mutant, HL250, that can be readily labelled when grown in radioactive fucose. Using this strain, I have identified a fucoprotein that fractionated with the cytosol and appeared to be the major fucosylated species in the cytosol. The oligosaccharide-peptide linkage was characterized and the fucoprotein was compared with fucosylated material derived from vesicles, and differentiated based upon several criteria.

Materials and Methods

Materials

L-[(5,6)-^{3}H]-fucose (60 Ci/mmol) was obtained from American Radiochemical Corporation and D-[(2,3)-^{3}H]mannose (24 Ci/mmol) from New England Nuclear. TS-1 was purchased from Research Product International; POPOP was from Mallinckrodt; SDS, leupeptin, aprotinin, phenyl methylsulfonyl fluoride, Triton X-100, dimethyldichlorosilane, MES, all nitro-phenyl substrates, mannose-6-phosphate, bovine serum albumin (fraction V), blue dextran, bromo phenol blue, and trypsin were from Sigma; glycine, benzene, ammonium acetate, PPO, and toluene were from Fisher. The concentrations of Triton X-100 and NP-40 are expressed as v/v, all others are expressed as w/v, unless specified otherwise.

Strains and Conditions of Growth

Dictyostelium discoideum strains Ax3 (from S. Free) and HL250 (from W.F. Loomis) were grown on HL-5, a complete medium that contains glucose, yeast extract, and proteose peptone (Loomis, 1971). Ax3 is the normal strain and HL250 is a mutant obtained from Ax3 by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis (Loomis, 1987). HL250 lacks the enzyme activity that converts GDP-mannose into GDP-fucose which results in a lack of cell fucose (González-Yanes et al., 1989; also see Chapter II). In all

experiments cells were collected at the logarithmic growth phase, with a cell density of 1 to 9 x 10^6 cells/ml. For metabolic labelling experiments, cells were grown for 4-6 doublings in 2-20 μCi/ml (0.03-0.26 μM) of L-[^{3}H]-fucose in FM medium, a minimal defined medium that lacks fucose (Franke and Kessin, 1977).

Cell Lysis and Fractionation

Logarithmically growing amoebae were harvested and washed in 50 mM MES (pH 7.4) and resuspended to a concentration of 2x10^8 cells/ml in the lysis buffer consisting of 0.25 M sucrose, 50 mM MES buffer (pH 7.4) supplemented with the protease inhibitors leupeptin (10 μg/ml), aprotinin (10 μg/ml), and PMSF (1 mM). When specified, cells were fractionated in the presence of a comprehensive cocktail of protease inhibitors which have been developed for the isolation of various proteolitically sensitive proteins in *Dictyostelium* (Goodloe-Holland & Luna, 1987; Stone, et al., 1987). All steps were carried out at 0-4°C. At once, the cells were gently lysed by forced passage through a 5 μm nuclepore polycarbonate filter, with a pore diameter slightly smaller than the diameter of the cells (Das and Henderson, 1986). This method routinely yielded more than 99% cell breakage as assessed by contrast phase microscopy. The lysate was clarified from unbroken cells and nuclei by a 2k xg centrifugation for 5 min, then

it was centrifuged at 100k xg for 1 hour, unless otherwise specified. The pellet (P100) was resuspended in lysis buffer by pipetting to the same volume as the supernatant (S100). For lysing vesicles, the P100 was sonicated using a Branson Sonifier Cell Disrupter 185.

Slug cells were plated for development as described earlier (West and Erdos, 1988), and harvested in buffer of Berger and Clark (as described in West and Brownstein, 1987) supplemented with 20 mM EDTA. Cells were dissociated in this buffer by passing 20 times through a long, 9 inches, pasteur pipette, followed by passing 20 times through a 23-gauge needle. EDTA was washed by resuspending cells in 50 mM MES, pH 7.4, titrated with NaOH. Cells were resuspended in lysis buffer and immediately lysed by passage through a 3 μm nuclepore polycarbonate filter (Das and Henderson, 1986). Cell lysates were then treated as described above for vegetative cells.

Gel Electrophoresis and Western Blotting

SDS-PAGE was carried out under reducing conditions essentially as described in West & Loomis (1985). Samples were resolved by 7-20% acrylamide linear gradient gels or 15% acrylamide gels and, initially, molecular weight assigned using low MW markers kit (Sigma). In later experiments, trypsin was used as a molecular weight marker. Following electrophoresis, the gels were cut immediately

and/or stained and destained and then cut into either 2.2 mm or 0.5 cm slices. Gel pieces were shaken and swollen overnight in a scintillation cocktail composed of 111.1 ml of tissue solubilizer (TS-1), 6.0 g PPO, 0.15 g POPOP, and 20 ml dH_2O to 1 l of toluene. Gel slices were counted and recounted until dpm were determined to be stable, usually 1-2 days later. For gel-purified material, the sample was run in a 7-20% linear gradient gel and the 21 kD area (approximately 1 cm below trypsin) cut out and electroeluted overnight using a Bio-Rad electroeluter following manufacturer's directions, except the Laemmli electrophoresis buffer used for SDS-PAGE (West and Loomis, 1985) was used instead of the recommended volatile buffer to avoid alkaline hydrolysis. Western blotting was carried out as previously described (West and Loomis, 1985).

Partial Purification of FP21 by Anion Exchange Chromatography

In preliminary studies, FP21 was partially purified by fractionation of metabolically labelled S100 fraction on a TSK DEAE-5PW 8 x 75-mm HPLC anion-exchange column (LKB) preequilibrated with 10 mM NH_4Ac, pH 7.0. Sample was dialyzed against 2 l of 10 mM NH_4Ac, pH 7.0, for several hours, and clarified by centrifugation at 10k x g for 10 min prior to injection. Protein was eluted using an increasing linear gradient (10 mM to 1 M NH_4Ac, pH 7.0) for 40 min at a rate of 0.75 ml per min, and the majority of FP21 was found

to elute at 0.5 M input buffer concentration. Fractions were analyzed by SDS-PAGE and counting of the gel slices.

Protein Concentration Assay

The Bio-Rad protein assay was used for determination of protein concentration, and bovine serum albumin used as standard.

Enzyme Assays

α-glucosidase-2 assays contained 100-300 μg protein, 8.6 mM p-nitrophenyl-α-D-glucoside, 0.1% Triton X-100, in 21 mM citrate-phosphate buffer (pH 7.5) at 37° (Borts and Dimond, 1981). Reaction was stopped after 1 hr by addition of Na_2CO_3 to a concentration of 0.5 M and the absorbance read at 420 nm. Glucose-6-phosphatase and mannose-6-phosphatase were measured by release of phosphate from mannose-6-phosphate, which has been previously shown to be a suitable substrate for both enzymes (Arion et al., 1976). Reaction mixtures contained 100-300 μg protein, 1 mM $MgCl_2$, 2 mM mannose-6-phosphate, 0.1% Triton X-100 in 10 mM MES (titrated with NaOH to a pH of 7.4) in a volume of 200 μl. After 20 min incubations at 30° reactions were stopped by adding 200 μl 20% ice-cold TCA (Snider et al., 1980). Tubes were centrifuged at 14k rpm, for 10 min in an Eppendorf table top microfuge and aliquots of the supernatants were assayed for P_i by the method of Chen et al. (1956). Acid

phosphatase was assayed as described in McMahon et al. (1977) except that Triton X-100 was included at a concentration of 0.1% and absorbance was measured at 420 nm, instead of 400 nm.

PNGase F Digestion

Gel-purified FP21 (3000-8000 dpm) was boiled in 0.5% SDS in water for 3 min. For digestion, the protocol of Tarentino et al. (1985) was followed. The sample was incubated in 104 mM sodium phosphate, pH 8.6, 10 mM EDTA, 10 mM 1,10-phenanthroline (stock solution of 100 mM in methanol), 2% NP-40, 0.21% SDS, and 20 U/ml PNGase F (Boehringer-Mannheim) in 300 μl for 22 h at 37°. Fetuin (B-grade, Calbiochem) and RNAse B (Sigma) were treated identically as controls and digestion was quantitative, or nearly quantitative, as determined by shifts in molecular weight in SDS-PAGE. In one trial FP21 was digested with trypsin prior to PNGase F digestion by incubating gel purified FP21 in 0.8 mg/ml trypsin, 1 mM $CaCl_2$ for 1 hr at 37°. The reaction was stopped by boiling in the presence of 1 mM PMSF and 10 μg/ml aprotinin for 3 min.

Pronase Digestion

Glycopeptides were prepared by exhaustive pronase digestion as described (Ivatt et al, 1984). In short, after gel purification and electroelution, the samples were dried

down and resuspended in water to a volume of 200 μl containing at least 5×10^3 dpm. In other experiments, 200 μl of the entire P100 fraction were left intact or made 0.1% Triton X-100. 200 μl of freshly dissolved 1% pronase (CalBiochem) in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, were added at 0, 24, and 48 hours. Incubation was at 50° and a few drops of toluene were added to prevent microbial growth. At 72 hours the reaction was stopped by incubating in a boiling water bath for 3 min.

Oligosaccharide Release by Alkaline-Borohydride Treatment

The oligosaccharide in FP21 was released by mild alkaline hydrolysis under reducing conditions, also known as ß-elimination. To approximately 5×10^3 dpm of gel purified protein, freshly made NaOH and $NaBH_4$ concentrated solutions were added in that order to yield a final concentration of 0.1 M and 1 M, respectively. Samples were incubated for 15 hours in a water bath at 45°. The reaction was stopped by the addition of acetic acid to a final concentration of 1 M. The samples were dried down by vacuum centrifugation, resuspended once in 1 ml 100 mM HAc, dried again, and resuspended twice in methanol and stored dry at -80° until ready to use.

The oligosaccharide was also released by strong alkaline-borohydride treatment. The method is that of Zinn et al. (1978) and very similar to the procedure for ß-

elimination, with some exceptions: NaOH and $NaBH_4$ were present at a final concentration of 1 and 4 M, respectively, and samples were incubated at 80° for 24 h. Reaction was terminated by diluting the sample twofold with water and adding acetic acid to a final concentration of 4 M. Borate salts were removed by methanol evaporation as described above.

P-4 Column Fractionation

Dry samples from ß-elimination and strong alkaline-borohydride treatment were resuspended in 800 μl of 50 mM pyridinium acetate (pH 5.5). Pyridinium acetate was made in the hood by mixing in water, to a final volume of 2 liters, 8.06 ml pyridine, 2.17 ml glacial acetic acid. After the previous reagents were dissolved, 0.4 g of sodium azide was added. The solution had a final pH of approximately 5.5. The buffer was degassed and stored under chloroform atmosphere. Samples from pronase digestion were centrifuged for 5 min at 14k rpm on a Eppendorf table top microfuge and the supernatant taken for P-4 chromatography. Oligosaccharides and glycopeptides were fractionated in a 0.9 cm x 1 m BioGel P-4 column (-400 mesh) equilibrated with 50 mM pyridinium acetate (pH 5.5) as the mobile phase. Prior to pouring, the column was acid washed overnight and siliconized by coating with 1% (v/v) dimethyldichlorosilane in benzene for 10 min. The column was calibrated with

glucose oligomers used as standards (Yamashita et al., 1982) that were derived from a dextran hydrolysate which was reduced with NaB^3H_4 (kindly provided by J. Baezinger). Twelve drop fractions (approximately 250 μl) were collected and counted using ScintiVerse LC (Fisher Scientific). All runs were performed at 37°. Recovery varied somewhat between runs, but it was between 30-80% of the loaded radioactivity. The void volume (Vo) was determined with blue dextran (at a concentration of 0.2%), and the inclusion volume (Vi) with either bromo phenol blue (0.2%) or [^{3}H]mannose (approximately 2,000 dpm). The relative elution coefficient (Rev) for each component was determined from the elution volume (Ve): Rev = (Ve-Vo)/(Vi-Vo).

Results

Analysis of Cellular Fucoproteins by SDS-PAGE

HL250 amoebae were grown in minimal defined media supplemented with L-[^{3}H]fucose. During logarithmic growth phase, cells were harvested, washed, and resuspended in 0.25 M sucrose buffer supplemented with protease inhibitors. Immediately, the cells were lysed and the lysate was clarified from unbroken cells and nuclei by centrifugation at 2k x g for 5 min. The resulting supernatant from this spin was centrifuged at 100k x g for 1 hour. Both fractions were analyzed by SDS-PAGE and the gels stained, cut into 2.2 mm slices, and counted. While total protein distributed in

a ratio of almost 1:1 (P100:S100), the specific activity (expressed as dpm/mg of protein) distributed roughly in a 6:1 ratio (P100:S100) (table 3-1). The majority of the radioactivity fractionated with the P100. However, the amounts of radioactivity recovered in the S100 were unexpected; 36% of the radioactivity in the S100 (this value varied in individual experiments from 30-68%) migrated as one peak at the 21 kD position, slightly ahead of trypsin, which was used as a molecular weight marker. On the other hand, the P100 showed two main broad peaks, one at 10-25 kD and another at 58-84 kD with 10% of the total radioactivity in the P100 migrating at the 21 kD level (figure 3-1). The proteinaceous nature of the S100 fucoprotein was confirmed by digestion with the proteases trypsin and pronase with quantitative recovery of radioactivity at lower MW positions in unfixed gels (results from pronase digestion shown in figure 3-2). The S100 fucosylated protein has been called FP21 for fucoprotein of 21 kD molecular weight.

The abundance of FP21 was estimated based on the specific activity of fucose and determined to be 10^3 molecules in FP21, assuming one fucose molecule per molecule of FP21. Based on the dilution of fucose specific activity in Ax3 (from table 2-3, Chapter II), there would be 4×10^5 molecules in Ax3. If there is one fucose per copy of FP21, and all FP21 molecules are fucosylated (evidence for quantitative FP21 fucosylation in Ax3 will be presented in

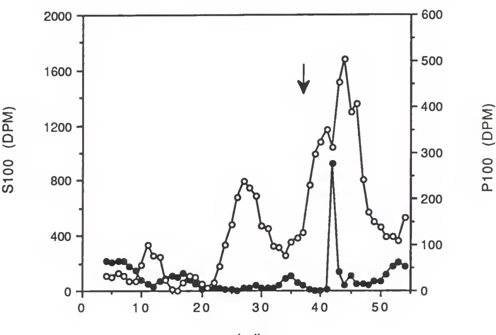
Table 3-1. Distribution of protein and radioactivity in S100 and P100 fractions.

	S100	P100
total protein (equivalents)	1	0.95
specific activity		
3 days	14 dpm/μg	88 dpm/μg
5 days	13 dpm/μg	225 dpm/μg

HL250 amoebae were grown in the presence of 2 μCi/ml of [^{3}H]fucose in FM media for the indicated period of time. Data are from one representative experiment. Cells were harvested, filter lysed, and fractionated into an S100 and P100.

Figure 3-1. Incorporation of $[^3H]$fucose into macromolecular species of the S100 and P100.

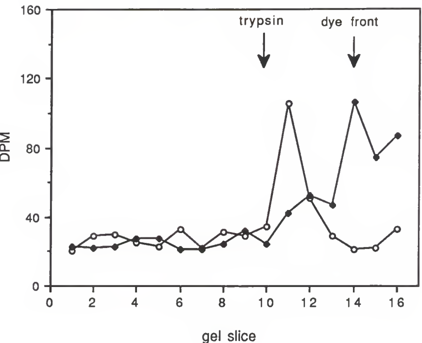
HL250 amoebae were metabolically labelled with 2 μCi/ml of $[^3H]$fucose, lysed, fractionated into an S100 and P100, and subjected to 7-20% linear gradient SDS-PAGE; the gel was sliced into 2.2 mm pieces and counted. 100 μg of protein were electrophoresed for the S100 and P100, respectively. Open circles, P100; closed circles, S100; arrow, migration of trypsin.



2000
1600
1200
800
400
0
S100 (DPM)
600
500
400
300
200
100
0
P100 (DPM)
0
10
20
30
40
50
gel slice

Figure 3-2. Proteinaceous nature of FP21.

Approximately 1,500 dpm of metabolically [^{3}H]fucose-labelled FP21 from HL250 was gel purified, as described in Materials and Methods, and subjected to either a mock or pronase digestion. Resulting digests were electrophoresed on a 15% SDS polyacrylamide gel, which was then sliced into 0.5 cm pieces and counted. Open circles, FP21; closed circles, FP21 digested with pronase.


trypsin
dye front
DPM
160
120
80
40
0
0
2
4
6
8
10
12
14
16
gel slice

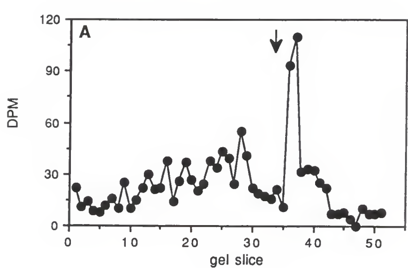
Chapter IV), and the number of copies of FP21 per cell is not affected by the mutation, then there may be a maximum of 4 x 10^5 copies of FP21 per cell.

Evidence that FP21 is Endogenous to the Cytosol

Fucosylation has been shown to occur in the Golgi apparatus in other organisms (Hirschberg and Snider, 1987; Kornfeld and Kornfeld, 1985), so I assessed the possibility that FP21 was a lumenal microsomal protein that leaked during the P100 and S100 isolation procedure. To guard the P100 from chemical lysis, the vesicles were prepared in a cocktail of protease inhibitors that contained additional inhibitors from those utilized in the standard fractionation protocol, and which have been developed for the isolation of various proteolitically sensitive proteins in *Dictyostelium* (Goodloe-Holland & Luna, 1987; Stone, et al., 1987). To address the possibility of mechanical disruption of the vesicles, the P100 was disrupted by sonication and recentrifuged at 100k x g for 1 h. Approximately 11% of the radioactivity was released and the supernatant of this centrifugation was analyzed by SDS-PAGE as described above (figure 3-3). Although several radioactive peaks were present in the supernatant of the second centrifugation, the radioactivity profile was different from the S100 suggesting that FP21 is not a protein released by disruption of the vesicle fraction. Although radioactivity which comigrated

Figure 3-3. Comparison of S100 and releasable P100 components.

HL250 amoebae were metabolically labelled with 2 μCi/ml of [^{3}H]fucose, lysed, fractionated into an S100 and P100. The P100 was sonicated and recentrifuged. After centrifugation, the resulting supernatant was examined by slicing 7-20% linear gradient SDS-PAGE into 2.2 mm and counting the gel pieces (panel B). Included for comparison, is the profile from the S100 radiolabelled species from the same preparation run on the same gel (panel A). 50 μg of protein were electrophoresed for the S100 and P100, respectively. Arrow, migration of trypsin.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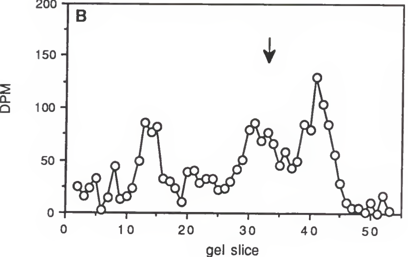
DPM
gel slice
120
90
60
30
0
0
10
20
30
40
50


B
DPM
gel slice
200
150
100
50
0
0
10
20
30
40
50

with FP21 was observed in the P100-derived supernatant, this material was a minority of the radioactivity released, and probably reflected the general heterogeneity of the P100 vesicle contents. Less than 1% of the total cell radioactivity that migrated at the 21 kD molecular weight position was released from the P100 by sonication, indicating that the remaining FP21 is recovered in the S100.

In a different approach, P100 from in vivo [^{3}H]fucose labelled cells was mixed with unlabelled post-nuclear supernatant and recentrifuged (table 3-2). More than 98% of the radioactivity sedimented with the P100, suggesting that once associated with the P100, radioactivity is not lost, unless vesicles are purposely disrupted, as in sonication.

The enzymes α-glucosidase-2, glucose-6-phosphatase, and acid phosphatase have been used as markers of the endoplasmic reticulum, Golgi apparatus and endoplasmic reticulum, and lysosomes, respectively (Borts and Dimond, 1981; McMahon et al., 1977). To examine the distribution of these enzyme markers in the high speed fractions, HL250 amoebae were harvested, homogenized, fractionated into an S100 and P100 and assayed for activity of the different marker enzymes. As seen in table 3-3, the majority of the activity was recovered in the P100, suggesting minimal contamination of the S100 by vesicles containing these enzymes. Less than 7% of the total α-glucosidase-2 activity

Table 3-2. Radioactivity recovered in the second S100 after different P100 treatments.

condition	% radioactivity recovered
untreated	2.2%
sonicated	11.3%
mixed*	1.4%

Cells were labelled in vivo by growing in 2 μCi/ml of [^{3}H]fucose in FM media for 3 days, lysed, and fractionated into an S100 and P100. The P100 was then subjected to different treatments and recentrifuged at 100k x g for 1 hr. The S100 from this second centrifugation was analysed for radioactivity. *P100 from metabolically labelled cells was mixed with unlabelled post-nuclear supernatant from cells grown in FM media and recentrifuged at 100k x g for 1 hr.

Table 3-3. Distribution of markers among S100 and P100 fractions.

	S100	P100
α-glucosidase-2	6.5%	93.5%
acid phosphatase	12.8%	87.2%
glucose-6-phosphatase	n.d.	100%

HL250 amoebae grown in HL-5 were fractionated into S100 and P100, and assayed for activity as described in Materials and Methods. Activity expressed as percentage of total activity detected in both fractions. n.d., not detectable.

was found in the S100; mannose-6-phosphatase was only detectable in the P100. More than 87% of the activity of the lysosomal enzyme acid phosphatase was detected in the P100. In *Dictyostelium*, this enzyme has been shown to be a soluble lumenal lysosomal protein (Dimond, et.el., 1981).

Further evidence that P100 vesicles are stable, closed structures comes from earlier studies from the laboratory. Prespore proteins SP75 and SP96 sediment at 100k x g unless cells are sonicated (West and Erdos, 1988). These spore coat proteins are contained in secretory vesicles (Erdos and West, 1989; West and Erdos, 1988), which appear to be intact since they are resistant to proteolysis by Proteinase K unless they are treated with 0.1% Triton X-100 (West et al., 1986; Q.H. Yang and C.M. West, unpublished data). In other studies, N-acetyl glucosaminyltransferase activity was inhibited by EDTA in the P100 fraction only when assayed in the presence of detergent, suggesting that Golgi-like vesicles in the P100 were closed (R.B. Mandell and C.M. West, unpublished observations)

FP21 is Unrelated to Other Known Cytoplasmic Proteins

The possible relationship of FP21 with other known *Dictyostelium* proteins was investigated. I examined the reactivity of antisera raised against discoidin I and II (Erdos and Whitaker, 1983) and against gp24 (Knecht et al., 1987) for FP21. On Western blots, antiserum against gp24

recognized a band that migrated with a slower mobility than metabolically labelled FP21 (not shown). The possibility of FP21 being the lectin discoidin was examined, since it is primarily present in the cytosol and exhibits weak affinity for L-fucose (Erdos and Whitaker, 1983; Bartles and Frazier, 1980). Metabolically labelled FP21 from HL250 was electrophoresed in a 15% SDS polyacrylamide gel, while a replicate lane was blotted onto nitrocellulose paper and immunoprobed using an anti-discoidin antiserum. The antiserum recognized a band of higher MW than FP21 with mobility slower than trypsin, reproducing results reported by others where discoidin I was shown to have a slower mobility than trypsin in 15% SDS polyacrylamide gels (Kohnken and Berger, 1987). Migration of purified discoidin in SDS-PAGE differed upon boiling of the sample, migrating as a tetramer (ca. 100 kD) when samples were not boiled, whereas metabolically labelled FP21 was found to migrate as a discrete peak of radioactivity ahead of trypsin regardless of boiling (Q.H. Yang and C.M. West, unpublished data).

FP21 could be partially purified by HPLC DEAE chromatography. Metabolically labelled S100 was fractionated on an anion exchange column and FP21 recovery monitored by counting of SDS-PAGE slices. FP21 eluted at 0.5 M NH_4Ac with an increase of 24-fold the specific activity relative to the starting sample (data not shown). Discoidin eluted earlier than FP21 from the HPLC DEAE column

and no radioactivity was found associated with discoidin, suggesting it does not bind to discoidin during purification. I conclude that FP21 is not related to discoidin or any discoidin isoforms, or gp24, and does not bind to any discoidin isoforms.

Oligosaccharide Studies

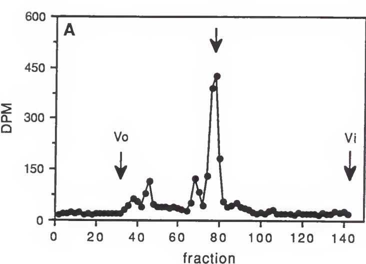
FP21 from Ax3 and HL250 yield similar size glycopeptides after pronase digestion. Pronase digested gel-purified FP21 that had been metabolically labelled from Ax3 and HL250 were compared (figure 3-4). More than 50% of the radioactivity from both sources eluted as a major peak with a relative elution coefficient (Rev) of 0.42 and 0.43, respectively (ca. 5.5 glucose units). In the case of FP21 derived from Ax3, the rest of the radioactivity eluted earlier in the void volume and distributed into minor peaks. The digestion products from HL250 FP21 yielded a major peak, with the rest of the radioactivity eluting earlier, and less than 10% eluting after the major peak, although some variability was observed in different runs regarding the minor peaks. Radioactivity eluting at an earlier position than the major peak may be explained by incomplete digestion. The minor amount of radioactivity that eluted at a later position may be due to breakdown of the oligosaccharide. These results indicate that the main glycopeptides derived from Ax3 and HL250 have the same

Figure 3-4. Gel filtration chromatography of FP21 glycopeptides.

Ax3 and HL250 vegetative cells were metabolically labelled with 2 μCi/ml of [^{3}H]fucose. Cells were lysed, fractionated into an S100 and P100, and FP21 isolated by SDS-PAGE and electroelution from the S100. The samples were exhaustively digested with pronase and analyzed by BioGel P-4 gel filtration. Data obtained from one representative experiment.

Panel A. Glycopeptides derived from Ax3 FP21, arrow identifies major peak with a Rev of 0.42. Vo, 32; Vi, 142.

Panel B. Glycopeptides derived from HL250 FP21, arrow identifies major peak with a Rev of 0.43. Vo, 26; Vi, 166.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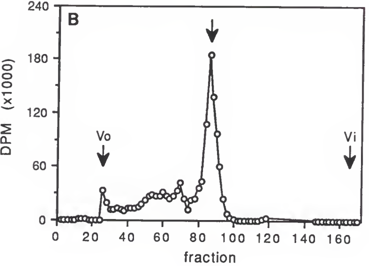
DPM
600
450
300
150
0
Vo
Vi
0
20
40
60
80
100
120
140
fraction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180
120
60
0
Vo
Vi
0
20
40
60
80
100
120
140
160
fraction

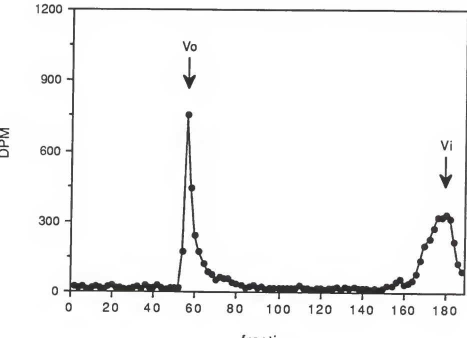
sizes, and suggest that both strains form in vivo the same oligosaccharide when grown in fucose-containing media.

Oligosaccharide in FP21 is O-linked. An enzymatic and a chemical approach were used to determine whether the fucose-containing oligosaccharide in FP21 is N-linked or O-linked. I first tried the enzyme PNGase F. The minimum requirement of PNGase F is a di-N-acetylchitobiose core (Chu, 1986; Tarentino et al., 1985). This enzyme has a broad specificity and can cleave most asparagine linked N-glycans (including high mannose and complex multibranched oligosaccharides) provided they are not located at the amino or carboxy termini (Chu, 1986). Gel-purified FP21 from metabolically labelled Ax3 was digested with PNGase F. As shown in figure 3-5, the fucose label eluted in the void volume, while control substrates were quantitatively digested as determined by SDS-PAGE (not shown). FP21 trypsinized prior to digestion with PNGase F also eluted in the void volume (not shown). The inability of PNGase F to release radioactivity from FP21 suggested that either the fucose-containing oligosaccharide was not N-linked or the oligosaccharide was insensitive to the enzyme.

I then considered the possibility that the oligosaccharide in FP21 was O-linked. Metabolically labelled gel purified FP21 was subjected to mild alkaline, reducing conditions, to release intact O-linked

Figure 3-5. Gel filtration chromatography of PNGase F digests.

Ax3 vegetative cells were metabolically labelled with 2 μCi/ml of [^{3}H]fucose, an S100 was prepared, and FP21 was isolated by SDS-PAGE and electroelution. FP21 digested with PNGase F as described in Materials and Methods, and analyzed by BioGel P-4 gel filtration. Nine drops fraction were collected, instead of the usual 12 drops; [^{3}H]mannose was used to determine Vi.


1200
900
600
300
0
DPM
Vo
Vi
0
20
40
60
80
100
120
140
160
180
fraction

oligosaccharides (ß-elimination). Under the conditions employed, N-linked oligosaccharides are insensitive to chemical release (Biermann, 1988). More than 95% of the radioactivity was released from Ax3 (Rev 0.48) and HL250 (Rev 0.49) in vivo labelled FP21 and it was resolved as one peak with a size of 4.8 glucose unit (figure 3-6, panels A and B). The oligosaccharide appeared to have been released by ß-elimination and thus is concluded to be O-linked. Consistent with the pronase digestion studies reported above, Ax3 and HL250 yielded a similar size oligosaccharide, supporting the idea that both strains produced the same oligosaccharide. The slightly smaller size of the oligosaccharide compared to the glycopeptide is consistent with the notion that the glycopeptide consisted of one or more amino acids and the oligosaccharide chain. If the glycopeptide resulting from pronase digestion consisted of the oligosaccharide attached to two or more amino acids, it was possible that mild alkaline hydrolysis under reducing conditions resulted in further hydrolysis of the remaining polypeptide backbone yielding one amino acid and the oligosaccharide. This would yield a smaller radioactive species, with concomitant increase in Rev. To investigate this possibility, I employed harsher chemical conditions. In vivo labelled FP21 from Ax3 was gel purified and subjected to strong alkaline hydrolysis in the presence of sodium borohydride, which cleaves N- and O-linked

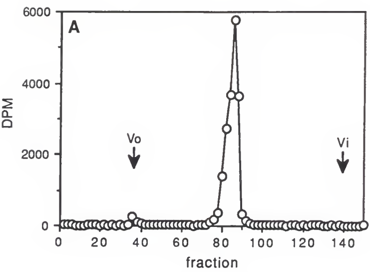
Figure 3-6. Gel filtration chromatography of FP21 oligosaccharides.

Vegetative cells were metabolically labelled with 2 μCi/ml of [^{3}H]fucose, an S100 was prepared, and FP21 was isolated by SDS-PAGE and electroelution. Gel purified FP21 from Ax3 and HL250 were subjected to ß-elimination or strong alkaline hydrolysis followed by fractionation by gel filtration chromatography. Data obtained from one representative experiment.

Panel A. ß-elimination of Ax3 FP21. Vo, 36; Vi, 141.

Panel B. ß-elimination of HL250 FP21. Vo, 30; Vi, 140.

Panel C. Alkaline hydrolysis of Ax3 FP21. Vo, 38; Vi, 134.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DPM
fraction
Vo
Vi
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4000
2000
0
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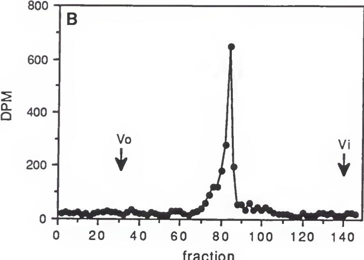
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fraction
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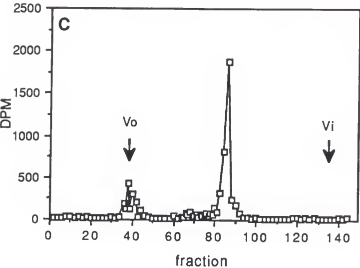
C
DPM
fraction
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Vi
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2000
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1000
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0 20 40 60 80 100 120 140

oligosaccharides (Zinn et al., 1978; Biermann, 1988). The results of this reaction are seen in figure 3-6, panel C. More than 75% of the radioactivity was released, eluting with a Rev of 0.49. Most of the remainder of the radioactivity eluted in the void volume. I expected to see a change in Rev by strong alkaline hydrolysis compared to mild alkaline hydrolysis if mild alkaline hydrolysis did not release the oligosaccharide, but hydrolysed the protein. The fact that similar results are obtained by mild and strong conditions suggested that ß-elimination occurred to release the oligosaccharide. Since mild alkaline hydrolysis had been shown earlier not to cleave N-linked sugars (Biermann, 1988), I conclude that the oligosaccharide in FP21 is linked via an O-linkage. The released oligosaccharide eluted as an asymmetrical peak, both by mild and strong alkaline hydrolysis. These results suggest that there is more than one type of oligosaccharide in FP21 that differ slightly in size. These results also suggest that the slight heterogeneity seen in the glycopeptide size may reflect amino acid heterogeneity.

Comparison of glycopeptides derived from vesicular and cytosolic material. To further address the possibility of FP21 arising by contamination of the S100 from the P100 fraction, glycopeptides derived from the Ax3 P100 were compared to gel purified pronase digested FP21 from Ax3. P100 derived glycopeptides were obtained in three different

manners. Metabolically labelled P100 was subjected to SDS-PAGE and the material that comigrated with FP21 was gel purified, pronase digested, and fractionated by gel filtration chromatography. Entire P100 from metabolically labelled cells was digested in the presence or absence of detergent, and analyzed by BioGel P-4 gel filtration. As seen earlier in figure 3-4, panel A, the S100 digest eluted mainly as a single peak with a Rev of 0.42; on the other hand, the digest from the FP21-comigrating P100 material, fractionated as a major peak of 0.50 Rev (figure 3-7, panel A). An analysis of glycopeptides from the entire P100 digestion showed a different elution profile from the FP21 digestion (figure 3-7, panel B). Note than in this chromatograph there is a minor peak of 0.51 Rev, consistent with the idea that the major peak seen in panel A is a minor component of the entire P100 glycopeptide repertoire. Also approximately 40% of the radioactivity eluted with the void volume, suggesting it may be resistant to pronase digestion. Digestion of the entire P100 fraction was carried out in the presence of Triton X-100, to determine if solubilization of the sample yielded a different digestion profile, by facilitating accessibility of the enzyme to the substrates (figure 3-7, panel C). The overall profile is similar, with more than 30% of the radioactivity eluting at the void volume, and a peak with a Rev of 0.51 is still a minor component of the glycopeptides released. West et al. (1986)

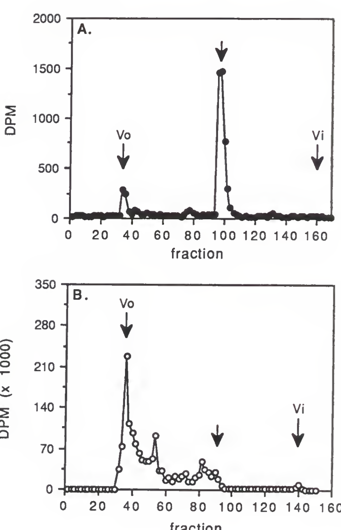
Figure 3-7. Gel filtration chromatography of P100 glycopeptides.

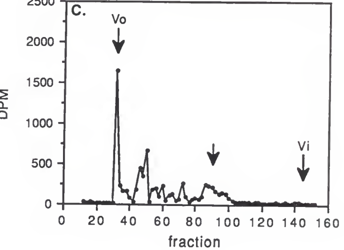
Vegetative Ax3 cells were metabolically labelled with 2 μCi/ml of [^{3}H]fucose. A P100 was prepared and the 21 kD MW material that comigrated with FP21 on SDS-PAGE was electroeluted and pronase digested. In another assay, entire P100 was pronase digested in the absence or presence of 0.1% Triton X-100. After pronase digestion, samples were subjected to BioGel P-4 fractionation. Arrow identifies the position with a Rev of 0.51.

Panel A. Glycopeptides from 21 kD MW P100-derived material. Vo, 34; Vi, 162.

Panel B. Glycopeptides from entire pronase-digested P100. Vo, 36; Vi, 142.

Panel C. Glycopeptides from entire P100 digested with pronase in the presence of Triton X-100. Vo, 32; Vi, 146.


A.
DPM
Vo
Vi
fraction
B.
DPM (x 1000)
Vo
Vi
fraction


C.
DPM
Vo
Vi
fraction

have reported the existence of pronase-resistant material in the particulate fraction of vegetative cells, so it seems possible that the pronase-resistant material eluting in the void volume is, or is related to, the smear previously described, although SDS-PAGE analysis will be needed to confirm this supposition.

<u>Fucose is Covalently Bound to FP21</u>

The fact that the radioactivity in FP21 was not released by boiling in SDS/ ß-mercaptoethanol, nor after boiling in SDS under reducing conditions followed by SDS-PAGE, suggested that ^{3}H was covalently bound to in vivo labelled FP21. Nevertheless, there have been reports of covalent-bound enzymatic intermediates in the literature (Scrimgeour, 1977). Although *ES* (enzyme-substrate) intermediates are very reactive and usually cannot be isolated without some sort of stabilization or chemical trapping technique, the possibility that FP21 is really a cytosolic fucosyltransferase that binds GDP-fucose or other fucose metabolites covalently was considered. The Rev of the radioactivity released by mild alkaline hydrolysis suggests that it is not related to GDP-fucose or fucose because it elutes with a different Rev than GDP-fucose or fucose. Additional evidence that ^{3}H is present as fucose was presented in Chapter II, where it was shown that more

than 95% of the macromolecular-associated radioactivity from metabolically labelled cells migrated as authentic fucose.

FP21 is Present in Migrating Slug Stage Cells

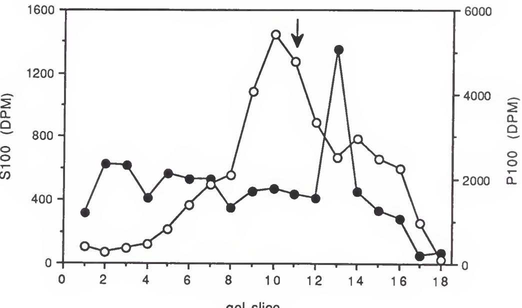
To investigate whether FP21 was present also in developing cells, HL250 amoebae were plated for development on nuclepore filters and 9 hours after plating the filters were lifted and cells were metabolically labelled by placement on 100 μCi of [^{3}H]fucose. After 5 hours of exposure to [^{3}H]fucose, filters were lifted again, carefully washed from ^{3}H label, and cells were allowed to continue development for two more hours. Cells were then harvested, disaggregated, and fractionated into an S100 and P100 fractions, run in an SDS-PAGE, and the gel cut and the pieces counted. As shown in figure 3-8, a fucosylated macromolecule is preferentially fucosylated in the cytosol and it has the same mobility in SDS-PAGE as FP21. Thus, it seems that FP21 fucosylation is not restricted to the growth phase of *Dictyostelium*.

Discussion

In this chapter I have presented evidence for the existence of a fucosylated single molecular weight species, which has been termed FP21 based on its mobility as determined by SDS-PAGE. This protein cofractionates with

Figure 3-8. Incorporation of [^{3}H]fucose into macromolecular species of slug stage cells.

HL250 cells were harvested, plated on filters for development, and exposed to [^{3}H]fucose for 5 h as described in the text. Cells were then collected, disaggregated, filter-lysed, and S100 and P100 fractions were prepared and analyzed by 7-20% linear gradient SDS-PAGE. 70 μg and 24.6 μg of protein were electrophoresed for the S100 and P100, respectively. Open circles, P100; closed circles, S100; arrow, migration position of trypsin.


1600
1200
800
400
0
S100 (DPM)
6000
4000
2000
0
P100 (DPM)
0
2
4
6
8
10
12
14
16
18
gel slice

the high speed supernatant, S100, being the major fucosylated species in this fraction. The recoverability of FP21 after TCA precipitation, HPLC anion exchange chromatography, boiling in SDS/ß-mercaptoethanol, and methanol/acetic acid fixation of the gels suggested a covalent nature for the association of radioactivity. It is unlikely that FP21 is a cytosolic fucosyltransferase that binds GDP-[^{3}H]fucose or another fucose metabolite covalently, since FP21 does not copurify with the cytosolic fucosyltransferase (C.M. West, unpublished results). Employing enzymatic and chemical analysis, the oligosaccharide in FP21 was examined and characterized as a small (4.8 glucose unit) oligosaccharide. The oligosaccharide appeared to be O-linked based on its insensitivity to PNGase F and the releasability from FP21 under alkaline, reducing conditions. Ax3 and HL250 produced FP21-derived glycopeptides and oligosaccharides of similar size, suggesting both strains carry out similar modifications in vivo, despite the starvation for fucose in HL250. Thus, it appears there are no competing reactions for the unfucosylated oligosaccharide, unlike the case for outer fucose in N-linked glycans from mammals (Paulson et al., 1978). Based on antiserum specificity, molecular weight, and/or fractionation by HPLC anion exchange chromatography, FP21 was shown to be a protein unrelated to discoidin or gp24. Finally, a 21 kD fucoprotein was present

in the cytosol of developing cells, suggesting FP21 was not limited to the vegetative stage in *Dictyostelium*, and was fucosylated during development.

I believe that FP21 is recovered in the S100 because it resides in the cytosol in living cells, and not as default location from ruptured vesicles, for several reasons. The S100 fraction was shown to be equivalent to the cytosol and essentially devoid of organellar markers. S100 and P100 fractions from [^{3}H]fucose metabolically labelled cells exhibited a different radioactive profile by SDS-PAGE. Sonication of the P100 fraction failed to release FP21 into the supernatant. FP21 appeared to be endogenous to the cytosol and not derived from organellar vesicles, because control experiments suggested there was no generalized breakage of vesicles during the preparation of the cytosolic fraction. In addition, as a control for contamination from P100 material, glycopeptides derived from comigrating 21 kD MW species from the P100 fraction were compared with FP21 glycopeptides. The fucopeptide in FP21 (ca. 5.5 glucose unit) does not seem to be a product of vesicular fucosylation, since it is not shared by macromolecules of 21 kD MW in the P100 fraction which yielded a major peak with a different Rev (ca. 4.3 glucose unit) than the one derived from FP21. When the entire P100 fraction was subjected to Pronase digestion, a heterogeneous mixture of fucosylated

species (in accordance with Tsurchin, et.al. 1989) was obtained with sizes unlike that of FP21 glycopeptides.

The designation of compartmentalization of a protein as cytosolic is difficult since the cytosol is the site of localization after disruption of organellar vesicles. This task is complicated in the case of glycoproteins and glycosylation enzymes, which are usually described as components of the secretory pathway. However, some glycoproteins have been identified as cytosolic, and generally accepted as such (Hart et al., 1989a; Hart et al., 1989b). My studies report the existence of a fucosylated cytosolic protein. However, these results do not exclude the possibility of FP21 being present in other locations topologically continuous with the cytosol, such as the nucleus, or being synthesized elsewhere and transported. Due to its small size, FP21 could, in theory, be able to diffuse freely into the nucleus.

Since glycoprotein fucosylation has been shown to take place in enclosed organelles of the secretory pathway (Hirschberg and Snider, 1987), the identification of FP21 in the cytosol raises the question of where in the cell is fucosylation of FP21 taking place. One scenario would have FP21 being fucosylated in organellar vesicles (presumably the Golgi apparatus) and subsequently transported to the cytosol, while another would postulate the presence of a fucosyltransferase that localized in the cytosol with FP21.

I consider these possible alternatives in the following chapter and present evidence for the presence of a fucosyltransferase in the cytosol responsible for FP21 fucosylation.

CHAPTER IV
EVIDENCE FOR A CYTOSOLIC FUCOSYLTRANSFERASE

Introduction

In the preceding chapter, I identified a fucosylated protein in the cytosol, FP21. The presence of FP21 in the cytosol challenges the prevailing belief that fucoproteins are restricted to the cell surface and lumenal compartments of the cell. Even though in the past three decades evidence has been accumulating on the presence of glycoproteins and fucoproteins in non-lumenal locations (Hart et al., 1989a; Hart et al., 1989b), to my knowledge, no one has shown the existence of a fucosyltransferase in the cytosol. One possibility is that fucosylation is restricted to the microsomes, and cytosolic fucoproteins are posttranslationally transported back across the membrane to the cytosol.

However, even though there is no previous evidence for cytosolic fucosylation there is precedent for glycosylation in the cytosol. Studies on the biosynthesis of nuclear pore proteins bearing O-GlcNAc suggested that the sugar was added to the proteins within 5 min of their synthesis and before they became associated with membranes (Davis and Blobel, 1987). These data suggested that the activity responsible

for the addition of O-GlcNAc was in the same topological compartment where translation takes place, the cytosol. Thus it is possible that fucosylation may take place in the cytosol, but it has escaped detection by previous investigators for a variety of reasons. One of the difficulties in assaying cytosolic enzymes is that the endogenous acceptors for the enzymes may be present in low quantities in the cell, complicating purification of large amounts for use as substrates. If acceptors are already fucosylated, in vitro assays that utilize endogenous acceptors would not detect enzymatic activity. Hart and coworkers have circumvented this problem with the use of synthetic peptides with a sequence based on O-GlcNAc glycosylation sites (Hart et al., 1989b). They have identified an enzymatic activity capable of O-linked GlcNAc transfer in rat hepatocytes that was recovered in both the soluble and membrane fractions (Haltiwanger et al., 1990). The membrane-associated activity was releasable by high salt treatment and oriented towards the cytosol, not the lumen of the vesicles (Haltiwanger et al., 1990).

With the help of the conditional fucosylation mutant HL250, I have addressed the existence of a fucosylation pathway in the cytosol. Total protein in HL250 is underfucosylated relative to the normal strain, so it was reasoned that it would be a useful strain to assay fucosylation in vitro due to the availability of

macromolecular acceptors. In this chapter I present evidence for a fucosyltransferase that partitions with FP21 in the cytosol. The fucosyltransferase was distinguished from vesicular fucosyltransferase activity by several criteria, and was characterized using hydrophobic synthetic analogs.

Materials and Methods

Materials

GDP-[U-^{3}H]fucose (6.6 Ci/mmol) and GDP-[U-^{14}C]fucose (250 mCi/mmol) were from New England Nuclear (more than 90% of the radiolabel was in the form of the ß anomer, as indicated by the manufacturer). Reagent grade KCl, $MnCl_2$, $CaCl_2$, $BaCl_2$ and $MgCl_2$ were from Mallinckrodt; GDP-ß-fucose from Biocarb (stored frozen as a concentrated stock); GDP-α-fucose (stored frozen as a concentrated stock), Tween-20, $CoCl_2$, Dowex-2 (2x8, minus 400, chloride form), Triton X-100, chymostatin, pepstatin, NBZ-phenylalanine, bovine serum albumin (BSA), and all p-nitro-phenyl acceptors were from Sigma; Na_2EDTA, $FeCl_3$, formic acid, and trichloroacetic acid were from Fisher. Cations and Na_2EDTA were stored as concentrated 500 mM solutions at 4°. Hydrophobic synthetic acceptors were generously provided by Monica Palcic. Making and regeneration of Dowex-2 formate form column was as described for Dowex-1 in Materials and Methods, Chapter II. The concentrations of Triton X-100 and NP-40 are expressed

as volume/volume (v/v), all others are expressed as weight/volume (w/v) unless specified otherwise.

Strains and Conditions of Growth

Dictyostelium discoideum strains Ax3 (from S. Free) and HL250 (from W.F. Loomis) were grown on HL-5, a complete medium that contains glucose, yeast extract, and proteose peptone (Loomis, 1971). Ax3 is the normal strain and HL250 is a mutant obtained from Ax3 by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis (Loomis, 1987). HL250 lacks the enzyme activity that converts GDP-mannose into GDP-fucose which results in a lack of cell fucose (González-Yanes et al., 1989; Chapter II). In all experiments cells were collected at the logarithmic growth phase, with a cell density of 1 to 9 x 10^6 cells/ml.

Cell Lysis and Fractionation

Vegetative and slug stage cells were fractionated into S100 and P100 fractions as described in Materials and Methods, Chapter III.

Fucosyltransferase Assay

Fucosyltransferase activity was assayed immediately after obtaining the S100 and P100 fractions. Fractions were found to be sensitive to freezing and thawing, and up to 70% of the fucosyltransferase activity could be lost. The

standard fucosyltransferase assay contains 30 μl of extract (100-350 μg of protein), 0.35 μM GDP-β-[^{14}C]-fucose, 5 mM $MgCl_2$, 0.25 mM NaF, and 5 mM ATP in 50 mM MES (titrated with NaOH to a pH of 7.4) in a 50 μl volume incubated at 30° for the specified amount of time. Endogenous macromolecules were used as acceptors. To terminate the assay, 1 ml of ice-cold 15% TCA was added to each sample along with 50 μg BSA (to serve as carrier protein) and the precipitate collected on 2.4 cm GF/C glass filters by vacuum filtration, washed with 10 ml 10% ice-cold TCA, 10 ml acetone, and counted after air-drying inside the vials for approximately 30 min using 10 ml of Bio-HP LC scintillation fluid (Fisher). Background was subtracted from experimental values, and was determined as the amount of TCA-precipitable radioactivity at time zero; it was usually between 20-40 dpm. When indicated, the disodium EDTA salt was used. In preliminary trials, 5 mM Mg^{++} was found to support maximal activity, and this concentration was used in all the assays unless indicated. GDP-fucose had been shown previously to be only slightly decomposed under similar conditions (Nuñez and Barker, 1976). Nevertheless, the extent of GDP-[^{14}C]fucose hydrolysis during the fucosyltransferase assay was examined by carrying out the reaction for two hours and separating products on a Dowex-2 formate column by sequentially eluting with 5 ml of water, 3 M formic acid and 15 M formic acid as described (Sommers and Hirschberg,

1982). After 2 hours of incubation less than 20% of the initial GDP-[^{14}C]fucose had been hydrolyzed. Incorporation of ^{14}C did not exceed 30% of the initial radioactivity on any given experiment. For analysis by gel electrophoresis of the endogenous acceptors of the in vitro fucosyltransferase activity, the reaction was terminated by 3 min boiling in sample buffer. For the determination of pH optima experiments, assays were buffered using concentrated solutions of MES previously adjusted to different pH values with either HCl or NaOH. The final pH value of each reaction was determined on an equivalent reaction mixture 100x the volume, without GDP-[^{14}C]fucose. Where indicated, the S100 was desalted on a 0.85 x 13 cm on a BioRad BioGel P-2 column (200-400 mesh) equilibrated with 50 mM MES, pH 7.4, titrated with NaOH, at 4° with a flow rate of 0.5 ml/min. If supplied, synthetic acceptors and/or FP21 were previously dried down onto the bottom of the assay tubes in a vacuum centrifuge. Acceptors were subsequently resuspended in water or in the reaction mixture. Results are expressed as average of two determinations (variations in the duplicates did not exceed 15% of the average value) or average of three measurements ± standard error of the mean (s.e.m.). Calculations of K_m and V_{max} were done by the Hanes single reciprocal plot ([S]/v vs. [S]) as discussed by Henderson (1985).

Incorporation into hydrophobic synthetic acceptors was determined as described by Palcic et al. (1988). The assay was carried out as for endogenous acceptors, but GDP-[^{3}H]fucose was used instead of GDP-[^{14}C]fucose and the reaction was terminated by the addition of 1 ml ice-cold water. The reaction mixture was loaded onto a C_{18} SepPak column (Waters) under vacuum, and eluted with 6 successive 5 ml aliquots of water, and four 5 ml aliquots of methanol. Eluates were counted by addition of 15 ml of ScintiVerse LC (Fisher). In initial trials I determined that the radioactivity eluted in the first methanol fraction, so in subsequent experiments only the first methanol fraction was used for determination of radioactivity incorporated. All extracts were assayed in the absence of exogenous acceptor and this value (usually about 20% of the dpm incorporated) subtracted from experimental value to determine substrate-dependent incorporation.

Purification of FP21

FP21 was purified in the following manner for preparations which were to be added back to cytosolic fractions to measure fucosylation acceptor activity. Starting with 8 x 10^{10} cells, an S100 cytosolic fraction was prepared from the mutant HL250. An aliquot of the fraction (approximately 0.7% of the total volume) was incubated with GDP-[^{14}C]fucose and allowed to fucosylate FP21 with

[^{14}C]fucose. Incorporation into FP21 was confirmed by electrophoresing an aliquot and counting of SDS-PAGE slices. The radiolabelled aliquot was mixed with the rest of the unlabelled preparation and subjected to $(NH_4)_2SO_4$ fractionation. The 70-80% cut was dissolved in and dialyzed against 100 mM NH_4Ac, applied to a 14 ml bed of the strong anion exchanger A25-QAE-Sephadex, and eluted with an ascending gradient up to 1.5 M NH_4Ac. [^{14}C]FP21 eluted at input buffer concentration of 0.49 M. This preparation was then concentrated and desalted on Centricon and/or Centriprep cartridges with nominal 10 kD MW cutoffs, and then applied to an HPLC gel filtration column (8 x 300 mm Toya Soda TSK GW-300) equilibrated in 100 mM NH_4Ac, with a flow rate of 0.5 ml/min. Sample was clarified by centrifugation at 10k x g for 10 min prior to injection. Radioactivity from the concentrated QAE-Sephadex eluate eluted between the 14 kD and 29 kD MW standards. Fractions were analyzed by SDS-PAGE using 15% polyacrylamide gels and counting of the gel slices. For addition of purified FP21 to cell extracts, HPLC gel filtration fractions were brought to dryness in a vacuum centrifuge, redissolved in dH_2O, and brought to dryness again, in the 1.5 ml microcentrifuge tube that was going to be used for the assay.

ß-elimination of In Vitro Labelled Acceptor

S100 extracts from HL250 were fucosylated in vitro. To corroborate that I obtained 21 kD MW fucosylated product from the in vitro reaction, 1/25th of the reaction was terminated by 3 min boiling in sample buffer and analyzed by SDS-PAGE. The remainder of the sample (containing approximately 10^4 dpm) was stored at -80° until ready to use. The reaction mixture was centrifuged for approximately 2 h in a centricon filter to reduce unused GDP-[^{14}C]fucose. After concentrating the volume to 200 µl, ß-elimination was carried out as described in Materials and Methods, Chapter III.

PNGase F Digestion of In Vitro Labelled FP21

FP21 was fucosylated in vitro as described above for ß-elimination. After the volume was concentrated, PNGase F digestion was carried out as described in Materials and Methods, Chapter III.

Results

Cytosolic Fucosyltransferase Activity

The presence of FP21 in the cytosol suggested that a fucosyltransferase might also be located there. To investigate this possibility, HL250 cells were fractionated to yield cytosolic supernatant (S100) and organelle (P100) fractions. The fractions were analyzed for their ability to

transfer [^{14}C] from GDP-[^{14}C]fucose into TCA precipitable endogenous material. As shown in figure 4-1, the cytosolic activity was dependent on time and protein content. The cytosolic fucosyltransferase activity had the properties of being enzyme-mediated. Table 4-1 shows the effect of boiling, denaturants and temperature on the cytosolic fucosyltransferase activity. While the non-ionic detergent Triton X-100 inhibited all activity, Tween-20 was only slightly inhibitory. 30° was the optimal temperature of those tested (22°, 30°, and 37°). Consistent with an enzyme-mediated process, only unlabelled GDP-ß-fucose was able to inhibit incorporation of radioactivity proportionate to its relative concentration (GDP-α-fucose was without effect), demonstrating the stereospecificity of the enzyme (lower section of table 4-1). It also implies a fucosyltransferase that catalyses an alpha-fucosyl linkage is being assayed.

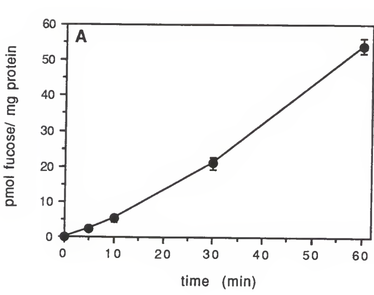
Earlier I explored the possibility of FP21 arising by contamination from the vesicular fraction. The same question was asked about the fucosyltransferase activity in the cytosol, since known fucosyltransferases are Golgi enzymes and activity was detectable in the P100 (see next section). Hence, I tried to deplete the S100 of fucosyltransferase activity by centrifuging at 170k x g for 2.5 hours (instead of 1 hr at 100k x g). This step was used to sediment any population of small or low density vesicles

Figure 4-1. Fucosylation of endogenous acceptors by S100 fraction.

Vegetative HL250 cells were harvested, homogenized, and an S100 obtained as described in detail in Materials and Methods. The indicated amount of S100 protein was incubated in the presence of 0.36 μM GDP-[^{14}C]fucose, 5 mM $MgCl_2$, 5 mM ATP, 0.25 mM NaF, in 50 mM MES, pH 7.4 for the indicated amount of time. Fucose incorporation was calculated from the amount of TCA-precipitable [^{14}C]radioactivity. Results expressed as the mean of three determinations ± s.e.m.

Panel A. Effect of time on fucosylation; 159 μg protein.

Panel B. Effect of protein concentration on fucosylation; 30 min assay.


A
pmol fucose/ mg protein
60
50
40
30
20
10
0
0
10
20
30
40
50
60
time (min)

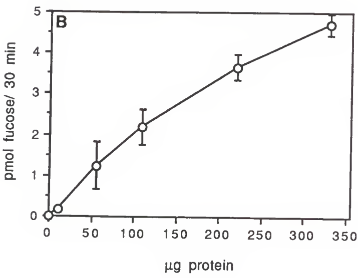

B
pmol fucose/ 30 min
5
4
3
2
1
0
0
50
100
150
200
250
300
350
μg protein

Table 4-1. Effect of different treatments on the cytosolic fucosyltransferase activity.

condition	GDP-[^{14}C]fucose (μM)	pmol fucose/mg/30 min		relative activity
		control	experimental	
no cation	0.36	17.3	0.97	0.06
5 mM EDTA	1.32	9.23	0.06	0.01
boiled	1.32	9.23	<0.01	<0.01
0.15% Triton X-100	0.36	18.5	0.22	0.02
0.1% Tween-20	0.36	16.6	10.5	0.63
10% ethanol	6	54.4	3.00	0.06
22°	6	31.2	22.8	0.73
37°	6	35.3	16.1	0.46
6 μM GDP-α-fucose	0.36	20.8	21.3	1.02
6 μM GDP-ß-fucose	0.36	20.8	1.09	0.05

HL250 vegetative cells were harvested, filter-lysed, and fractionated into an S100 and P100 fractions. The S100 was assayed for fucosyltransferase activity in the presence of 100-300 μg protein, the noted concentration of GDP-[^{14}C]fucose, 5 mM ATP, 0.25 mM NaF, 5 mM $MgCl_2$, in 50 mM MES, pH 7.4, for 30 min at 30° (standard conditions), unless other conditions are specified. Additives were added at the noted concentration; when EDTA was present divalent cations were omitted from the reaction mixture; the experiments performed at 22° contained 10 mM $MgCl_2$ and 10 mM $MnCl_2$; those at 37° were supplemented with 10 mM $MgCl_2$. The results are a compilation of different experiments carried out at the given GDP-[^{14}C]fucose concentrations. Relative activity refers to the activity exhibited under experimental conditions compared to control conditions. Fucose incorporation into endogenous acceptors was calculated from the amount of TCA-precipitable [^{14}C]radioactivity. Results are the average of two determinations.

that were not sedimented before (if existent) and that could have contained fucosyltransferase activity. To preserve the intactness of the P100 vesicles, additional protease inhibitors (Goodloe-Holland and Luna, 1987) from those routinely used, were utilized during cell fractionation. After these measures activity was still recovered in the cytosol at similar levels (table 4-2). Additional evidence supporting the notion that the vesicles in the P100 are not damaged during filter lysis and centrifugation is presented in Chapter III in the section of the origin of FP21. Taken together, these results suggest that vesicles were not measurably damaged during the isolation procedure; therefore, the fucosyltransferase activity is probably endogenous to the cytosol.

Comparison Between the P100 and S100 Fucosyltransferase Activities

In order to compare the S100 and P100 activities, I examined the identity of endogenous acceptors and the effects of divalent cations, pH, and varying GDP-fucose concentration on both fucosyltransferase activities. The criteria of differential behavior has previously been used to differentiate glycosyltransferases, since it is assumed that under similar conditions, enzymes should behave in a similar fashion (Campbell and Stanley, 1984; Galland et al., 1988). Activities were measured in the presence of detergent to circumvent any potential problem in substrate

Table 4-2. Failure to sediment S100 fucosyltransferase activity.

	S100	P100
100k x g, 1h	943	444
170k x g, 2.5h	1131	484

Cells were lysed, centrifuged, and fractionated into S100 and P100. In this experiment lysis buffer (described in Materials and Methods, pH 8.0) was supplemented with 1 mM chymostatin, 5 μg/ml pepstatin, and 2 mM NBZ-phenylalanine. Fractions were assayed immediately for [^{14}C] incorporation from GDP-[^{14}C]fucose and expressed as total dpm incorporated in 30 min into TCA insoluble endogenous acceptors. Reaction mixtures contained 0.36 μM GDP-fucose, 5 mM $MgCl_2$, 240 μg protein, and were incubated for 30 min. Results are expressed as average of two determinations.

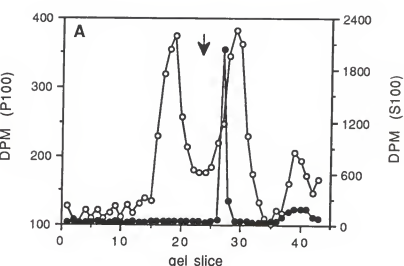
or cation accessibility. Tween-20 at a concentration of 0.1% was chosen because it did not inhibit considerably the activity in the S100 (table 4-1).

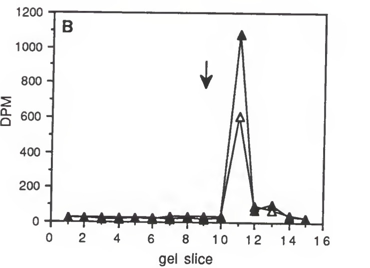
I first examined the profile of in vitro endogenous acceptors by SDS-PAGE analysis. Standard S100 and P100 fractions from HL250 cells were isolated and added to fucosyltransferase reaction mixtures. After 90 min of incubation, reactions were boiled in sample electrophoresis buffer, subjected to SDS-PAGE, and the gels cut and counted. The profile of radiolabel incorporation in vitro by the S100 was similar to that observed in metabolic labelling experiments, with more than 70% of the radioactivity migrating as one discrete peak with a MW of 21 kD (compare figure 4-2, panel A with figure 3-1). Incorporation into 21 kD MW material by the S100 fraction varied from 70-95% in different experiments, with the remainder of the radioactivity migrating near the dye front or top of the gel. In the P100 fraction, radioactivity distributed in two peaks, similar to what was seen in metabolically labelled P100. To determine if in vitro fucosylated protein had the same apparent MW as metabolically labelled FP21, Ax3 gel-purified FP21 was mixed with in vitro fucosylated HL250 S100, as described in the figure legend (figure 4-2, panel B). The migration of in vitro fucosylated material coincided with that of metabolically labelled FP21. I

Figure 4-2. SDS-PAGE profile of endogenous acceptors fucosylated in vitro.

Panel A. SDS-PAGE profile of S100 and P100 endogenous acceptors fucosylated in vitro. HL250 cells in logarithmic growth phase were harvested, homogenized, and an S100 and P100 prepared. Both fractions were fucosylated in vitro in the presence of 4.4 μM and 8.8 μM of GDP-[^{14}C]fucose for the S100 and the P100, respectively, 5 mM $MgCl_2$, for 90 min. Fucosyltransferase reactions were stopped by boiling in SDS/ß-mercaptoethanol sample buffer and resolved by 7-20% linear gradient SDS-PAGE; the gel was sliced into 2.2 mm pieces and counted. Electrophoresis was from left to right. 138 μg and 150 μg of protein were electrophoresed for the S100 and P100, respectively. Open circles, P100 endogenous acceptor species; closed circles, S100 endogenous acceptor species; arrow, migration of trypsin.

Panel B. Comigration of in vitro fucosylated FP21 with metabolically labelled FP21 on SDS-PAGE. Ax3 vegetative cells grown in [^{3}H]fucose were harvested, lysed, and fractionated into an S100 and P100. The S100 was subjected to SDS-PAGE and Ax3 FP21 was gel purified and electroeluted. Independently, HL250 amoebae were harvested, homogenized, fractionated, and the S100 obtained from the fractionation fucosylated in vitro in the presence of 0.36 μM GDP-[^{14}C]fucose, 5 mM MgCl, 140 μg protein, for 30 min. Reaction was stopped by mixing with gel purified Ax3 [^{3}H]FP21 followed by boiling in SDS/ß-mercaptoethanol. Samples were coelectrophoresed in a 15% SDS-polyacrylamide gel, and the gel cut into 0.5 cm slices and counted. Open circles, Ax3 [^{3}H] metabolically labelled gel purified FP21; closed circles, [^{14}C] in vitro labelled mutant S100 extract.


A
DPM (P100)
DPM (S100)
gel slice
400
300
200
100
2400
1800
1200
600
0
10
20
30
40


B
DPM
gel slice
1200
1000
800
600
400
200
0
2
4
6
8
10
12
14
16

interpret these results as an indication that the activity that fucosylates FP21 in vivo is being assayed in vitro.

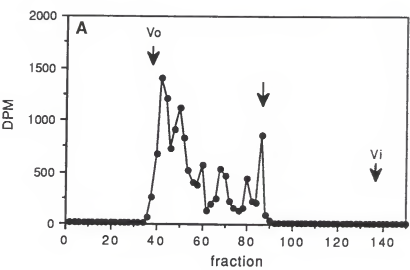
FP21 was fucosylated in vitro by incubating HL250 S100 fractions in the presence of GDP-[^{14}C]fucose, and the oligosaccharide fucosylation in vitro was examined as before for in vivo fucosylated FP21. In vitro fucosylated FP21 was digested with PNGase F (not shown) or subjected to mild alkaline hydrolysis and analyzed by gel filtration (figure 4-3, panel A). As was the case with metabolically labelled FP21, PNGase F failed to release radioactivity and in vitro labelled FP21 digested with PNGase F eluted in the void volume. The digestion of the control substrates, fetuin and ribonuclease B, was confirmed by SDS-PAGE. On the other hand, approximately 20% of the radioactivity eluted with a Rev of 0.50, the elution position of the metabolically fucosylated oligosaccharide produced by Ax3 (reproduced for comparison in figure 4-3, panel B, from figure 3-6, panel A). The remainder of the radioactivity fractionated as material of larger size. These results suggested that the in vitro fucosylated oligosaccharide in FP21 was also O-linked. The reasons for the discrepancies in size between in vivo and in vitro fucosylated oligosaccharides are not known, but may be due to incomplete release of the oligosaccharide, accompanied by partial hydrolysis of the polypeptide. Alternatively, it may indicate the presence of oligosaccharides of various sizes.

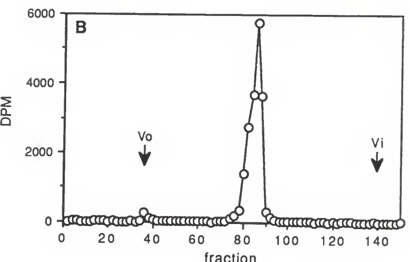
Figure 4-3. BioGel P-4 gel filtration chromatography of in vitro labelled FP21 oligosaccharide.

HL250 S100 extracts were incubated in vitro in the presence of GDP-[^{14}C]fucose, desalted, subjected to ß-elimination (as described in Materials and Methods), and analyzed by gel filtration. For comparison, the profile resulting from ß-elimination of in vivo labelled Ax3 gel purified FP21 is presented in panel B (was panel A in figure 3-6, Chapter III).

Panel A. ß-elimination of in vitro fucosylated FP21; arrow identifies peak with Rev of 0.50. Vo, 38; Vi, 136.

Panel B. ß-elimination of Ax3 FP21. Vo, 36; Vi, 141.


A
Vo
Vi
DPM
fraction
2000
1500
1000
500
0
20
40
60
80
100
120
140


B
Vo
Vi
DPM
fraction
6000
4000
2000
0
20
40
60
80
100
120
140

To compare the S100 and P100 fucosyltransferase activities, HL250 vegetative cells were harvested, homogenized, and fractionated into an S100 and P100. Fractions were assayed for fucosyltransferase activity under different conditions in the presence of Tween-20 (table 4-3). The fractions differed in that the S100 fucosyltransferase activity was approximately threefold more efficient (on a per protein basis) than the P100 under standard conditions (which contained 0.36 μM GDP-fucose and 5 mM $MgCl_2$, see Materials and Methods). A major difference between the bulk activities was their sensitivity to the presence of divalent cations. In the absence of any added cation, the P100 retains more than one fourth the activity exhibited in the presence of Mg^{++}, while the activity in the S100 was almost negligible. The presence of EDTA does not inhibit further the activity in the P100. One interpretation is that the fucosyltransferase activity in the S100 is dependent on added Mg^{++}, while the activity in the P100 is present in the absence and presence of Mg^{++}, being stimulated by the cation. An alternative explanation, is that there are multiple enzymes in the P100, which differ in their requirements for divalent cations.

Sensitivity to pH is a feature exhibited by enzymes, including fucosyltransferases (Foster, et al. 1991; Kumazaki and Yoshida, 1984). HL250 vegetative cells were harvested, lysed, and fractionated into S100 and P100 fractions. Both

Table 4-3. Comparison between the S100 and P100 fucosyltransferase activities.

condition	pmol fucose/mg protein/45 min	
	S100	P100
$MgCl_2$*	6.95±2.60	1.71±0.45
no cation	0.18±0.08	0.68±0.10
EDTA	0.05±0.03	0.64±0.10

HL250 amoebae were harvested, filter-lysed, and centrifuged to prepare S100 and P100 fractions. Fucosyltransferase reaction mixtures contained 0.36 μM GDP-[^{14}C]fucose, 0.1% Tween-20, and 135 μg or 138 μg of protein from the S100 and P100, respectively, in the absence of added divalent cations. $MgCl_2$ and EDTA were present at 5 mM. *this is the standard assay, as described in Materials and Methods. Results are the average of three measurements ± s.e.m.

fractions were assayed for fucosyltransferase activity at various pH values as described in Materials and Methods. The pH profiles of the S100 and P100 fucosyltransferase activities in the presence of Tween-20 are shown in figure 4-4. Activity was maximal for the S100 from pH 6.8 to 7.8 and for the P100 from pH 6.4 to 7.8. At pH 9.6 the activity in the S100 was inhibited more than 20-fold compared to maximum ($p<0.05$), whereas the activity in the P100 was only inhibited threefold ($p<0.05$). The pH-dependent activity profiles were not affected by the exclusion of Tween-20 (not shown). Thus, while the general profile is similar for the activities from the S100 and P100, the cytosolic activity was more sensitive to alkaline pH than the P100 activity.

The dependence of S100 and P100 fucosyltransferase activities on the GDP-fucose concentration in the presence of Tween-20 was also studied. S100 and P100 fractions were prepared from HL250 vegetative cells and assayed for fucosyltransferase activity under standard conditions, at increasing concentrations of GDP-fucose in the presence of 0.1% Tween 20 (figure 4-5). The apparent K_m for GDP-fucose was 1.7 μM and 38.2 μM for the S100 and P100, respectively. The apparent V_{max} for the S100 was 42.7 pmol fuc/ mg protein/ 30 min and 122 pmol fuc/ mg protein/ 30 min for the P100. As evidenced by the lower apparent K_m (22-fold lower), the affinity of the S100 fucosyltransferase for GDP-fucose was higher than the one from the P100 activity. This accounts

Figure 4-4. Effect of pH on S100 and P100 fucosyltransferase activities in the presence of Tween-20.

S100 and P100 fractions prepared from vegetative HL250 cells were assayed for fucosyltransferase activities in the presence of Tween-20 at different pH values (pH values determined as described in materials and methods). GDP-[^{14}C]fucose concentration was 0.36 μM; $MgCl_2$, 5 mM; Tween-20, 0.1%. The assay was carried out for 30 min, and 150 μg and 228 μg of protein were supplied from the S100 and P100, respectively. Fucose incorporation was calculated from the amount of TCA-precipitable [^{14}C]radioactivity. Results expressed as the mean of three determinations ± s.e.m.

Panel A. Effect of pH on S100 fucosyltransferase activity in the presence of Tween-20.

Panel B. Effect of pH on P100 fucosyltransferase activity in the presence of Tween-20.

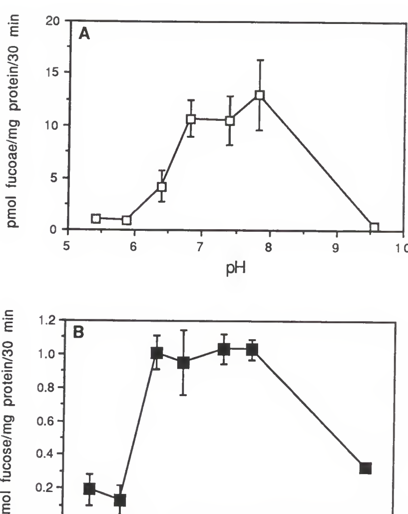

A
pmol fucoae/mg protein/30 min
20
15
10
5
0
5
6
7
8
9
10
pH
B
pmol fucose/mg protein/30 min
1.2
1.0
0.8
0.6
0.4
0.2
0.0
pH

Figure 4-5. Effect of GDP-fucose concentration on S100 and P100 fucosyltransferase activities in the presence of Tween-20.

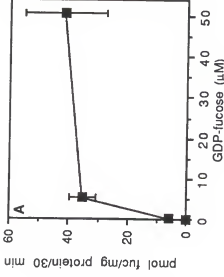
HL250 amoebae were harvested, homogenized, fractionated into an S100 and P100 and fucosylated in vitro at varying concentrations of GDP-[^{14}C]fucose. Reactions were carried out for 30 min in the presence of 5 mM $MgCl_2$, 0.1% Tween-20, 240 μg of protein for the S100, and 432 μg of protein for the P100. Fucose incorporation was calculated from the amount of TCA-precipitable [^{14}C]radioactivity. Results expressed as the mean of three determinations ± s.e.m.

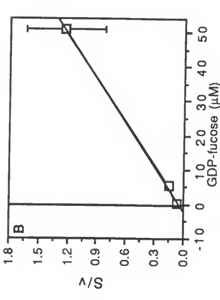
Panel A. Effect of GDP-fucose concentration on S100 fucosyltransferase activity in the presence of Tween-20.

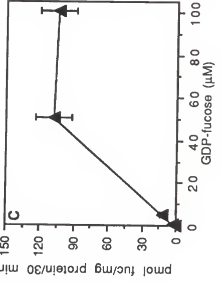
Panel B. Hanes single reciprocal plot ([S]/v vs. [S]) for the S100 fucosyltransferase activity in the presence of Tween-20; apparent K_m=1.7 μM, apparent V_{max}=42.7 pmol/mg protein/30 min.

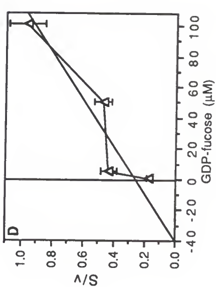
Panel C. Effect of GDP-fucose concentration on P100 fucosyltransferase activity in the presence of Tween-20.

Panel D. Hanes single reciprocal plot ([S]/v vs. [S]) for the S100 fucosyltransferase activity in the presence of Tween-20; apparent K_m=38.2 μM, apparent V_{max}=122 pmol/mg protein/30 min.


A
pmol fuc/mg protein/30 min
GDP-fucose (μM)


B
S/v
GDP-fucose (μM)


C
pmol fuc/mg protein/30 min
GDP-fucose (μM)


D
S/v
GDP-fucose (μM)

for the higher activity of the S100 fraction in the presence of detergent at the concentration of GDP-fucose used in most assays (0.36 μM), despite the higher V_{max} of the P100.

Since the above results were obtained in the presence of detergent, I investigated the effect of GDP-fucose concentration on the fucosyltransferase activities in the intact fractions. It was reasoned that, in the case of the P100, it would give us some insight into the overall fucosylation process, including uptake of GDP-fucose into the vesicles. S100 and P100 fractions were prepared from HL250 vegetative cells and assayed for fucosyltransferase activity under standard conditions at increasing concentrations of GDP-fucose (figure 4-6). An apparent K_m of 0.44 μM and V_{max} of 25.5 pmol fuc/ mg protein/ 30 min was calculated for the S100. For the P100 an apparent K_m of 28.3 μM and V_{max} of 233 pmol fuc/ mg protein/ 30 min was determined.

The fucosyltransferase activities from the S100 and P100 differed in the acceptor species that were fucosylated, the sensitivity to high pH, divalent cation dependence, and apparent affinity for GDP-fucose. These differences in enzymatic behavior support a model for separate compartmentalization of the two fucosyltransferase activities; the S100 activity is free in the cytosol and the P100 activity is in a membrane bound organelle.

Figure 4-6. Effect of GDP-fucose concentration on intact S100 and P100 fucosyltransferase activities.

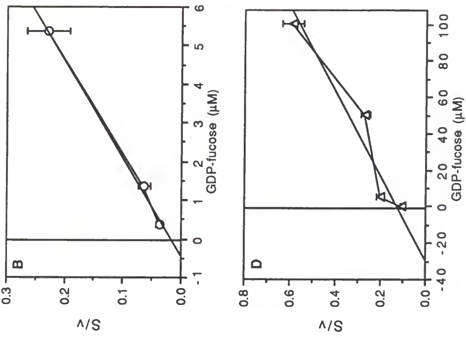
HL250 amoebae were harvested, homogenized, fractionated into an S100 and P100 and fucosylated in vitro at varying concentrations of GDP-[^{14}C]fucose. Reactions were carried out for 30 min in the presence of 5 mM $MgCl_2$, 240 μg of protein for the S100, and 432 μg of protein for the P100. Fucose incorporation was calculated from the amount of TCA-precipitable [^{14}C]radioactivity. Results expressed as the mean of three determinations ± s.e.m.

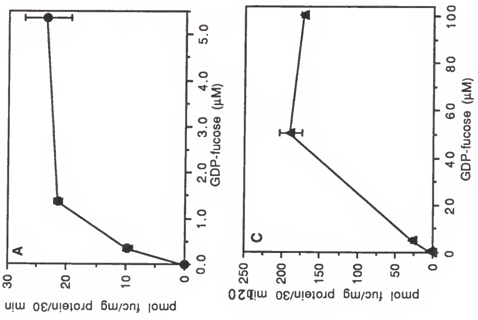
Panel A. Effect of GDP-fucose concentration on intact S100 fucosyltransferase activity.

Panel B. Hanes single reciprocal plot ([S]/v vs. [S]) for the S100 fucosyltransferase activity; apparent K_m=0.44 μM, apparent V_{max}=25.5 pmol/mg protein/30 min.

Panel C. Effect of GDP-fucose concentration on intact P100 fucosyltransferase activity.

Panel D. Hanes single reciprocal plot ([S]/v vs. [S]) for the S100 fucosyltransferase activity; apparent K_m=28.3 μM, apparent V_{max}=233 pmol/mg protein/30 min.


B
S/v
GDP-fucose (μM)
D
S/v
GDP-fucose (μM)


A
pmol fuc/mg protein/30 min
GDP-fucose (μM)
C
pmol fuc/mg protein/30 min
GDP-fucose (μM)

Fucosyltransferase Activity Cannot be Detected In Vitro in Ax3 S100 Extracts

Normal growing cells expressed [^{3}H]fucose metabolically labelled FP21 and the main glycopeptide and oligosaccharide products released by pronase digestion and ß-elimination, respectively, were indistinguishable from those derived from the mutant HL250 (Chapter III). All the studies reported above on the cytosolic fucosyltransferase activity were carried out in HL250 because in vitro transfer of fucose from GDP-fucose to endogenous acceptors cannot be detected in Ax3 S100 fractions by TCA-precipitation, SDS-PAGE analysis, or C_{18} SepPak fractionation (see Materials and Methods for description of methods). A plausible explanation for the lack of activity in the Ax3 would be that the S100 from Ax3 contained an inhibitor for the activity. To investigate this possibility, I performed several mixing experiments. Vegetative Ax3 and HL250 cells were harvested, filter-lysed and fractionated into an S100 and P100. The S100 fractions were assayed individually or mixed in different ratios before assaying for fucosyltransferase activity. The fractions were used intact or desalted prior to assay (table 4-4). There was no evidence for an inhibitor since experiments in which S100 fractions from Ax3 and HL250 were mixed in different ratios showed activity commensurate to the HL250 contribution (table 4-4). Dilution of labelled GDP-fucose with endogenous unlabelled GDP-fucose is not an explanation

Table 4-4. Fucosyltransferase activity in Ax3 S100 fraction.

Contribution		Intact[a]		Desalted[b]		
Ax3	HL250	DPM	rel. activity°	DPM	rel. activity	th. value*
1	0	3	<0.01	8	<0.01	0
0	1	1842	1.00	2715	1.00	1
1	1	1030	0.56	963	0.35	0.50
4	1	435	0.24	218	0.08	0.20
1	4	1425	0.77	2098	0.77	0.80

Ax3 S100 was mixed with mutant (HL250) S100 and assayed for fucosyltransferase activity as described for the standard assay for 30 min. Results are the average of two determinations. The samples were either intact or desalted prior to assay. rel. activity, relative activity; th. value, theoretical value. A total of 30 μl of extract was used in each assay and mixing was done relative to volume contributed by each S100 fraction. 1:1 ratio was 15 μl:15 μl; 1:4, 6 μl:24 μl; 4:1, 24 μl:6 μl. °relative activity calculated using HL250 activity as a unit. *theoretical value based solely on contribution from HL250 fraction. [a]Ax3 S100 was at a concentration of 5 μg of protein/μl, and HL250 at 6 μg/μl. [b]Ax3 protein concentration at 8.5 μg/μl and HL250 at 6 μg/μl.

either because activity was not detected in Ax3 after desalting through a P-2 column, while the HL250 S100 retained activity (table 4-4). Another explanation for the lack of activity in the Ax3 S100 is that FP21 from Ax3 was quantitatively fucosylated in vivo, leaving no acceptor sites for the reaction in vitro. Evidence using purified FP21 from HL250 supports this conclusion (see next section).

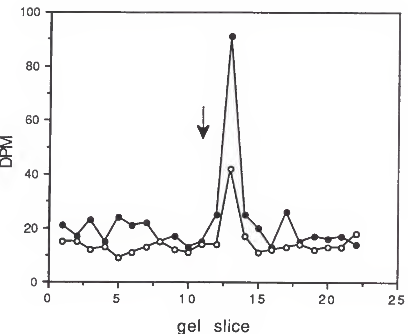
Fucosyltransferase Activity Is Detected in Ax3 S100 Fraction Upon Addition of Mutant FP21

The absence of cytosolic fucosyltransferase activity in Ax3 S100 extracts could be explained as a result of quantitative fucosylation of FP21 in the living cell. It was reasoned that if this model was correct, then addition of mutant FP21 to Ax3 extracts would lead to incorporation into FP21. FP21 was trace-labelled in vitro using GDP-[^{14}C]fucose, and partially purified by ammonium sulfate precipitation, QAE-ion exchange chromatography, and HPLC gel filtration. Fractions from the gel filtration step were counted and examined by SDS-PAGE and those that contained FP21 were pooled, brought to dryness, dissolved in water, and added to Ax3 S100 extract. In vitro fucosylation was determined as [^{3}H] incorporated into TCA-insoluble material in the presence of GDP-[^{3}H]fucose. Since FP21 was trace-labeled with [^{14}C]fucose, the relative amount of the acceptor added was estimated from [^{14}C] dpm. West et al. (unpublished results) showed that incorporation was

proportional to the amount of [^{14}C] radioactivity added. Since this study did not analyze the in vitro fucosylated species by SDS-PAGE, it cannot be concluded that Ax3 was able to fucosylate FP21. To investigate which MW species served as acceptor for the Ax3 cytosolic fucosyltransferase, the experiment was repeated using a new batch of partially purified [^{14}C]FP21. In vitro labelled FP21 eluted in consecutive fractions 20 and 21 during HPLC gel filtration chromatography, as confirmed by SDS-PAGE. Ax3 S100 was added to an aliquot of fraction 21 that had previously been dried on the bottom of the assay tube and assayed for fucosyltransferase activity in the presence of GDP-[^{3}H]fucose and Mg^{++}. The reaction was stopped by boiling in sample electrophoresis buffer, resolved by SDS-PAGE, and the gel sliced and counted. Figure 4-7 shows the comigration on SDS-PAGE of the in vitro [^{3}H] label resulting from fucosylation by Ax3 with the trace [^{14}C] labelled FP21 from fraction 21. There was no [^{3}H] radioactivity incorporation into any MW species when the Ax3 S100 fraction was incubated in the absence of purified HL250 FP21. In conclusion, Ax3 S100 has a cytosolic fucosyltransferase activity that utilizes the same acceptor as the mutant cytosolic fucosyltransferase.

Figure 4-7. Fucosylation of mutant FP21 by Ax3 S100 fraction.

HL250 amoebae were harvested, homogenized, and fractionated into S100 and P100 fractions. Unlabeled HL250 S100 was mixed with an aliquot of in vitro [^{14}C]fucosylated HL250 S100. FP21 was purified from the S100 fraction by $(NH_4)SO_2$ precipitation, QAE-ion exchange chromatography, and HPLC gel filtration (described in detail in Materials and Methods). [^{14}C]FP21 eluted in fractions 20 and 21 of the HPLC gel filtration chromatography, as confirmed by SDS-PAGE. Ax3 S100 was added to an aliquot of fraction 21 that was previously dried down in the bottom of an assay tube in a vacuum centrifuge to serve as acceptor in the in vitro Ax3 S100 fucosyltransferase reaction. The reaction mixture contained 0.15 μM GDP-[^{3}H]fucose, 5 mM $MgCl_2$, 349 μg of Ax3 S100 protein, was incubated for 60 min and the reaction stopped by boiling in SDS/ß-mercaptoethanol electrophoresis buffer. Sample was resolved on a 15% SDS-polyacrylamide gel, which was cut into 0.5 cm slices and counted. No [^{3}H] radioactivity was incorporated into any MW species when the wild type Ax3 S100 fraction was incubated in the absence of added FP21 from mutant source (not shown). Open circles, [^{14}C] label derived from in vitro labelled purified FP21 from HL250; closed circles, [^{3}H] radioactivity from in vitro fucosylation by the Ax3 S100 fraction.



100
80
60
40
20
0
DPM
0
5
10
15
20
25
gel slice

Cytosolic Fucosyltransferase Preferentially Fucosylated a Type I Acceptor

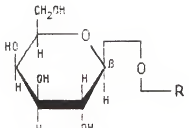
The size of the fucose-containing oligosaccharide in FP21 (determined in Chapter III to be 4.8 glucose units) implies there is more than one sugar residue, so the acceptor site on FP21 may be another sugar. As a first step to determine whether the cytosolic fucosyltransferase could fucosylate model acceptor analogs utilized by known fucosyltransferases, the S100 from Ax3 and mutant origin were screened for activity towards hydrophobic model acceptors. The incorporation of radioactivity into synthetic sugar acceptors that contained 8-methoxy carbonyloctyl, or methyl nonanoate, [$CH_3(CH_2)_7COOCH_3$, referred to as R throughout the text] as the hydrophobic tail by the S100 was determined by the C_{18} Sep-Pak method, which employs a hydrophobic interaction column. Unreacted GDP-fucose does not interact with the column, eluting in the water wash while the glycolipid acceptor is eluted from the column with methanol. As shown in table 4-5, only the type I acceptor analog (known as lacto-N-biose I or galß1,3GlcNAcß-R) sustained activity in the Ax3 S100. In contrast, type II (known as N-acetyllactosamine or galß1,4-GlcNAc-R) and ß-gal-R were not suitable acceptors (see figure 4-8 for structures). The mutant S100 was also active with galß1,3GlcNAcß-R, but only one tenth as active (on a per protein basis) as Ax3.

Table 4-5. Utilization of 8-methoxycarbonyloctyl synthetic acceptors by cytosolic fucosyltransferase activity from Ax3 and HL250.

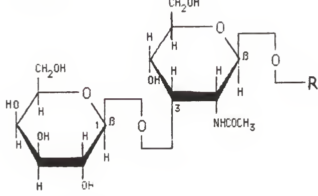
substrate	concentration (mM)	pmol fucose/mg/h Ax3	 HL250
ß-gal-R	1	und.	n.d.
galß1,3GlcNAcß-R	0.15	0.38	0.039
galß1,4GlcNAcß-R	0.15	und.	n.d.

Incorporation was determined by the C_{18} Sep-Pak assay (see Materials and Methods). Experiment performed by C.M. West and is the result of one determination. und, undetectable; n.d., not determined; -R is $-(CH_2)_8COOCH_3$; GDP-[^{3}H]fucose concentration, 0.15 µM.

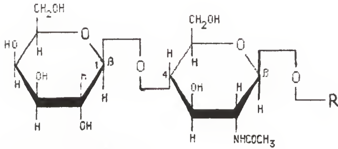
Figure 4-8. Haworth projections of the structures of the synthetic glycolipid acceptors.



1. ß-gal-R



2. galß(1,3)GlcNAcß-R



3. galß(1,4)GlcNAcß-R

R= $(CH_2)_8COOCH_3$

8-methoxycarbonyloctyl synthetic acceptors are not available commercially, so I investigated the possibility that other hydrophobic glycosides, which can be readily obtained from commercial sources, would serve as acceptors. Some of these phenyl derivatives have been shown by others to be suitable acceptors for a fucosyltransferase (Potvin, et al., 1990; Palcic et al., 1988). S100 fractions from HL250 and Ax3 were assayed for fucosyltransferase activity in the presence of p-nitro-phenyl glycoside derivatives; fucosylation of endogenous substrates was monitored by TCA-precipitation, and fucosylation of p-nitro-phenyl glycosides by the Sep-Pak method (table 4-6). Inhibition of incorporation of radioactivity into FP21 in HL250 was examined by TCA precipitation of endogenous acceptors. Millimolar concentrations of these compounds failed to inhibit significantly incorporation. Likewise, none of the p-nitro-phenyl glycosides served as acceptor for the mutant nor the Ax3 cytosolic fucosyltransferase activity when assayed by the C_{18} Sep-Pak method. Thus it is concluded, that from the acceptor candidates examined, only galß1,3GlcNAcß-R is a suitable acceptor under the conditions used.

The activity responsible for fucosylation of the type I acceptor analog in Ax3 was examined by varying the concentration of acceptor or the concentration of nucleotide sugar donor. The type I acceptor analog was fucosylated

Table 4-6. Evaluation of the suitability of p-nitro-phenyl glycosides as acceptors for cytosolic fucosyltransferase activity in HL250 and Ax3 S100.

treatment	concentration	DPM HL250 (TCA)	DPM HL250 (SepPak C_{18})	DPM Ax3 (SepPak C_{18})
control		1003	<3	<3
p-nitro-ø-α-D-glucose	10 mM	996	21	4
p-nitro-ø-ß-D-glucose	10 mM	1020	19	n.d.
p-nitro-ø-α-D-GlcNAc	10 mM	1066	26	10
p-nitro-ø-ß-D-GlcNAc	10 mM	1032	31	18
p-nitro-ø-ß-D-galactose	10 mM	1059	38	3
p-nitro-ø-ß-D-glc tetraacetate	10 mM	1134	24	10

S100 fractions from Ax3 and HL250 were assayed for fucosyltransferase activity and fucose incorporation determined by TCA-precipitation or SepPak C_{18} method (see Materials and Methods for details). *n*=1; n.d., not determined; ø, phenyl; 0.89 µM GDP-[^{14}C]fucose; 0.1% Tween-20; 619 µg protein; 60 min assay.

with an apparent K_m of approximately 1 mM for the acceptor and 1.6 μM for GDP-fucose (C.M. West, unpublished results). The similarity in the apparent K_m for GDP-fucose for the type I and FP21 fucosyltransferase suggested that the same enzyme may be responsible for both reactions. The fact that HL250 was able to fucosylate so poorly the analog when compared to Ax3, suggested that the availability of endogenous substrate (FP21) inhibited incorporation into the synthetic acceptor, and supported the idea of the same enzyme fucosylating both substrates. The notion was further reinforced by reduction of type I analog fucosylation in Ax3 S100 extracts by purified FP21. Fractions 20 and 21 from the HPLC gel filtration chromatograph (see section above on reconstitution of Ax3 fucosyltransferase activity by purified FP21) reduced incorporation of radioactivity into type I acceptor (table 4-7). Even though reduction was not strictly proportional, it was evident that it increased with increasing amounts of FP21. Taken together the results of this section and the preceding one, it appears that Ax3 possesses a fucosyltransferase activity in the cytosol capable of fucosylating FP21 and the type I analog acceptor.

Cytosolic Fucosyltransferase Activity is Present in Migrating Slug Stage Cells

Cytosolic FP21 was detectable by metabolic labelling in slug stage HL250 cells (Chapter III). Reasoning that a fucosyltransferase responsible for its modification would be

Table 4-7. Reduction of fucosylation of acceptor type I analog by purified FP21.

fraction	relative amount added	[^{3}H]fucose incorporated (dpm/mg protein/h)
	0	499
20	1x	387
20	4x	318
21	1x	436
21	4x	<20

Transfer of [^{3}H] from GDP-[^{3}H]fucose into 4 μg (0.145 mM) of type I acceptor analog by Ax3 S100 was measured using the C_{18} Sep-Pak method (see Materials and Methods). Data are the results of one determination. 349 μg protein of Ax3 S100; GDP-[^{3}H]fucose concentration, 0.15 μM; 60 min assay.

present at this stage of development, I assayed developing cells for their ability to incorporate [^{14}C] from GDP-[^{14}C]fucose into endogenous acceptors. HL250 cells were plated for development and at slug stage harvested, disaggregated, fractionated into an S100 and P100, and assayed for fucosyltransferase activity as described in Materials and Methods. For comparison, fractions from Ax3 slugs were assayed (table 4-8). As seen with amoebae cells, HL250 S100 was active whereas the Ax3 S100 did not incorporate radioactivity. On the other hand, the P100 was active in both strains. Thus, it seems that the cytosolic fucosyltransferase is not restricted to the growth phase and is present in developing cells.

Discussion

The presence of FP21 in the cytosol suggested that a fucosyltransferase might also be located in the cytosol. An S100 fucosyltransferase activity was detected which was both time- and protein concentration-dependent. The activity was strictly divalent cation dependent. Incorporation of radioactivity was sensitive to temperature, certain detergents, and ethanol. A variety of sugars and sugar-derivatives failed to inhibit activity, except GDP-ß-fucose, which inhibited in a dose-responsive manner. The activity could not be sedimented by higher centrifugation force in the presence of an extensive list of protease inhibitors.

Table 4-8. In vitro fucosyltransferase activity of HL250 and Ax3 slug extracts.

strain	pmol fucose/mg protein/30 min S100	P100
HL250	40.6	36.3
Ax3	<0.1	30.7

Normal and mutant amoebae were allowed to develop, harvested, disaggregated, filter-lysed, and fractionated into an S100 and P100. Intact fractions were assayed for fucosyltransferase activity in the presence of 0.36 μM GDP-[^{14}C]fucose, 5 mM $MgCl_2$, 12-48 μg of protein, for 30 min. Results are the average of two determinations.

The endogenous acceptor utilized by the S100 fucosyltransferase was a protein which comigrated with FP21 by SDS-PAGE. I compared the acceptor for the in vitro fucosyltransferase reaction with metabolically labelled FP21 from Ax3 cells by SDS-PAGE. There was one main radioactive peak, revealing in vivo and in vitro fucosylated acceptors with the same mobility on polyacrylamide gels. These results suggested that a cytosolic fucosyltransferase existed that utilized FP21 as its primary acceptor species in vitro, and may be responsible for fucosylation of FP21.

To investigate the origin of the S100 fucosyltransferase, I compared it to the bulk P100 fucosyltransferase activity, since the cytosol is the default location of lumenal enzymes released by rupture of vesicles. If both activities were indeed different, I expected to detect enzymatic differences. Initially, I examined the SDS-PAGE profiles of in vitro fucosylated acceptors and found they were very similar to those obtained from metabolic labelling. Incorporation by endogenous acceptors was at the 21 kD MW position for the S100, and in the P100 radioactivity migrated as two separate, broad peaks.

In order to compare directly the soluble and the sedimentable activities, I assayed the S100 and P100 in the presence of detergent to overcome any differences in accessibility for GDP-fucose by the fucosyltransferases. I

determined that the S100 fraction was dependent on divalent cations, while the P100 was active in the absence of cations and in the presence of the chelator EDTA. The activities in both fractions were maximal at a similar pH range, but the cytosolic fucosyltransferase was more sensitive to higher pH than the P100 fucosyltransferase activity. Glycosyltransferase activities have commonly been found to be dependent on the presence of divalent cations. In the case of fucosyltransferases, however, there are precedents for α1,2, α1,3, and α1,3/1,4 fucosyltransferases which are active in the absence of cations, and are either stimulated or inhibited by different cations (Beyer and Hill, 1980; Campbell and Stanley, 1984; Foster et al.,1991; Stroup et al., 1990; Zatz and Barondes, 1971).

The apparent affinity for GDP-fucose differed greatly for S100 and P100 activities. The S100 fucosyltransferase activity had a higher affinity for GDP-fucose than the P100 activity when both were assayed in the presence of Tween-20. The lower apparent K_m for the cytosolic fucosyltransferase explained why activity is higher in the S100 at the low concentration of GDP-fucose used in most assays, 0.36 μM. At 0.36 μM the concentration of GDP-fucose was near its apparent K_m for the S100 fucosyltransferase (1.7 μM), but well below the apparent K_m for the P100 enzyme (38.2 μM). The dependence on GDP-fucose concentration was also examined in the intact fractions to gain some insight into the

overall fucosylation process in the P100 fraction, including transport into the intact vesicles. The apparent Michaelis constants for the P100 activity in the presence of Tween-20 and in the intact fraction were 38.2 μM and 28.3 μM, respectively. The similarity of the apparent K_m values suggested that the GDP-fucose transporter in the P100 vesicles had a similar or lower K_m relative to that of the bulk P100 fucosyltransferase activity, since if it had a much higher apparent K_m, GDP-fucose transport would have been rate limiting. The GDP-fucose transporter from rat liver Golgi-enriched vesicles has an apparent K_m of 7.5 μM (Sommers and Hirschberg, 1982). The apparent K_m for the cytosolic fucosyltransferase is relatively low compared to that of the bulk P100 activity. Though the relative concentrations of GDP-fucose in the cytosol and vesicles are not known, vesicles have the ability to concentrate GDP-fucose relative to the outside (Perez and Hirschberg, 1986). Thus it is not unreasonable to predict that a cytosolic fucosyltransferase would have a higher affinity for GDP-fucose since the concentration of GDP-fucose is probably lower in the cytosol than in the vesicles.

The studies described in this chapter concerning the P100, characterized the bulk activity in the fraction and cannot differentiate among different fucosyltransferases that may be present. The fucosyltransferase activity in the P100 may be a product of different fucosyltransferases with

different specificities. This may be the case in *Dictyostelium* because, even though fucosyltransferases have not been well characterized in this organism, various fucosyltransferases have been localized to microsomes in other eukaryotes (Hirschberg and Snider, 1987; Kornfeld and Kornfeld, 1985). The fact that I was able to differentiate the bulk activity in the P100 from the cytosolic fucosyltransferase supported the idea that the fucosyltransferase in the S100 is unrelated to the P100 activity and thus endogenous to the cytosol.

However, my observations do not rule out other possibilities. For example, the cytosolic fucosyltransferase could have derived from vesicles but was preferentially lost during isolation and the remaining enzyme, though with distinct properties from the majority of the P100 activity, is in the minority. The inability of EDTA to inhibit activity further when compared to no addition of divalent cations to the P100, may mean that the enzyme does not need cations at all. Conversely, since the bulk activity in the P100 is stimulated by cations, it is possible that the activity retains tightly bound cations which EDTA cannot remove. Another possibility is that the acceptor, FP21, is not present in the P100, either due to a cytosolic compartmentalization, or to leakage from the vesicles. A definitive confirmation that the cytosolic fucosyltransferase is different from any fucosyltransferase

activity in the P100 will require characterization of the purified fucosyltransferases from the S100 and P100.

The results obtained from my investigation are based on biochemical evidence in which a soluble fucosyltransferase partitioned with the cytosol. Other investigators have identified glycosylated proteins in the cytosol and/or nucleus and have searched for an enzyme responsible for the addition of the sugar (Haltiwanger et al., 1990). Their biochemical studies showed that an activity capable of adding GlcNAc to protein was recovered in both the soluble and membrane fractions (Haltiwanger et al., 1990). However, they showed that the membrane-associated activity was releasable by high salt treatment and was oriented towards the cytosol, not the lumen of the vesicles. Thus, it is possible that a fraction of this newly discovered cytosolic fucosyltransferase stayed associated with vesicles but since it was in a minority, remained masked by other P100 fucosyltransferases. As more synthetic acceptors become available, latency experiments in the presence and absence of detergent can be done to address this question. Alternatively, it is possible that a fucosyltransferase with enzymatic properties similar to the cytosolic fucosyltransferase is present in the lumen of P100 vesicles. Still this will not contradict my findings and will imply that there are two similar enzymes that reside in distinct compartments, as has been reported for another enzyme (Lewin

et al., 1990). In any event, I interpret the data presented as evidence for a fucosyltransferase in the cytosol of *Dictyostelium discoideum.*

The fact that Ax3 produced fucosylated FP21 suggested that, as it occurred in the mutant, the normal strain may have a cytosolic fucosyltransferase responsible for FP21 fucosylation. However, while activity was not detectable in Ax3 S100 fraction, it could be reconstituted by addition of mutant FP21, indicating that Ax3 possessed a cytosolic fucosyltransferase equivalent to the mutant fucosyltransferase.

In order to characterize the fucosyl linkage catalyzed by the cytosolic fucosyltransferase, several acceptors were used. Activity with synthetic acceptors was about an order of magnitude higher for the Ax3 extract, which may be attributed to competitive inhibition by the unfucosylated FP21 in the mutant. Of those tested, the only suitable acceptor was found to be a type I analog, 8-methoxycarbonyloctyl galß1,3GlcNAcß. Since the type II analog [8-methoxycarbonyloctyl galß1,4GlcNAcß] did not work as acceptor, it appears that the cytosolic fucosyltransferase may be an α1,4fucosyltransferase.

The cytosolic fucosyltransferase preferentially recognized a type I analog, suggesting it was an α1,4fucosyltransferase that lacked α1,3 activity. This activity would differ from other α1,4fucosyltransferase

described, which exhibit α1,3 activity as well (Kukowska-Latallo et al, 1990; Stroup et al., 1990). However, there are some limitations to the studies employing synthetic acceptors. To conserve synthetic acceptors, which were not commercially available, the concentration of the acceptors was well below the K_m (0.145 mM, while the apparent K_m was determined to be approximately 1 mM). The possibility still exists that the enzyme is able to use 8-methoxycarbonyloctyl galß1,4GlcNAcß as acceptor, but will only be evident at higher concentrations. Tentatively, an α1,4 specificity is being assigned to the cytosolic fucosyltransferase, but definitive proof will require characterization of the enzyme purified to homogeneity.

Finally, slug stage extracts were examined for fucosyltransferase activity, because it was found by metabolic labelling experiments in Chapter III that a fucosylated protein of 21 kD fractionated with the S100. The S100 and P100 fractions from HL250 had considerable activity, but from the Ax3 fractions only the P100 showed activity, consistent with the results from vegetative cells. The detection of a cytosolic fucosyltransferase in slug-stage cell extracts is consistent with their ability to fucosylate FP21 in vivo as determined by metabolic labelling. The apparent absence of activity in Ax3 cells indicated that, as found for vegetative stage cells, FP21 was quantitatively fucosylated.

CHAPTER V
SUMMARY AND CONCLUSIONS

Summary of Results

Fucosylation has generally been regarded as a modification restricted to the secretory compartment, however, there is evidence of fucosylated macromolecules in the nucleus and cytosol (see Chapter I). In the present study, I identified a novel fucosylation pathway in the cytosol of *Dictyostelium discoideum*. In the next three paragraphs a short summary is presented of the results reported in this dissertation, followed by a proposed model of fucosylation in the cytosol.

In chapter II the mutant HL250 was characterized as a conditional fucosylation mutant. The results are summarized as follows: 1) Spores and vegetative cells from the mutant strain contained negligible amounts of macromolecular-associated and total cell fucose when compared to the normal strain, Ax3, as determined chemically in acid hydrolysates. 2) The phenotype was conditional to growth in the absence of fucose. When vegetative cells were grown in fucose-supplemented media, they expressed macromolecular fucose conjugates. The fucose specific activity of the medium was not diluted relative to the intracellular fucose. 3)

Mutant extracts were incapable of carrying out the conversion of GDP-mannose to GDP-fucose in vitro. In other organisms, this pathway is the sole pathway of GDP-fucose synthesis in the absence of extracellular fucose. The low fucose biochemical phenotype can be explained by the model that the conversion pathway is defective. HL250 cells and extracts in vitro can still fucosylate, showing that GDP-fucose transport and fucosyltransferase(s) are still active. Although the possibility remains that there are other genetic defects in this mutagenized strain, there is no reason to suspect that other genes of the fucosylation pathway have been affected.

After determining that the source of macromolecular fucose in HL250 grown in normal medium was derived from extracellular fucose, I examined the compartmentalization of fucosylation. The results of the experiments described in Chapter III show the existence of a fucosylated protein in the cytosol and are summarized as follows: 1) The major fucosylated species in the S100 is FP21. It is present in both Ax3 and HL250. 2) Analysis of FP21 revealed that the oligosaccharide in FP21 was O-linked with a size of 4.8 glucose units. 3) FP21 appears to be endogenous to the cytosol, and not derived from a sedimentable compartment during preparation of the extracts. 4) Glycopeptides released from FP21 by pronase digestion differ from 21 kD MW

P100-derived glycopeptides, which reinforced the notion that contaminating P100 material was not the source of FP21.

The presence of a cytosolic fucosylated protein suggested the existence of a cytosolic fucosylation pathway. In vitro analysis of subcellular fractions led to the detection of a fucosyltransferase activity in the cytosol. The results of this investigation described in Chapter IV are summarized as follows: 1) Using a fucosylation assay dependent on endogenous acceptor substrates, I detected fucosyltransferase activity in cytosolic and vesicular fractions. 2) Activities from S100 and P100 fractions differed in the acceptor species fucosylated, their sensitivities to alkaline pH and divalent cations, and affinities for GDP-fucose, as evidenced by differences in apparent K_m. I consider these results to be an indication that the S100 fucosyltransferase did not arise from vesicles by rupturing during cell fractionation. 3) The cytosolic fucosyltransferase activity was absolutely dependent on availability of a non-fucosylated acceptor. Accordingly, in vitro cytosolic fucosylation could be detected in mutant extracts, but not in Ax3 fractions. However, cytosolic fucosyltransferase activity was reconstituted in Ax3 fractions by addition of purified mutant FP21. 4) A fucosyltransferase activity was detected in the S100 with the use of synthetic hydrophobic acceptors. Based on the utilization of these acceptors, the activity was determined

to be an $\alpha 1,4$fucosyltransferase lacking $\alpha 1,3$ activity. 5) Fucosylation of the type I acceptor analog (galβ1,3GlcNAcβ-8-methoxycarbonyloctyl) was inhibited by addition of purified FP21, suggesting the same activity was responsible for fucosylation of both molecules.

Based on the results obtained in my studies, I propose a model for fucosylation in *Dictyostelium*, acknowledging the existence of a fucosyltransferase in the cytosol that fucosylates a cytosolic protein, FP21. There are fucosyltransferases in vesicles and in the cytosol; the preferential acceptor for the cytosolic fucosyltransferase is FP21. This model is appealing because all of the elements necessary for fucosylation, biosynthesis of GDP-fucose, a fucosyltransferase, and the acceptor, compartmentalize in the cytosol. The model also concurs with emerging views of glycosylation in the cytosol (Hart et al., 1989a; Hart et al., 1989b). Initially I showed that *Dictyostelium* possesses a GDP-fucose conversion pathway similar to that reported earlier for other organisms (Kornfeld and Ginsburg, 1966; Liao and Barber, 1971; Ripka et al., 1986). It was shown that *Dictyostelium* can convert GDP-mannose into GDP-fucose, and that when this biosynthetic pathway is defective, GDP-fucose is formed from fucose supplied in the extracellular medium by the salvage pathway. This is the first time evidence has been presented that suggests *Dictyostelium* has GDP-fucose biosynthetic pathways

similar to those found in bacteria (Kornfeld and Ginsburg, 1966), a higher plant (Liao and Barber, 1971), and mammalian cells (Ripka et al., 1986; Reitman et al., 1980).

However, this model is not the only one that could account for the data obtained during the course of my investigation. Alternatively, the presence of FP21 in the cytosol could be explained by fucosylation in vesicles and rapid posttranslational transport to the cytosol. The absence of FP21 and FP21-like glycopeptides in the vesicular fraction was interpreted earlier as evidence for the absence of FP21 in the P100. However, it does not rule out the possibility that FP21 was fucosylated in vesicles and soon thereafter transported back into the cytosol, but was not detected because it did not accumulate in the P100. The presence of a fucosyltransferase in the cytosol would then be accounted by leakage from the vesicular fraction. Clearly, this model must then explain the export of FP21 into the cytosol by novel and unknown mechanisms. Another model that would account for my results is that both FP21 and the fucosyltransferase detected in the S100 leaked into the supernatant during fractionation. Since known vesicular markers were shown to remain in the P100, this model would require FP21, from all fucoconjugates in the P100, to be released preferentially. It would also require the leakage of a fucosyltransferase capable of fucosylating FP21. Nevertheless, in order to distinguish between the model

proposed and the other possible models, additional studies are needed.

Future Studies

The results presented in this dissertation lay the ground work for future studies with immense possibilities. With the help of a strain with a conditional fucosylation mutation, I was able to recognize the presence of a fucosylation pathway that otherwise may have gone undetected. Future research should focus on FP21 or the cytosolic fucosyltransferase.

Studies on FP21

The first question to be addressed will be the compartmentalization of FP21 using an independent approach from that followed in my studies. An initial step would be to raise antibodies against FP21. A protocol to purify FP21 is being improved in the laboratory, and should prove useful for this purpose. An antibody against FP21 will be useful for immunolocalization of the acceptor in fixed cells. Currently, FP21 is detected by SDS-PAGE as a fucose-labelled 21 kD MW species. In the P100, the presence of other fucoconjugates of similar MW on SDS-PAGE could mask FP21, although, as discussed earlier, it appears that FP21 is not present in the P100 fraction. However, if FP21 was entirely released into the S100 fraction during cell fractionation

that would explain its absence in the P100. Alternatively, a higher MW precursor may exist in the P100. Immunolocalization of FP21 will help clarify this point. An antibody against FP21 will help in localizing FP21 in other compartments of the cell, if present, such as in the nucleus or nuclear membranes.

Another aspect of interest is the other sugar residues present in the FP21 oligosaccharide. Due to its size (4.8 glucose units) I suspect the carbohydrate moiety is truly an oligosaccharide, containing more than one sugar residue. There is evidence for a peptide-GlcNAc transferase in the cytosol of rat hepatocytes (Haltiwanger et al., 1990), so it is possible that the oligosaccharide is O-linked to the polypeptide backbone via a GlcNAc residue. The fact that the FP21 radioactive peak released by alkaline hydrolysis was not symmetric, suggested there is more than one type of oligosaccharide. The first step would be to separate and purify the oligosaccharides. For this purpose, a longer P-4 column could be used. Alternatively, the oligosaccharides could be separated by other chromatographic methods (Townsend et al., 1989; Beniak et al., 1988). Once separated, the oligosaccharides can be examined by nuclear magnetic resonance spectrometry.

Fucosylated FP21 was present in the cytosol of vegetative and developing cells, but at this moment the relative levels of expression at different developmental

times, nor if its preferentially expressed in any cell type during development, have been determined. Ideally, it would be useful to produce antibodies with specificity for the glycosylated protein, and specificity for the peptide moiety of FP21 (similar to other mAb produced in the laboratory; see West et al., 1986). Using these antibodies, fucosylation of FP21 during development could be followed by immunoprecipitation of FP21.

Studies on the Cytosolic Fucosyltransferase

Another aspect of my project was the evidence presented for a novel fucosyltransferase that appears to be cytosolic and seems to differ from the bulk sedimentable fucosyltransferase activity. The first question to be addressed will be the compartmentalization of the enzyme. The cytosolic enzyme could be purified by conventional methods (Beyer et al., 1980; Foster et al., 1991; Martin et al., 1987). Once purified, antibodies could be raised against the enzyme and used for immunolocalization of the fucosyltransferase. Currently, a purification protocol is being developed in the laboratory. If localization of the enzyme is done by immunofluorescence and the enzyme is a soluble cytosolic protein, it should be possible to observe a cytosolic distribution of the enzyme and an absence from intracellular vesicles. However, if the immunofluorescence pattern shows labelling of vesicles, the results will need

to be examined more carefully. It is possible that the enzyme fucosylates the cytosolic acceptor, FP21, while being membrane-associated, but facing the cytosol. There is a membrane-associated glycosyltransferase that utilizes cytosolic acceptors (Haltiwanger et al., 1990). If a portion of the fucosyltransferase pool was to partition to the outside of the vesicles, the activity on intact vesicles should fucosylate the type I analog. Fucosylation of this synthetic acceptor by intact P100 vesicles should be dependent on added Mg^{++}.

Another approach to study the cytosolic fucosyltransferase is to clone and sequence the enzyme, avoiding purification of the protein. To date, only two fucosyltransferases have been sequenced, one encodes an α1,3/1,4fucosyltransferase and the other an α1,3fucosyltransferase (Kukowska-Latallo et al., 1990; Goelz et al., 1990). There is 57% identity between the two enzymes at the C-terminus, for a stretch of two-thirds the length of the protein (Goelz et al., 1990). Both enzymes appear to be type II transmembrane proteins, each composed of a short amino-terminal cytoplasmic domain with no discernible signal sequence, and a putative single transmembrane signal/anchor domain (Kukowska-Latallo et al., 1990; Goelz et al., 1990). The sequenced fucosyltransferases possessed N-linked glycosylation sites, and one of them was shown to be a glycoprotein (Kukowska-

Latallo et al., 1990). Since the fucosyltransferase reported in my studies appears to be cytosolic, it would be important to determine what is the relationship between microsomal and cytosolic fucosyltransferases. All fucosyltransferases utilize the same sugar nucleotide donor, GDP-fucose, so it is likely that the GDP-fucose binding site would be similar for all enzymes. In addition, comparisons among the fucosyltransferases may reveal important information regarding intracellular targeting and possible evolutionary relationships. There is evidence that a retaining sequence allows glycosyltransferases to remain in the Golgi apparatus and endoplasmic reticulum (Paulson and Colley, 1989). The fact that the fucosyltransferase reported in these studies localizes to the cytosol raises the possibility that the fucosyltransferase would lack the targeting and retaining sequences.

In order to compare the cytosolic fucosyltransferase with the sequenced fucosyltransferases (Kukowska-Latallo et al., 1990; Goelz et al., 1990), it will be necessary to sequence the cytosolic fucosyltransferase. The aforementioned enzymes were cloned using a gene transfer system in which cloned cDNAs determined the expression of the enzyme in a recipient host that did not express such activity. It could be possible to do the same for the cytosolic fucosyltransferase, using the type I analog synthetic acceptor to screen for activity of transfected

clones. A suitable host to express the cytosolic fucosyltransferase cDNA would be a mutant *Dictyostelium* strain, although there are no such mutants available at the present. On the other hand, yeast could be used, since it has been shown yeast cells do not carry out fucosylation (Kukuruzinska et al., 1987). One of the complications that may arise in trying to screen for clones expressing the cytosolic fucosyltransferase is the transfection of microsomal fucosyltransferases. It remains to be determined whether the P100 fucosyltransferase activity is capable of fucosylating the type I analog. If the activity in the P100 does not utilize the type I analog [galß(1,3)GlcNAcß-8-methoxycarbonyloctyl] as acceptor, then clones can be screened using the synthetic acceptor. However, if there are fucosyltransferases in the P100 that utilize the type I analog as acceptor, it will be necessary to differentiate the activity in vitro before the transfection experiments. P100 extracts will be assayed for the ability to fucosylate galß1,3GlcNAcß-8-methoxycarbonyloctyl. If the fraction fucosylates the acceptor, the sensitivity to cations will be examined for possible differences with the cytosolic fucosyltransferase activity. If the fraction is active in the presence of EDTA, fucosyltransferase positive clones may be screened in the presence and absence of Mg^{++}. Those that express activity only in the presence of Mg^{++} may represent positive clones. In the event that the activity in the P100

is dependent on Mg^{++} in a fashion similar to the cytosolic fucosyltransferase, other inhibitors should be tried, including tunicamycin and N-ethylmaleimide (Galland et al., 1988; Campbell and Stanley; 1984).

REFERENCES

Abeijon, C. and C.B. Hirschberg. 1990. Topography of initiation of N-glycosylation reactions. *J Biol. Chem.* 265:14691-14695.

Aparicio, J., G.W. Erdos, and C.M. West. 1990. Spore coat is altered in *mod* B glycosylation mutants of *Dictyostelium discoideum*. *J. Cell. Biochem.* 42:255-266.

Arion, W.J., L.M. Ballas, A.J. Lange, and B.K. Wallin. 1976. Microsomal membrane permeability and the hepatic glucose-6-phosphatase system. Interactions of the system with D-mannose- 6-phosphate and D-mannose. *J. Biol. Chem.* 251:4901-4907.

Barondes, S.H. and P.L. Haywood. 1979. Comparison of developmentally regulated lectins from three species of cellular slime mold. *Biochim. Biophys. Acta* 550:297-308.

Bartles, J.R. and W.A. Frazier. 1980. Preparation of ^{125}I-discoidin I and the properties of its binding to *Dictyostelium discoideum* cells. *J. Biol. Chem.* 255:30-38.

Beniak, B., J. Orr, I. Brockhausen, G. Vella, and C. Phoebe. 1988. Separation of neutral reducing oligosaccharides derived from glycoproteins by HPLC on a hydroxylated polymeric support. *Anal. Biochem.* 175:96-105.

Bennett, G., C.P. Leblond, and A. Haddad. 1974. Migration of glycoprotein from the Golgi apparatus to the surface of various cell types as shown by radioautography after labeled fucose injection into rats. *J. Cell Biol.* 60:259-284.

Beyer, T.A. and R.L. Hill. 1980. Enzymatic properties of the ß-galactoside α1-2 fucosyltransferase from porcine submaxillary gland. *J. Biol. Chem.* 255:5373-5379.

Beyer, T.A., J.E. Sadler, and R.L. Hill. 1980. Purification to homogeneity of the H blood group ß-galactoside α1-2 fucosyltransferase from porcine submaxillary gland. *J. Biol. Chem.* 255:5364-5372.

Biermann, C.J. 1988. Hydrolysis and other cleavages of glycosidic linkages in polysaccharides. *Adv. Carbohydr. Chem. Biochem.* 46:251-271.

Borts, R.H. and R.L. Dimond. 1981. The a-glucosidases of *Dictyostelium discoideum*. *Develop. Biol.* 87:176-184.

Campbell, C. and P. Stanley. 1984. The chinese hamster ovary glycosylation mutants LEC11 and LEC12 express two novel GDP-fucose:N-acetylglucosaminide 3-α-L-fucosyltransferase enzymes. *J. Biol. Chem.* 259:11208-11214.

Chen, P.S., Jr., T.Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Analyt. Chem.* 28:1756-1758.

Chu, F.K. 1986. Requirements of cleavage of high mannose oligosaccharides in glycoproteins by peptide N-glycosidase F. *J. Biol. Chem.* 261:172-177.

Darnell, J., H. Lodish, and D. Baltimore. 1986. Molecular Cell Biology. Scientific American Books, Inc., New York.

Das, O.P. and E.J. Henderson. 1986. A novel technique for gentle lysis of eukaryotic cells: isolation of plasma membranes from *Dictyostelium discoideum*. *Biochim. Biophys. Acta* 736:43-56.

Davis, L.I. and G. Blobel. 1987. Nuclear pore complex contains a family of glycoproteins that includes p62: glycosylation through a previously unidentified cellular pathway. *Proc. Natl. Acad. Sci. USA* 84:7552-7556.

Dimond, R.L., R.A. Burns, and K.B. Jordan. 1981. Secretion of lysosomal enzymes in the cellular slime mold *Dictyostelium discoideum*. *J. Biol. Chem.* 256:6565-6572.

Erdos, G.W. and C.M. West. 1989. Formation and organization of the spore coat of *Dictyostelium discoideum*. *Exp. Mycol.* 13:169-182.

Erdos, G.W. and D. Whitaker. 1983. Failure to detect immunocytochemically reactive endogenous lectin on the cell surface of *Dictyostelium discoideum*. *J. Cell Biol.* 97:993-1000.

Finne, J., T. Krusius, R.K. Margolis, and R.U. Margolis. 1979. Novel mannitol-containing oligosaccharides obtained by mild alkaline borohydride treatment of a chondroitin sulfated proteoglycan from brain. *J. Biol. Chem.* 254:10295-10300.

Flowers, H.M. 1981. Chemistry and biochemistry of D- and L-fucose. *Adv. Carbohydr. Chem. Biochem.* 39:279-345.

Foster, C.S., D.R.B. Gillies, and M.C. Glick. 1991. Purification and characterization of GDP-L-fuc-N-acetyl-ß-D-glucosaminide α1-3fucosyltransferase from human neuroblastoma cells. Unusual substrate specificities of the tumor enzyme. *J. Biol. Chem.* 266:3526-3531.

Franke, J. and R. Kessin. 1977. A defined minimal medium for axenic strains of *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA* 74:2157-2161.

Galland, S., A. Degiuli, J. Frot-Coutaz, and R. Got. 1988. Transfer of N-acetylglucosamine to endogenous glycoproteins in the nucleus and in non-nuclear membranes of rat hepatocytes: electrophoretic analysis of the endogenous acceptors. *Biochem. Intl.* 17:59-67.

Goelz, S.E., C. Hession, D. Goff, B. Griffiths, R. Tizard, B. Newman, G. Chi-Rosso, and R. Lobb. 1990. ELFT: a gene that directs the expression of an ELAM-1 ligand. *Cell.* 63:1349-1356.

Goldberg, A.H., L.C. Yeoman, and H. Busch. 1978. Chromatin-associated glycoproteins of normal rat liver and Novikoff hepatoma ascites cells. *Cancer Res.* 38:1052-1056.

González-Yanes, B., R.B. Mandell, M. Girard, S. Henry, O. Aparicio, M. Gritzalli, R.D. Brown, Jr., G.W. Erdos, and C.M. West. 1989. The spore coat of a fucosylation mutant in *Dictyostelium discoideum*. *Develop. Biol.* 133:576-587.

Goodloe-Holland, C.M. and E.J. Luna. 1987. Purification and characterization of *Dictyostelium discoideum* plasma membranes. *Meth. Cell Biol.* 28:215-229.

Gregg, J.H. and G.C. Karp. 1978. Patterns of cell differentiation revealed by L-[^{3}H]fucose incorporation in *Dictyostelium*. *Exptl. Cell Res.* 112:31-46.

Hallgreen, P., A. Lundblad, and S. Svensson. 1975. A new type of carbohydrate-protein linkage in a glycopeptide from normal human urine. *J. Biol. Chem.* 250:5312-5314.

Haltiwanger, R.S., G.D. Holt, and G.W. Hart. 1990. Enzymatic addition of O-GlcNAc to nuclear and cytoplasmic proteins. *J. Biol. Chem.* 265:2563-2568.

Hardy, M.R., R.R. Townsend, and Y.C. Lee. 1988. Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection. *Anal. Biochem.* 170:54-62.

Hart, G.W., R.S. Haltiwanger, G.D. Holt, and W.G. Kelly. 1989a. Glycosylation in the nucleus and cytoplasm. *Annu. Rev. Biochem.* 58:841-874.

Hart, G.W., R.S. Haltiwanger, G.D. Holt, and W.G. Kelly. 1989b. Nucleoplasmic and cytoplasmic glycoproteins. *Ciba Found. Symp.* 145:102-118.

Henderson, P.J.F. 1985. Statistical analysis of enzyme kinetic data. In: Techniques in the Life Sciences, vol. B1/II, Protein and Enzyme Biochemistry, BS114:1-48, Elsevier Scientific Publishers, Ltd., Ireland.

Hirschberg, C.B., M. Perez, M.D. Snider, W.L. Hanneman, J. Esko, and C.R.H. Raetz. 1982. Autoradiographic detection and characterization of a chinese hamster ovary cell mutant deficient in fucoproteins. *J. Cell. Physiol.* 111:255-263.

Hirschberg, C.B. and M.D. Snider. 1987. Topography of glycosylation in the rough endoplasmic reticulum and golgi apparatus. *Ann. Rev. Biochem.* 56:63-87.

Ivatt, R.L., O.P. Das, E.J. Henderson, and P.W. Robbins. 1984. Glycoprotein biosynthesis in *Dictyostelium discoideum*: developmental regulation of the protein-linked glycans. *Cell* 38:561-567.

Jackson, S.P. and R. Tjian. 1988. O-Glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. *Cell* 55:125-133.

Kan, F.W.K. and P. Pinto da Silva. 1986. Preferential association of glycoproteins to the euchromatin regions of cross-fractured nuclei is revealed by fracture-label. *J. Cell Biol.* 102:576-586.

Kawasaki, T. and I. Yamashina. 1972. Isolation and characterization of glycopeptides from rat liver nuclear membrane. *J. Biochem.* 72:1517-1525.

Knecht, D.A., D.L. Fuller, and W.F. Loomis. 1987. Surface glycoprotein, gp24, involved in early adhesion. *Develop. Biol.* 121:277-283.

Kohnken, R.E. and E.A. Berger. 1987. Assay and characterization of carbohydrate binding by the lectin discoidin I immobilized on nitrocellulose. *Biochemistry* 25:3949-3957.

Kornfeld, R.H. and V. Ginsburg. 1966. Control of synthesis of guanosine 5'-diphosphate D-mannose and guanosine 5'-diphosphate L-fucose in bacteria. *Biochim. Biophys. Acta* 117:79-87.

Kornfeld, R. and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631-664.

Kornfeld, K., M.L. Reitman, and R. Kornfeld. 1981. The carbohydrate-binding specificity of pea and lentil lectins. Fucose is an important determinant. *J. Biol. Chem.* 256:6633-6640.

Kukowska-Latallo, J.F., R.D. Larsen, R.P. Nair, and J.B. Lowe. 1990. A cloned human cDNA determines expression of a mouse stage-specific embryonic antigen and the Lewis blood group (α1,3/α1,4)fucosyltransferase. *Genes Dev.* 4:1288-1303.

Kukuruzinska, M.A., M.L.E. Bergh, and B.J. Jackson. 1987. Protein glycosylation in yeast. *Ann. Rev. Biochem.* 56:915-944.

Kumazaki, T. and A. Yoshida. 1984. Biochemical evidence that secretor gene, *Se*, is a structural gene encoding a specific fucosyltransferase. *Proc. Natl. Acad. Sci. USA* 81:4193-4197.

Lam, T.Y. and C.-H. Siu. 1981. Synthesis of stage-specific glycoproteins in *Dictyostelium discoideum* during development. *Develop. Biol.* 83:127-137.

Levy-Wilson, B. 1983. Glycosylation, ADP-ribosylation, and methylation of *Tetrahymena* histones. *Biochemistry* 22:484-489.

Lewin, A.S., V. Hines, and G.M. Small. 1990. Citrate synthase encoded by the CIT2 gene of *Saccharomyces cerevisiae* is peroxisomal. *Mol. Cell Biol.* 10:1399-1405.

Liao, T.-H. and G.A. Barber. 1971. The synthesis of guanosine 5'-diphosphate L-fucose by enzymes of a higher plant. *Biochim. Biophys. Acta* 230:64-71.

Lichtsteiner, S. and U. Schibler. 1989. A glycosylated liver-specific transcription factor stimulated transcription of the albumin gene. *Cell* 57:1179-1187.

Lis, H. and N. Sharon. 1986. Lectins as molecules and as tools. *Annu. Rev. Biochem.* 55:35-67.

Loomis, W.F. 1971. Sensitivity of *Dictyostelium discoideum* to nucleic acid analogues. *Exptl. Cell Res.* 64:484-486.

Loomis, W.F. 1987. Genetic tools for *Dictyostelium discoideum. Methods Cell Biol.* 28:31-65.

Magner, J.A., W. Novak, and E. Papagiannes. 1986. Subcellular localization of fucose incorporation into mouse thyrotropin and free a-subunits: studies employing subcellular fractionation and inhibitors of the intracellular translocation of proteins. *Endocrinology* 119:1315-1328.

Margolis, R.U., K. Lalley. W.-L. Kiang, C. Crockett, and R.K. Margolis. 1976. Isolation and properties of a soluble chondroitin sulfate proteoglycan from brain. *Biochem. Biophys. Res. Comm.* 73:1018-1024.

Martin, A., M.-C. Biol, M. Richard, and P. Louisot. 1987. Purification and separation of two soluble fucosyltransferase activities of small intestinal mucosa. *Comp. Biochem. Physiol.* 87B:725-731.

McMahon, D., M. Miller, and S. Long. 1977. The involvement of the plasma membrane in the development of *Dictyostelium discoideum. Biochim. Biophys. Acta* 465:224-241.

Nicolson, G., M. Lacorbière, and P. Delmonte. 1972. Outer membrane terminal saccharides of bovine liver nuclei and mitochondria. *Exptl. Cell Res.* 71:468-473.

Nuñez, H.A. and R. Barker. 1976. The metal ion catalyzed decomposition of nucleoside diphosphate sugars. *Biochemistry* 15:3843-3847.

Palcic, M.M., L.D. Heerze, M. Pierce, and O. Hindsgaul. 1988. The use of hydrophobic synthetic glycosides as acceptors in glycosyltransferase assays. *Glycoconjugate J.* 5:49-63.

Paulson, J.C. and K.J. Colley. 1989. Glycosyltransferases. Structure, localization, and control of cell type-specific glycosylation. *J. Biol. Chem.* 264:17615-17618.

Paulson, J.C., J.-P. Prieels, L.R. Glasgow, and R.L. Hill. 1978. Sialyl- and fucosyltransferases in the biosynthesis of asparaginyl-linked oligosaccharides in glycoproteins. Mutually exclusive glycosylation by ß-galactoside α2-6 sialyltransferase and N-acetylglucosaminide α1-3 fucosyltransferase. *J. Biol. Chem.* 253:5617-5624.

Perez, M. and C.B. Hirschberg. 1986. Transport of sugar nucleotides and adenosine 3'-phosphate 5'-phosphosulfate into vesicles derived from the Golgi apparatus. *Biochim. Biophys. Acta* 864:213-222.

Poole, S., R.A. Firtel, and E. Lamar. 1981. Sequence and expression of the discoidin I gene family in *Dictyostelium discoideum*. *J. Mol. Biol.* 153:273-289.

Potvin, B., R. Kumar, D.R. Howard, and P. Stanley. 1990. Transfection of a human α-(1,3)fucosyltransferase gene into chinese hamster ovary cells. Complication arise from activion of endogenous α-(1,3)fucosyltransferases. *J. Biol. Chem.* 265:1615-1622.

Rajan, V.P., R.D. Larsen, S. Ajmera, L.K. Ernst, and J.B. Lowe. 1989. A cloned human DNA restriction fragment determines expression of a GDP-L-fucose:ß-D-galactoside 2-a-L-fucosyltransferase in transfected cells. *J. Biol. Chem.* 264:11158-11167.

Reeves, R. and D. Chang. 1983. Investigations of the possible functions for glycosylation in the high mobility group proteins. Evidence for a role in nuclear matrix association. *J. Biol. Chem.* 258:679-687.

Reeves, R., D. Chang, and S.-C. Chung. 1981. Carbohydrate modifications of the high mobility group proteins. *Proc. Natl. Acad. Sci. USA* 78:6704-6708.

Reitman, M.L., I.S. Trowbridge, and S. Kornfeld. 1980. Mouse lymphoma cell lines resistant to pea lectin are defective in fucose metabolism. *J. Biol. Chem.* 255:9900-9906.

Richard, M., A. Martin, and P. Louisot. 1975. Evidence for glycosyl-transferases in rat liver nuclei. *Biochem. Biophys. Res. Comm.* 64:108-114.

Ripka, J., A. Adamany, and P. Stanley. 1986. Two chinese hamster ovary glycosylation mutants affected in the conversion of GDP-mannose to GDP-fucose. *Arch. Biochem. Biophys.* 249:533-545.

Ripka, J. and P. Stanley. 1986. Lectin-resistant CHO cells: selection of four new pea lectin-resistant phenotypes. *Somatic Cell Mol. Genet.* 12:51-62.

Satir, B.H., C. Srisomsap, M. Reichman, and R.B. Marchase. 1990. Parafusin, an exocytic-sensitive phosphoprotein, is the primary acceptor for the glucosylphosphotransferase in *Paramecium tetraurelia* and rat liver. *J. Cell Biol.* 111:901-907.

Scrimgeour, K.G. 1977. Chemistry and Control of Enzyme Reactions. Academic Press, London and New York.

Sève, A.-P., J. Hubert, D. Bouvier, C. Bourgeois, P. Midoux, A.-C. Roche, and M. Monsigny. 1986. Analysis of sugar-binding sites in mammalian cell nuclei by quantitative flow microfluorometry. *Proc. Natl. Acad. USA* 83:5997-6001.

Snider, M.D., L.A. Sultzman, and P.W. Robbins. 1980. Transmembrane location of oligosaccharide-lipid synthesis in microsomal vesicles. *Cell* 21:385-392.

Sommers, L.W. and C.B. Hirschberg. 1982. Transport of sugar nucleotides into rat liver Golgi. A new Golgi marker activity. *J. Biol. Chem.* 257:10811-10817.

Spielman, J., S.R. Hull, Z.Q. Sheng, R. Kanterman, A. Bright, K.L. Carraway. 1988. Biosynthesis of a tumor cell surface sialomucin. Maturation and effects of monensin. *J. Biol. Chem.* 263:9621-9629.

Stanley, P. 1984. Glycosylation mutants of animal cells. *Ann. Rev. Genet.* 18:525-552.

Stein, G.S., R.M. Roberts, J.L. Davis, W.J. Head, J.L. Stein, C.L. Thrall, J. van Veen, and D.W. Welch. 1975. Are glycoproteins and glycosaminoglycans components of the eukaryotic genome? *Nature* 258:639-641.

Stone, D.B., P.M.G. Curmi, and R.A. Mendelson. 1987. Preparation of deuterated actin from *Dictyostelium discoideum*. *Meth. Cell Biol.* 28:215-229.

Stroup, G.B., K.R. Anumula, T.F. Kline, and M.M. Caltabiano. 1990. Identification and characterization of two distinct α-(1-3)-L-fucosyltransferase activities in human colon carcinoma. *Cancer Res.* 50:6787-6792.

Tarentino, A.L., C.M. Gómez, and T.H. Plummer, Jr. 1985. Deglycosylation of asparagine-linked glycans by peptide:N-glycosidase F. *Biochemistry* 24:4665-4671.

Townsend, R.R., M.R. Hardy, D.A. Cumming, J.P. Carver, and B. Bendiak. 1989. Separation of branched sialylated oligosaccharides using high-pH anion-exchange chromatography with pulsed amperometric detection. *Anal. Biochem.* 182:1-8.

Tschursin, E., G.R. Riley, and E.J. Henderson. 1989. Differential regulation of glycoprotein sulfation and fucosylation during growth of *Dictyostelium discoideum*. *Differentiation* 40:1-9.

Virtanen, I. and J. Wartiovaara. 1976. Lectin receptor sites on rat liver cell nuclear membranes. *J. Cell Sci.* 22:335-344.

Weinstein, J., E.U. Lee, K. McEntee, P.-H. Lai, and J.C. Paulson. 1987. Primary structure of ß-galactoside α2,6-sialyltransferase. Conversion of membrane-bound enzyme to soluble forms by cleavage of the NH_2-terminal signal anchor. *J. Biol. Chem.* 262:17735-17743.

West, C.M. and S.A. Brownstein. 1988. EDTA treatment alters protein glycosylation in the cellular slime mold *Dictyostelium discoideum*. *Exptl. Cell Res.* 175:26-36.

West, C.M. and G.W. Erdos. 1988. The expression of glycoproteins in the extracellular matrix of the cellular slime mold *Dictyostelium discoideum*. *Cell Differ.* 23:1-6.

West, C.M. and G.W. Erdos. 1990. Formation of the *Dictyostelium* spore coat. *Dev. Genet.* 11:492-506.

West, C.M., G.W. Erdos, and R. Davis. 1986. Glycoantigen expression is regulated both temporally and spatially during development in the cellular slime molds *Dictyostelium discoideum* and *L. mucoroides*. *Mol. Cell. Biochem.* 72:121-140.

West, C.M. and W.F. Loomis. 1985. Absence of a carbohydrate modification does not affect the level or subcellular localization of three membrane glycoproteins in *mod*B mutants of *Dictyostelium discoideum*. *J. Biol. Chem.* 260:13803-13809.

Yamashita, K., T. Mizuochi, and A. Kobata. 1982. Analysis of oligosaccharides by gel filtration. *Meth. Enzymol.* 83:105-126.

Yurchenko, P.D. and P.H. Atkinson. 1975. Fucosyl-glycoprotein and precursor pools in HeLa cells. *Biochemistry* 14:3107-3114.

Yurchenko, P.D., C. Ceccarini, and P.H. Atkinson. 1978. Labeling complex carbohydrates of animal cells with monosaccharides. *Meth. Enzymol.* 50:175-204.

Zatz, M. and S.H. Barondes. 1971. Particulate and solubilized fucosyltransferases from mouse brain. *J. Neurochem.* 18:1625-1637.

Zinn, A.B., J.S. Marshall, and D.M. Carlson. 1978. Preparation of glycopeptides and oligosaccharides from thyroxine-binding globulin. *J. Biol. Chem.* 253:6761-6767.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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