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An IgG Monoclonal Antibody against *Dictyostelium discoideum* Glycoproteins Specifically Recognizes Fucα1,6GlcNAcβ in the Core of *N*-Linked Glycans

LOCALIZED EXPRESSION OF CORE-FUCOSYLATED GLYCOCONJUGATES IN HUMAN TISSUES*

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Core fucosylation of *N*-linked oligosaccharides (GlcNAcβ1,4(Fucα1,6)GlcNAcβ1-Asn) is a common modification in animal glycans, but little is known about the distribution of core-fucosylated glycoproteins in mammalian tissues. Two monoclonal antibodies, CAB2 and CAB4, previously raised against carbohydrate epitopes of *Dictyostelium discoideum* glycoproteins (Crandall, I. E. and Newell, P. C. (1989) *Development* 107, 87–94), specifically recognize fucose residues in α1,6-linkage to the asparagine-bound GlcNAc of *N*-linked oligosaccharides. These IgG3 antibodies do not cross-react with glycoproteins containing α-fucoses in other linkages commonly seen in *N*- or *O*-linked sugar chains. CAB4 recognizes core α1,6 fucose regardless of terminal sugars, branching pattern, sialic acid linkage, or polylactosamine substitution. This contrasts to lentil and pea lectins that recognize a similar epitope in only a subset of these structures. Additional GlcNAc residues found in the core of *N*-glycans from dominant Chinese hamster ovary cell mutants LEC14 and LEC18 progressively decrease binding. These antibodies show that many proteins in human tissues are core-fucosylated, but their expression is localized to skin keratinocytes, vascular and visceral smooth muscle cells, epithelia, and some extracellular matrix-like material surrounding subpopulations of lymphocytes. The availability of these antibodies now allows for an extended investigation of core fucose epitope expression in development and malignancy and in genetically manipulated mice.

L-Fucosyl residues in α1,6-linkage to the innermost GlcNAc ("core fucose") are relatively common in mammalian *N*-glycans. The enzyme GDP-L-fucose:2-acetamido-2-deoxy-β-D-glucoside (Fuc→Asn-linked GlcNAc) 6-α-L-fucosyltransferase, which catalyzes the transfer of L-fucose from GDP-fucose to the Asn-linked GlcNAc, has been purified from human skin fibroblasts (1) and substrate specificity studied in the porcine liver enzyme (2). The enzyme has recently been cloned from porcine brain (3). A great deal of attention has been drawn to the modifications near the nonreducing terminus of oligosaccharides with the success of demonstrating biological functions, *e.g.* sialyl Lewis^x in selectin binding (4), mannose 6-phosphate in lysosomal enzyme targeting (5), sialic acids in protein recognition (6), polysialic acids in neuronal development (7), and *N*-acetylgalactosamine 4-sulfate in pituitary hormonal regulation (8). In contrast, the biological significance of core modifications in *N*-glycans has not been clearly elucidated.

The presence of a core fucose residue greatly enhances recognition of *N*-linked sugar chains by lentil (*Lens culinaris* agglutinin) and pea (*Pisum sativum* agglutinin) lectins (9). Bourne *et al.* (10, 11) demonstrated that core fucose binds within a small crevice of *Lathyrus ochrus* isolectin II, but in its absence the Manα1,3Man arm of the oligosaccharide is in an energetically less favorable conformation that prevents strong binding. Thus fucose promotes the glycan to assume the critical conformation required for lectin binding. More recently, Stubbs *et al.* (12) showed that core fucose greatly influences the conformation and flexibility of the Manα1,6Man antenna of the biantennary oligosaccharide from porcine fibrinogen. These studies suggest that core fucose residues could play important roles in defining oligosaccharide conformations needed for specific carbohydrate-protein interactions. For example, core fucosylation is required for polysialylation of neural cell adhesion molecule by the specific polysialic acid synthase (13) and is involved in regulation of de-*N*-glycosylation by mammalian peptide *N*-glycosidases (14).

The expression of many oligosaccharides is known to be highly regulated in a tissue- and cell-specific manner, reflecting the differential regulation of glycosyltransferases (15). Enhanced core fucosylation of proteins such as α_1-fetoprotein and α_1-protease inhibitor in germ cell tumors, hepatocellular carcinomas, and other neoplasms (16–19) suggests that this modification may be restricted in normal human tissues. However, little is known about the tissue distribution of core-fucosylated glycoproteins in humans.

The literature is replete with histochemical studies that use lectins to detect glycoconjugate expression in tissues (for recent reviews see Refs. 20–22). Cytochemical staining obtained with *L. culinaris* agglutinin and *P. sativum* agglutinin is considered as chiefly indicating the presence of core-fucosylated glycans, although fucosylation only serves to enhance binding of these lectins to the trimannosyl core of complex oligosaccharides. Most of the lectin histochemistry studies of adult and embryonic mammalian tissues include *L. culinaris* agglutinin and *P. sativum* agglutinin as part of lectin "mixtures" (23–28), but in most cases the binding patterns have been similar to those obtained with concanavalin A. Lectin binding studies also have other inherent shortcomings, since many lectins with the same nominal specificity show different staining intensities for the

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same cell or tissue structure (29).

Monoclonal antibodies are more sensitive and specific than lectins, but many of the established carbohydrate-specific monoclonal antibodies are low affinity IgM types with significant cross-reactivities. IgG monoclonal antibodies with increased specificity and sensitivity would be more advantageous for *in situ* localization of oligosaccharides in tissues and for detection by immunoassays. Also, with the advent of new *in vivo* genetic approaches for elucidating oligosaccharide function (30), transgenic expressions or deletions of glycosyltransferases require high quality reagents to assess tissue-specific distribution of oligosaccharides. IgG monoclonal antibodies that recognize specific linkages should have a decided advantage over lectins that are often defined by their monosaccharide specificities.

During our study of a library of carbohydrate-specific monoclonal antibodies made against *Dictyostelium discoideum* glycoproteins, we found two IgG antibodies that specifically recognized fucose residues linked α1,6 to the Asn-bound GlcNAc of *N*-linked oligosaccharides. We used these antibodies to study the expression of core-fucosylated glycoconjugates in human tissues, and we find that they may have a much more restricted cell type localization than previously believed.

EXPERIMENTAL PROCEDURES

Fucosylated BSA[1] neoglycoproteins were generously provided by Dr. Ole Hindsgaul of the University of Alberta, Edmonton, Alberta, Canada. They were prepared and analyzed for sugar content as described previously (31, 32). *tert*-Butoxycarbonyl-L-tyrosine oligosaccharides from porcine fibrinogen (pFg) and reducing oligosaccharides from recombinant erythropoietin (EPO) were generous gifts from Dr. Kevin Rice, University of Michigan. They were characterized by proton NMR and fast atom bombardment-mass spectrometry or a combination of two-dimensional high pressure liquid chromatography mapping and enzymatic digestions (33, 34). Horseradish peroxidase (HRP), honeybee (*Apis mellifera*) venom phospholipase A_2 (PLA_2), pineapple stem bromelain, ovalbumin, pFg, porcine thyroglobulin (pTg), human lactoferrin, human α_1-acid glycoprotein, polyclonal rabbit anti-HRP, HRP-agarose, PLA_2 agarose, *Aspergillus* β-xylosidase, biotinylated *L. culinaris* agglutinin, *P. sativum* agglutinin, biotinylated anti-mouse IgG, avidin peroxidase, and immunoglobulin isotyping kit were purchased from Sigma. Biotinylated *Ulex europeus* agglutinin I lectin was from Vector Laboratories, Burlingam, CA. Streptavidin-biotin kit was from Dako, Carpenteria, CA. Goat anti-mouse IgG alkaline phosphatase conjugate was from Promega, Madison WI. *L. culinaris* agglutinin-alkaline phosphatase conjugate was obtained from E-Y Laboratories, San Mateo, CA. Chicken liver α-L-fucosidase was from Oxford Glycosystems, NY. Lumiphos 530 was from Lumigen Inc. Southfield, MI. Proteinase K was obtained from Boehringer Mannheim. Human tissues were obtained from the Tissue Core Facility of the Cancer Center, University of California, San Diego.

Production of Anti-Dictyostelium Monoclonal Antibodies

Production of monoclonal antibodies CAB2 and CAB4 against cell surface proteins of *D. discoideum* was described earlier (35). Immunoglobulin isotyping was done as per the manufacturer's instructions.

Immunoassays

Spectrophotometric ELISA—Reference glycoproteins or fucosylated BSA conjugates were immobilized on 96-well microtiter plates, and the wells were blocked with 3% BSA in Tris-HCl saline (TBS) overnight. They were washed and the antigens then allowed to react with the CAB antibodies at concentrations of 4 μg/ml IgG, in TBS containing 1% BSA and 0.1% Tween 20 for 1 h at room temperature. The plates were then washed and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG, followed by development with *p*-nitrophenyl phosphate substrate. They were read at 405 nm on an ELISA plate reader.

Chemiluminescence ELISA—pFg was coated onto the wells of FluoroNunc Maxi-sorb plates and blocked with 1% gelatin in phosphate-buffered saline (PBS). The wells were incubated with CAB4 at a concentration of 250 ng/ml in TBS containing 1% BSA and 0.2% Tween 20 for 2 h at 37 °C, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG. The plates were then developed with Lumiphos-530 and were read on an Anthos-LUCY1 luminometer.

Preparation of Human Tissue Homogenates

Human tissues were homogenized with a BioHomogenizer in 50 mM Tris-HCl, pH 7.5, containing 0.1 M 2-mercaptoethanol and 1% SDS. Suspensions were centrifuged at 650 $\times$ *g* for 15 min, and the postnuclear supernatants were harvested and centrifuged further for 30 min at 100,000 $\times$ *g*. After protein estimation, the supernatants were stored frozen until analysis.

CHO Mutant Cell Lysates

Cell lysates from LEC10, LEC14, LEC18, and Lec13 CHO cell mutants were kindly provided by Dr. Pamela Stanley, Albert Einstein College of Medicine, New York, NY. Cell extracts were made in 1.5% Triton X-100, and postnuclear supernatants were analyzed by CAB4 in immunoblots.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Proteins were separated by SDS-polyacrylamide gel electrophoresis in 12.5% polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked overnight with 10% skimmed milk in TBS or 3% BSA in TBS, washed with TBS containing 0.05% Tween 20, and incubated with either of the CAB antibodies at concentrations of 4 μg/ml IgG for reference proteins, 1 μg/ml for human tissue extracts, and 400 ng/ml for CHO cell lysates or with 2.5 μg to 5 μg/ml *L. culinaris* agglutinin-alkaline phosphatase conjugate for 1 h at room temperature. For the immunoblots this was followed by reaction with alkaline phosphatase-conjugated goat anti-mouse IgG. Bound proteins were visualized by incubating with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate.

Affinity Purification, Fractionation, and Characterization of Rabbit Anti-HRP

Antibodies to HRP are predominantly directed against core modifications on its *N*-glycans, specifically Xylβ1,2Manβ-, and Fucα1,3GlcNAcβ-Asn. Commercial rabbit polyclonal anti-HRP (purified IgG fraction) was affinity purified on a column of HRP-agarose and further fractionated into anti-Fucα1,3GlcNAc and anti-Xylβ1,2Manβ- components by a second affinity purification on a PLA_2 column as described (36). The bound anti-fucose component reacted with all plant glycoproteins carrying Fucα1,3GlcNAc in the core, and with PLA_2 which also carries the same modification, but it did not recognize Fucα1,6GlcNAcβ in the core of mammalian *N*-glycans. The anti-xylose component that was isolated from the run-through fraction of the PLA_2 column was repurified on the same column and was found to be completely free of the anti-fucose reactivity assayed with PLA_2. Details of the purification and characterization of these antibodies will be described elsewhere.

Enzyme Digestions

α-L-Fucosidase—One μg of honey bee venom PLA_2 or pFg were each incubated with 4 milliunits of chicken liver α-L-fucosidase at 37 °C for 20 h, in a total volume of 50 μl in 100 mM citrate/phosphate buffer, pH 6.0. A control tube containing each protein was incubated without added enzyme. (The digestion was done with and without prior denaturation of the proteins using 0.1% SDS, 100 °C for 2 min, and the results were essentially the same for both treatments.) After heat inactivation of the enzyme (100 °C, 5 min), the control and digested proteins were tested for binding to CAB4 antibody using ELISAs. In addition, control and digested PLA_2 were also tested against the anti-Fucα1,3GlcNAc component of anti-HRP to determine the specificity of the enzyme.

β-Xylosidase—One μg of HRP was incubated with 50 milliunits of *Aspergillus* β-xylosidase at 37 °C for 16 h, in 50 μl of 100 mM phosphate buffer, pH 6.0, containing 100 μM dithiothreitol. A control was incubated without added enzyme. After heat inactivation of the enzyme, the control and digested proteins were tested for binding to CAB4 using an ELISA. Efficiency of digestion was monitored by checking loss of reactivity of the digested protein with the purified anti-Xylβ1,2Manβ- fraction of anti-HRP.

[1] The abbreviations used are: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; PLA_2, phospholipase A_2; pFg, porcine fibrinogen; pTg, porcine thyroglobulin; EPO, erythropoietin; CHO, Chinese hamster ovary; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

TABLE I
Reactivity of CAB antibodies with some reference glycoproteins

Reactivities of all proteins except bovine fetuin are shown in Fig. 1 and/or Fig. 2. References for structural elucidation of each of these glycans are given in parentheses.

Glycoprotein	*N*-Glycan structure
Reactive	
Honey bee venom phospholipase A2	Oligomannose glycan, with core Fucα1,3GlcNAcβ/core Fucα1,6GlcNAcβ/ difucosylation at the core (37)
Porcine fibrinogen	Complex biantennary glycan, with core Fucα1,6GlcNAcβ (33)
Human lactoferrin	Complex biantennary glycan, with core Fucα1,6GlcNAcβ and outer Fucα1,3GlcNAcβ (38)
Non-reactive	
Horseradish peroxidase	Oligomannose glycan, with core Fucα1,3GlcNAcβ and Xylβ1,2Manβ (39)
Pineapple stem bromelain	Oligomannose glycan, with core Fucα1,3GlcNAcβ and Xylβ1,2Manβ (40)
Chicken egg albumin	Hybrid glycan, intersecting GlcNAc, no core substitution (42)
Human α_1-acid glycoprotein	Complex bi-, tri-, and tetraantennary glycans, with outer Fucα1,3GlcNAcβ and no core substitutions (43)
Bovine fetuin	Complex triantennary glycan, with no core substitutions (41)

Isolation of Glycopeptides—Core α1,3-fucosylated glycopeptides, core α1,6-fucosylated glycopeptides, and non-fucosylated glycopeptides from HRP, pTg/pFg, and ovalbumin respectively, were prepared from 50 mg of each protein by digesting with 2.5–5 mg of proteinase K in 0.2 M Tris-HCl buffer, pH 7.5, for 24 h. The reaction mixture was boiled for 10 min and centrifuged. The glycopeptides were lyophilized and purified on a Bio-Gel P2 column equilibrated with 0.1 M ammonium formate, pH 6.0. Fractions were assayed for neutral sugar, and void fractions were pooled and repeatedly lyophilized from water to remove ammonium formate. The glycopeptides were then reconstituted in water. Neutral sugar was measured by phenol sulfuric acid method, and total sugar concentration was calculated from the established structure of *N*-linked oligosaccharides from each protein.

Immunostaining of Tissues

Cryostat sections of human tissues (5-micron thickness) were cut and air-dried. Sections were fixed in 10% buffered formalin for 20 min followed by removal of the endogenous peroxidase with 0.03% hydrogen peroxide if necessary, and by blocking of nonspecific binding sites with 10% normal goat serum in PBS containing 1% BSA. Five-micron paraffin sections were deparaffinized and rehydrated before proceeding with the immunostaining. After washing, the antibodies were overlayered onto serial tissue sections at predetermined dilutions (usually between 1 and 10 μg/ml), and the slides were incubated in a humid atmosphere for 30 min at room temperature or overnight at 4 °C. The labeled streptavidin biotin kit from Dako was used as per the manufacturer's instructions or with PBS or TBS washes between every step, and biotinylated anti-mouse IgG was then applied for 10 min followed by either alkaline phosphatase or peroxidase-linked streptavidin for 10 min. After another wash, the appropriate substrate was added, and the slides were incubated in the dark for 20 min. After a wash in buffer, they were counterstained with hematoxylin, mounted, and viewed using an Olympus BH2 microscope. Lectin staining was carried out using biotinylated *U. europeus* agglutinin I, *L. culinaris* agglutinin, or *P. sativum* agglutinin. Incubation with the lectins was carried out in TBS containing 1% BSA and 1 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$, followed by alkaline phosphatase-conjugated streptavidin and Fast Red as the developer.

RESULTS

Characterization of the Epitope Recognized by CAB2 and CAB4 as Core Fucα1,6GlcNAc—CAB2 and CAB4 are members of a group of IgG monoclonal antibodies generated against cell surface glycoproteins of the slime mold *D. discoideum* (35). Reactivity of these antibodies to *Dictyostelium* cells or cell ghosts was lost or reduced by periodate treatment or digestion with endoglycosidase F, indicating that they were directed against *N*-linked oligosaccharide epitopes (35). In the present study, they were found to be of the IgG3 subclass. When tested in ELISAs against a panel of glycoproteins with established glycan structures (Table I), both the antibodies reacted with the following: 1) PLA_2 which has an oligomannose structure core substituted by fucose residues linked either α1,3 or α1,6 (or is occasionally difucosylated) (37); 2) pFg which has complex biantennary oligosaccharides, core-substituted with fucose linked α1,6 to GlcNAc (33); and 3) human lactoferrin which has complex biantennary oligosaccharides core-substituted with fucose linked α1,6 to GlcNAc, and additional fucose residues linked α1,3 to GlcNAc on the antennae (38). The antibodies did not bind to core α1,3-fucosylated plant proteins such as HRP (39) or pineapple stem bromelain (40). The absence of binding to the plant glycoproteins did not result from interference by a β1,2 xylose residue in the core region of these sugar chains, since β-xylosidase digestion of HRP did not increase CAB4 reactivity (Fig. 1). The effectiveness of this digestion is evident from >75% reduction in binding of an affinity purified antibody (see "Experimental Procedures") against β1,2 xylose (data not shown). CAB4 does not bind to non-core-fucosylated proteins such as 1) bovine fetuin, which has triantennary oligosaccharides, (41); or 2) ovalbumin, which has hybrid oligosaccharides, with an intersecting GlcNAc residue (42); or 3) human α_1-acid glycoprotein, which has complex bi-, tri-, and tetraantennary oligosaccharides, with some fucose residues linked α1,3 to an outer GlcNAc but lacks core fucose substitutions (43). These results indicated that the CAB2 and CAB4 antibodies are probably recognizing core Fucα1,6GlcNAc on *N*-linked glycans.

Fig. 1 shows CAB4 antibody binding to increasing amounts of pFg, PLA_2, HRP, and β-xylosidase-treated HRP measured by ELISA. Linearity was evident up to 100 ng with PLA_2 and pFg, with a lower detection limit of 2–5 ng. No reactivity was seen even with 250 ng of either native or dexylosylated HRP. Similar results were seen with CAB2 (not shown).

The specificity of both these antibodies was also established by Western blots (Fig. 2). Since the binding pattern for both antibodies is identical, data are shown only for CAB4. A nonrelevant monoclonal antibody served as a negative control. The antibodies recognized only core Fucα1,6GlcNAc containing proteins in the blots. Background binding seen with ovalbumin and bromelain was eliminated at higher antibody dilutions (<2 μg/ml). pFg, like other fibrinogens, is composed of three different polypeptides, Aα (69 kDa), Bβ (57 kDa), and γ (51 kDa) chains. The Bβ and γ chains carry core-fucosylated biantennary *N*-glycans (33) and are recognized by the CAB antibodies, but the non-glycosylated Aα chain is not. In addition, a higher molecular mass (79 kDa) band is also intensely stained by the antibody. Since fibrinogens are notoriously heterogeneous, this may represent catabolic intermediates of fibrinogen which are often present in plasma or other contaminating binding proteins from commercial pFg.

Binding Is Reduced When Core Fucα1,6GlcNAc-containing Proteins Are Defucosylated—Digestion of PLA_2 or pFg with chicken liver α-L-fucosidase, which cleaves fucose in α1→6, →2,

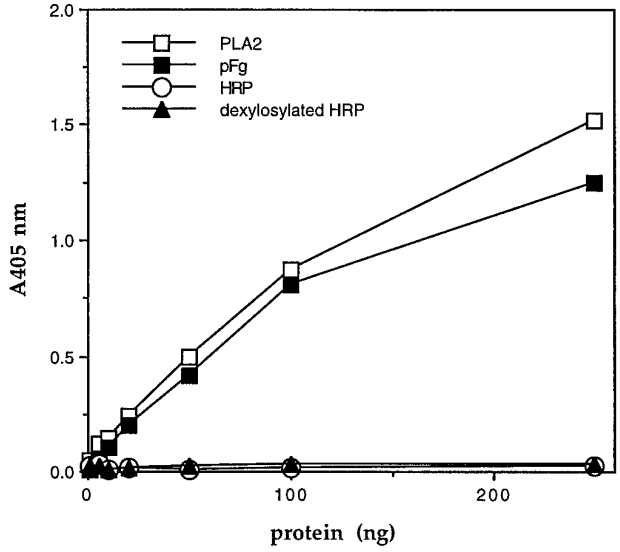

FIG. 1. **Characterization of the epitope recognized by CAB antibodies as core Fuc α1,6GlcNAc.** HRP, dexylosylated HRP, pFg, and bee venom PLA_2 were coated onto microtiter plates at concentrations ranging from 1 to 250 ng. Wells were incubated with CAB4 (4 μg/ml IgG) and then with goat anti-mouse IgG alkaline phosphatase. Plates were developed with *p*-nitrophenyl phosphate substrate and read at 405 nm. Similar results were obtained with CAB2 antibody (not shown).

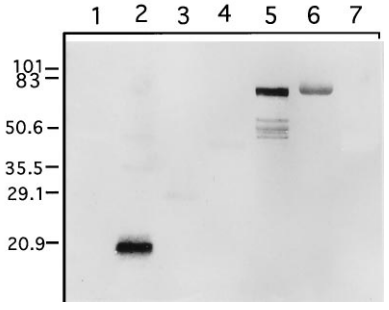

FIG. 2. **CAB antibodies recognize only core Fucα1,6GlcNAc containing proteins in Western blots.** 1 μg each of seven reference glycoproteins was separated on 12.5% polyacrylamide gels and blotted onto nitrocellulose membranes. After blocking, the membranes were probed with CAB4 (4 μg/ml IgG) followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG and developed with BCIP/NBT. *Lane 1*, HRP; *lane 2*, bee venom PLA2; *lane 3*, pineapple stem bromelain; *lane 4*, ovalbumin; *lane 5*, pFg; *lane 6*, human lactoferrin; *lane 7*, human α_1-acid glycoprotein. CAB2 showed identical staining pattern, and staining with an unrelated antibody was negative (not shown).

→3, →4 linkages, reduces binding >80% (Fig. 3). pFg has only core Fucα1,6GlcNAc, but PLA_2 also has core Fucα1,3GlcNAc. The digestion did not cleave core α1,3 fucose residues in PLA_2 since there was minimal loss of reactivity when probed with the affinity purified anti-Fucα1,3GlcNAc fraction of anti-HRP (Fig. 3, *inset*).

Binding of CAB4 to pFg Is Inhibited by pTg/pFg Glycopeptides and Reducing Oligosaccharides from Erythropoietin but Not by HRP or Ovalbumin Glycopeptides—Biantennary glycopeptides containing core-substituted Fucα1,6GlcNAc were generated from pTg (44) or pFg. These glycopeptides, or ones from HRP (core Fucα1,3GlcNAc) and ovalbumin (lacking core Fucα1,6GlcNAc), were then compared for their ability to inhibit CAB4 binding to pFg in spectrophotometric or chemiluminescent ELISA. The latter method was adopted when <1 nmol of free inhibitory oligosaccharides was available. As shown in Fig. 4, *A* and *B*, each assay gave comparable results. In both assays, HRP and ovalbumin glycopeptides did not block antibody binding, but pTg/pFg glycopeptides progressively inhibited CAB4 binding to pFg, again showing the specificity for core Fucα1,6GlcNAc. Biantennary core-fucosylated oligosaccharide from EPO (*EPO1*, Fig. 4*B*) and pFg (not shown) were equally effective inhibitors, showing that GlcNAc-asparagine linkage is probably not required for recognition.

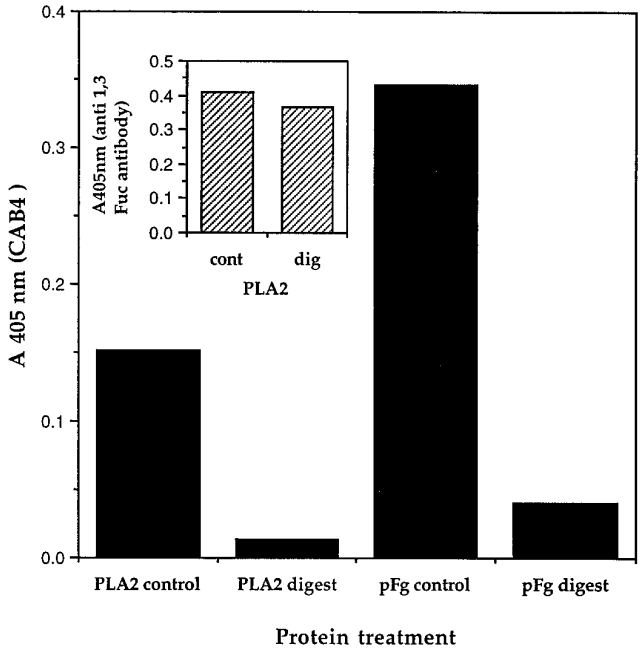

FIG. 3. **Binding of CAB antibodies to pFg or PLA_2 is decreased when the proteins are defucosylated.** 1 μg of PLA_2 or pFg was digested with chicken liver α-L-fucosidase overnight as indicated under "Experimental Procedures." A control tube for each protein without added enzyme was also incubated simultaneously. 100 ng protein each of control and digest were analyzed in an ELISA for binding to CAB4. The *inset* shows minimal loss of reactivity of PLA_2 with the anti-Fucα1,3GlcNAc fraction of anti-HRP, after digestion with the same fucosidase.

The Antibodies Do Not Recognize Fucose Residues in Other Linkages Such as Those Seen in Lewis Antigens—To confirm that the CAB antibodies did not recognize fucose residues in α1→2, →3, or →4 linkages to GlcNAc or Gal, we tested a panel of fucosylated oligosaccharide-BSA conjugates using the spectrophotometric immunoassay described in Fig. 1. Since the number of oligosaccharides per mol of BSA varied, all were normalized to the same molar content of glycan. The results in Table II show comparison of the binding of the various Fuc glycans to the reactivity of 1.5 and 25 pmol of pFg. The first quantity of protein is within the linear range of the assay for pFg, and the second is >10-fold above the linear range, but all the neoglycoproteins were read within the linear range of the assay. The antibodies did not recognize fucose residues in $Lewis^a$, $Lewis^b$, or $Lewis^y$, 3′-sialyl $Lewis^a$ or 3′-sialyl $Lewis^x$ (<0.1%). $Lewis^x$, Fucα1,3GlcNAcβ, Fucα1,4GlcNAcβ, and Fucα1,2Galβ1,3GlcNAcβ were very weakly recognized (<1.0%). This weak reactivity of the different Fuc glycans suggests that not only the fucose residue but the surrounding glycan and the linkage are important for recognition by the antibody.

CAB4 Recognizes Core Fucα1,6GlcNAc Found on Many Known Oligosaccharides—A chemiluminescence immunoassay described in Fig. 4*B* was used to test inhibition of CAB4 binding to pFg by a wide variety of structurally characterized core Fucα1,6GlcNAc-containing glycans. The results using two concentrations of each are shown in Table III. There is little difference between the inhibitions seen using *tert*-butoxycarbonyl-L-tyrosine-linked pFg biantennary chains terminated ei-

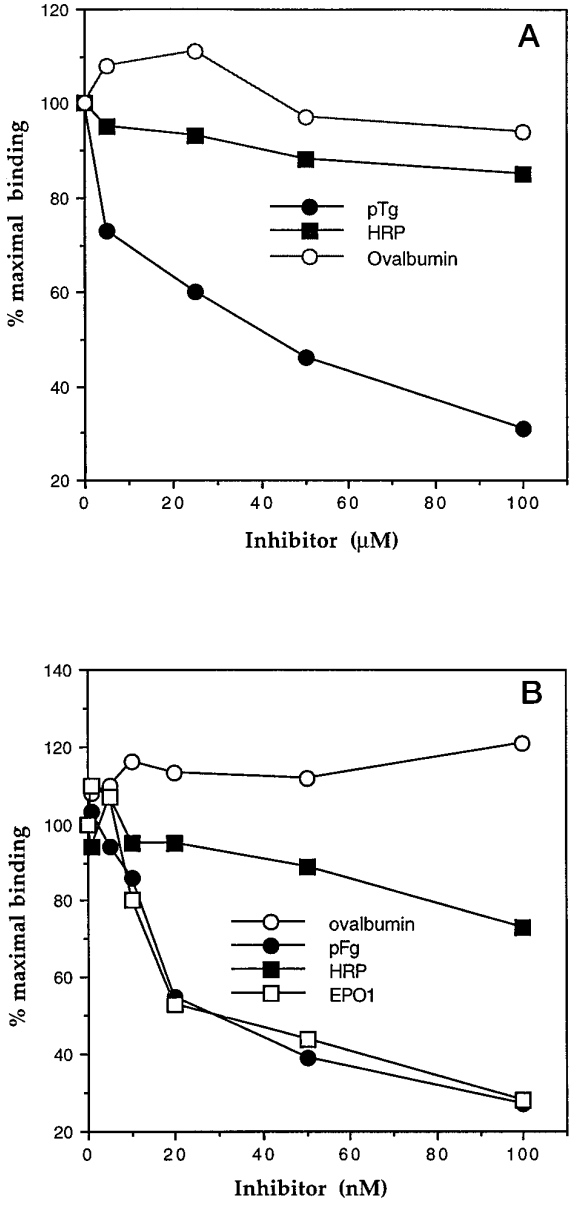

FIG. 4. **Binding of CAB4 to pFg is inhibited by pTg/pFg glycopeptides and biantennary EPO oligosaccharides but not HRP or ovalbumin glycopeptides.** 25 ng (*A*) or 10 ng (*B*) of pFg was coated onto an ELISA microwell plate (*A*) or FluoroNunc Maxi-sorb plate (*B*) and the wells were incubated with CAB4 (4 μg/ml IgG (*A*) or 250 ng/ml IgG (*B*)) in the presence of varying concentrations of ovalbumin, HRP, or pTg/pFg glycopeptides, or biantennary EPO oligosaccharide. The plates were developed with alkaline phosphatase-conjugated anti-mouse IgG, and *p*-nitrophenyl substrate (*A*) or Lumiphos 530 (*B*). Binding in the absence of inhibitor was considered 100%.

ther by Siaα2,6, Galβ1,4,GlcNAcβ1,2 or the typical trimannosyl core. Oligosaccharide chains from recombinant EPO give similar inhibitions to those seen using the biantennary chains from pFg. The EPO glycans include those with α2,3 Sia (EPO1), polylactosamine repeats (EPO5), triantennary chains with different branching patterns (EPO4), and tetraantennary chains (EPO8). Inhibition of CAB4 binding by the tri- and tetra-branched chains is significant since neither *P. sativum* agglutinin nor *L. culinaris* agglutinin lectins recognize core fucose when it is presented on tetraantennary and triantennary chains disubstituted on the α1,3 core mannose residue (45). These results show that CAB4 is not only highly specific but that it also recognizes a broader range of oligosaccharides than the lectins commonly used to detect core fucosylation.

Modifications in the N-Glycan Core Decrease Binding of CAB4 to Proteins in Immunoblots—The above results clearly show that the outer structures of sugar chains do not influence the binding of CAB4 to Fucα1,6GlcNAc, but it is possible that modifications in the core region, such as GlcNAc addition to the "bisecting" location (GlcNAcβ1, 4Manβ-), or β1,2 linked to Manβ-, or possibly (GlcNAcβ/α1, 6)GlcNAcβ1,4GlcNAc-Asn might block or reduce antibody binding. Each of these structures was recently reported to occur in CHO mutant cell lines LEC10, LEC14, and LEC18, respectively, due to the activation of quiescent GlcNAc-transferases (46–48). These mutants were originally selected for their resistance to ricin (LEC10) or pea lectin (LEC14 and LEC18). To determine if these modifications affected CAB4 binding, immunoblots of total cell lysates from wild-type CHO cells or from mutants LEC10, -14, and -18 were tested in immunoblots (Fig. 5). Bisecting GlcNAc (LEC10) did not significantly alter binding compared with the parental strain, whereas GlcNAcβ1,2Manβ- (LEC14) and substitution on the distal GlcNAc (LEC18) showed a progressive decrease in reactivity. However, detection of this difference required careful titration of the antibody; a 2-fold increase in antibody concentration eliminated the difference from the control (not shown). All of the bands were specific as shown by the competition with 50 μM pFg glycopeptides. As a positive control, lysates from Lec13 cells which cannot synthesize GDP-Fuc from GDP-Man and have fewer fucosylated chains (49) showed considerably reduced binding. Residual bands observed with Lec13 are also seen when specific CAB4 binding in lysates of parental cells is blocked by pFg (Fig. 5) or when Lec13 lysates are probed with *L. culinaris* agglutinin (not shown). This residual binding could be due to the ability of Lec13 cells to scavenge fucose, which could partially correct their phenotype (49). These results suggest that the currently known *N*-glycan core modifications have modest inhibitory effects on CAB4 binding.

Detection of Core-fucosylated Proteins on Immunoblots: A Wide Range of Proteins Carry the Modification—Western blots of proteins from different human tissues showed that many proteins were core-fucosylated (Fig. 6*A*) especially in the brain, heart, colon, ovary, placenta, and skin. Staining was less intense in the liver and kidney at the antibody dilutions (1 μg/ml) used but was appreciable at higher concentrations. Specific proteins were stained in the lung, tonsil, and spleen. Binding was abrogated when the blots were probed with the antibody in presence of 200 μM pTg glycopeptides or 50 μM pFg showing that binding was specific. Examples of these inhibitions are shown for heart, spleen, and skin.

Fig. 6*B* shows a comparison of *L. culinaris* agglutinin and immunoblots for brain, heart, skin, and placenta. An alignment of protein bands stained either by *L. culinaris* agglutinin or CAB4 shows that, depending on the tissue, approximately 35–50% of the bands corresponded to one another, although intensities varied; however, at a 20-fold lower molar concentration, the antibody produces considerably sharper bands and a lower background compared with the lectin.

Immunolocalization of Core-fucosylated Proteins in Normal Adult Human Tissues: Proteins Modified by Core Fucosylation Are Selectively Localized—We used CAB2 and CAB4 to localize the expression of core-fucosylated proteins in adult human tissues. Frozen and paraffin sections of heart, lung, liver, colon, pancreas, spleen, thymus, tonsil, ovary, skin, placenta, brain, and adrenal were examined. Although this modification is widespread, we obtained distinct and localized staining patterns with the antibodies. Since binding patterns were essentially the same for both the frozen and paraffin sections, only examples of frozen sections are presented in Fig. 7.

TABLE II

CAB antibodies do not recognize α-fucose residues in other linkages

The reactivity of pFg is considered 100% for each concentration. 1.5 pmol is within the linear range for pFg and gave a net A_{405} of 0.322. 1.5 pmol of Fuc-glycans gave a net A_{405} <0.011.

BSA-conjugate	Glycans/per BSA molecule	Structure	% binding *versus* pFg oligosaccharides	
			1.5 pmol	25 pmol[a]
1	17	Galβ1,3GlcNAcβ 4 ↑ Fucα1 (Lewis[a])	2.5	0.075
2	18	Fucα1,2Galβ1,3GlcNAcβ	1.8	0.17
3	18	Fucα1,2Galβ1,3GlcNAcβ 4 ↑ Fucα1 (Lewis[b])	2.2	0.05
4	38	Fucα1,4GlcNAcβ	3.3	0.56
5	16	Galβ1,4GlcNAcβ 3 ↑ Fucα1 (Lewis[x])	1.5	0.3
6	16	Fucα1,2Galβ1,4GlcNAcβ 3 ↑ Fucα1 (Lewis[y])	1.5	0.05
7	15	Fucα1,3GlcNAcβ	2.6	0.9
8	13	3′-SialylLewis[a]	2.9	0.05
9	9	3′-SialylLewis[x]	1.0	0.065

[a] 25 pmol is beyond the linear range for pFg, but extrapolation yields a A_{405} of 5.35. At 25 pmol, most Fuc-glycans gave a net A_{405} of <0.01. Their reactivity relative to that of pFg has been calculated from the extrapolated value for pFg.

In the skin, the antibodies stained the cytoplasm of keratinocytes and were especially prominent in the granular layer of the epidermis (Fig. 7*a*). Cells in the underlying dermis were not stained. The antibodies selectively stained smooth muscle cells in five different tissues. In the heart, smooth muscle of the coronary arterioles was stained (Fig. 7*b*). In the lungs, only smooth muscle cells surrounding the pulmonary arteriole were stained but not the smooth muscle cells in the wall of terminal or respiratory bronchiole (Fig. 7*c*). The bronchiolar epithelium and the endothelium of the blood vessels were also not stained. In the liver, the antibodies stained the smooth muscle cells lining the hepatic arteriole in the portal triad (Fig. 7*d*), but the endothelia of vessels, epithelium of bile duct, parenchymal, and Kupffer cells were negative. In the colon, smooth muscle cells of the muscularis mucosa were selectively stained (Fig. 7*e*), whereas the mucosal epithelium and the smooth muscle of the muscularis externa were negative. In the ovary, the antibodies stained the smooth muscle cells of the arteriolar walls, but the germinal epithelium, follicles, and corpus luteum were not stained (Fig. 7*f*).

In the palatine tonsil the squamous epithelium lining was positive, as was some extracellular matrix-like material (Fig. 7*g*) surrounding a subpopulation of lymphocytes. This was also seen in the spleen and thymus (Fig. 7*h*). In the brain, the white matter of the cerebellum was stained (not shown).

The specificity of CAB4 binding in tissue sections was confirmed by inhibition with pFg glycopeptides at 50 μM or less (not shown).

In contrast to the staining with the antibodies, biotinylated *L. culinaris* agglutinin or *P. sativum* agglutinin did not stain specific recognizable structures above background in both cryostat and paraffin-embedded sections (not shown). As a positive control, biotinylated *U. europeus* agglutinin I, which is specific for outer branch fucose in α1,2 linkage to Galβ1,4GlcNAc (50), distinctly stained vascular endothelial cells in a variety of tissues (not shown), in agreement with prior studies (51).

DISCUSSION

D. discoideum is a simple eukaryotic amoeba that can be induced to develop into a multicellular organism in response to lack of nutrients. Many of the glycans expressed during development in *Dictyostelium* are highly immunogenic (52), although this organism does not synthesize complex-type oligosaccharides found in mammalian cells (53). In *Dictyostelium*, α-L-fucose residues are presumed to be present both in the peripheral and in the core regions of neutral oligosaccharides. Those in the core are probably bound to the proximal GlcNAc on *N*-linked oligosaccharides since they are resistant to endoglycosidase H digestion (54, 55). Using the anti-Fucα1,3GlcNAc fraction of rabbit anti-HRP and the CAB4 antibody which recognizes core Fucα1,6GlcNAc, we have now obtained more direct evidence for the occurrence and developmental regulation of both types of core fucosylation in the glycoproteins of *D. discoideum*.[2]

In the synthesis of mammalian *N*-linked oligosaccharides, addition of fucose is believed to be a terminal event occurring exclusively on complex or hybrid structures (56). Identification of core fucose residues in the high mannose type glycoproteins of *D. discoideum* appears to be inconsistent with existing *in vitro* substrate specificity studies on mammalian core α1,6-fucosyltransferases which do not use oligomannose *N*-glycans as acceptors (1, 2). Although this could be explained as specificity difference, there is growing evidence that fucosylated oligomannose structures do occur in mammalian cells. Lin *et al.* (57) identified fucose residues in α1,6 linkage to core GlcNAc in the *N*-glycans of GlcNAc-transferase 1-deficient Lec-1 CHO cells, which cannot synthesize complex or hybrid *N*-glycans. More recently, Endo *et al.* (58) documented the presence of novel fucosylated high mannose type sugar chains in the oligosaccharides of the rat hepatoma alkaline phosphatase. It is possible that the substrate specificities of the fucosyltrans-

[2] G. Srikrishna, L. Wang, and H. H. Freeze, in preparation.

TABLE III

CAB4 recognizes core Fucα1,6GlcNAc on many known oligosaccharides

pFg was immobilized onto microtiter plates, and *t*-butoxycarbonyl (Tyr-Boc) pFg oligosaccharide conjugates or EPO oligosaccharides were used as competitors in the binding of CAB4 to pFg at 20 or 100 nM in chemiluminescent assays. Results are expressed as mean % of control binding, defined as 100%, in the absence of inhibitor. Each value is the mean of two experiments, using quadruplicate determinations for each concentration.

	Inhibitor	Concentration	Mean residual binding
		nM	%
	NeuAcα2–6Galβ1–4GlcNAcβ1–2Manα1 \ 6 Fucα1 \ 6 Manβ1–4GlcNAcβ1–4GlcNAcβ1-Tyr-Boc 3 NeuAcα2–6Galβ1–4GlcNAcβ1–2Manα1 /	20 100	44 27
	Galβ1–4GlcNAcβ1–2Manα1 \ 6 Fucα1 \ 6 Manβ1–4GlcNAcβ1–4GlcNAcβ1-Tyr-Boc 3 Galβ1–4GlcNAcβ1–2Manα1 /	20 100	48 36
	GlcNAcβ1–2Manα1 \ 6 Fucα1 \ 6 Manβ1–4GlcNAcβ1–4GlcNAcβ1-Tyr-Boc 3 GlcNAcβ1–2Manα1 /	20 100	60 46
	Manα1 \ 6 Fucα1 \ 6 Manβ1–4GlcNAcβ1–4GlcNAcβ1-Tyr-Boc 3 Manα1 /	20 100	58 41
EPO1	NeuAcα2–3Galβ1–4GlcNAcβ1–2Manα1 \ 6 Fucα1 \ 6 Manβ1–4GlcNAcβ1–4GlcNAc 3 NeuAcα2–3Galβ1–4GlcNAcβ1–2Manα1 /	20 100	44 32
EPO4	NeuAcα2–3Galβ1–4GlcNAcβ1–2Manα1 \ 6 Fucα1 \ 6 Manβ1–4GlcNAcβ1–4GlcNAc 3 NeuAcα2–3Galβ1–4GlcNAcβ1–2Manα1 / 4 NeuAcα2–3Galβ1–4GlcNAcβ1 / NeuAcα2–3Galβ1–4GlcNAcβ1 \ 6 NeuAcα2–3Galβ1–4GlcNAcβ1–2Manα1 \ 6 Fucα1 \ 6 Manβ1–4GlcNAcβ1–4GlcNAc 3 NeuAcα2–3Galβ1–4GlcNAcβ1–2Manα1 /	20 100	64 36
EPO5	NeuAcα2–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1 \ 6 NeuAcα2–3Galβ1–4GlcNAcβ1–2Manα1 \ 6 Fucα1 \ 6 Manβ1–4GlcNAcβ1–4GlcNAc 3 NeuAcα2–3Galβ1–4GlcNAcβ1–2Manα1 / 4 NeuAcα2–3Galβ1–4GlcNAcβ1 /	20 100	53 34
EPO8	NeuAcα2–3Galβ1–4GlcNAcβ1 \ 6 NeuAcα2–3Galβ1–4GlcNAcβ1–2Manα1 \ 6 Fucα1 \ 6 Manβ1–4GlcNAcβ1–4GlcNAc 3 NeuAcα2–3Galβ1–4GlcNAcβ1–2Manα1 / 4 NeuAcα2–3Galβ1–4GlcNAcβ1 /	20 100	47 34

ferase in the *in vitro* studies do not adequately reflect those *in vivo*.

CAB2 and CAB4 were previously found to recognize *N*-linked glycans on *Dictyostelium* glycoproteins (35). In the present work, immunoassays, immunoblots, enzyme digestion, and inhibition studies all showed that these antibodies specifically recognize fucose residues in α1,6 linkage to the most proximal GlcNAc of *N*-linked oligosaccharides (Figs. 1–4). They did not cross-react with proteins or neoglycoproteins that contained Fuc in other linkages or in other positions commonly seen in *N*-

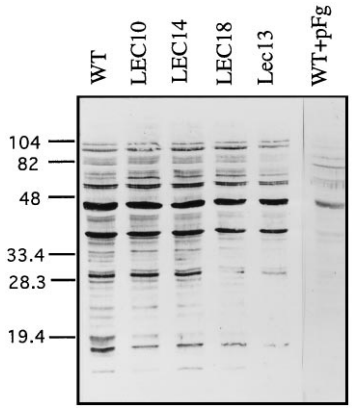

FIG. 5. **Other core modifications have minimal effect on the binding of CAB4 to proteins.** 10 μg each of LEC10, LEC14, LEC18, and Lec13 mutant CHO cell extracts, and the parent CHO cell extract (*WT*) were separated by electrophoresis on 12.5% SDS-polyacrylamide gels, blotted onto nitrocellulose membranes, and blocked with 10% milk in TBS. The membranes were incubated with CAB4 (400 ng/ml IgG). They were then developed with alkaline phosphatase-conjugated goat anti-mouse IgG and BCIP/NBT substrate. The *last lane* on the *right* shows inhibition of the reactivity of wild-type extract with 50 μM pFg.

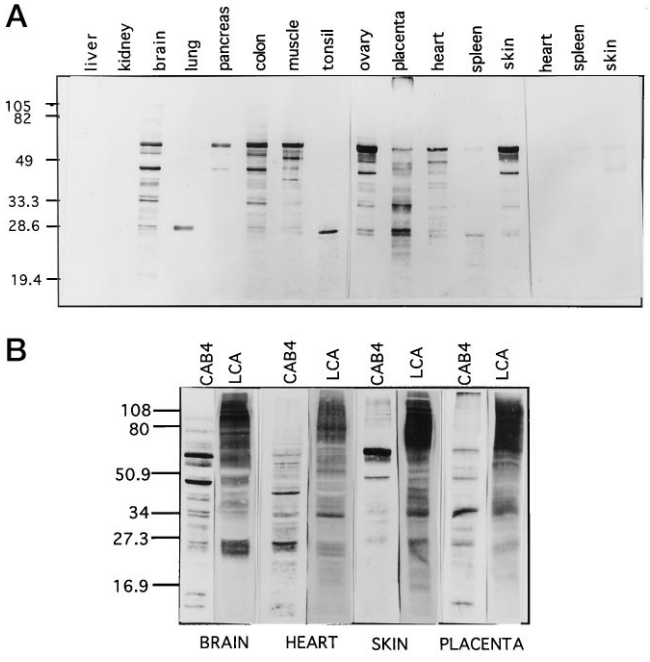

FIG. 6. *A*, Western blots of human tissue extracts probed with CAB4. 50 μg of protein extract from each tissue was separated by electrophoresis on 12.5% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. The membranes were incubated with CAB4 (1 μg/ml IgG), followed by development as described in Fig. 5. The *last three lanes* on the *right* represent incubations done in the presence of 200 μM pTg glycopeptides. *B*, a comparison of *L. culinaris* agglutinin lectin blots and immunoblots of four different tissues. 50 μg of protein was separated in two different lanes for each tissue extract on SDS-polyacrylamide gels and electroblotted as described above. Lanes for each tissue were cut and incubated either with CAB4 (1 μg/ml IgG) or with *L. culinaris* agglutinin-alkaline phosphatase (5.0 μg/ml). The antibody blots were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG, and both blots were developed with BCIP/NBT.

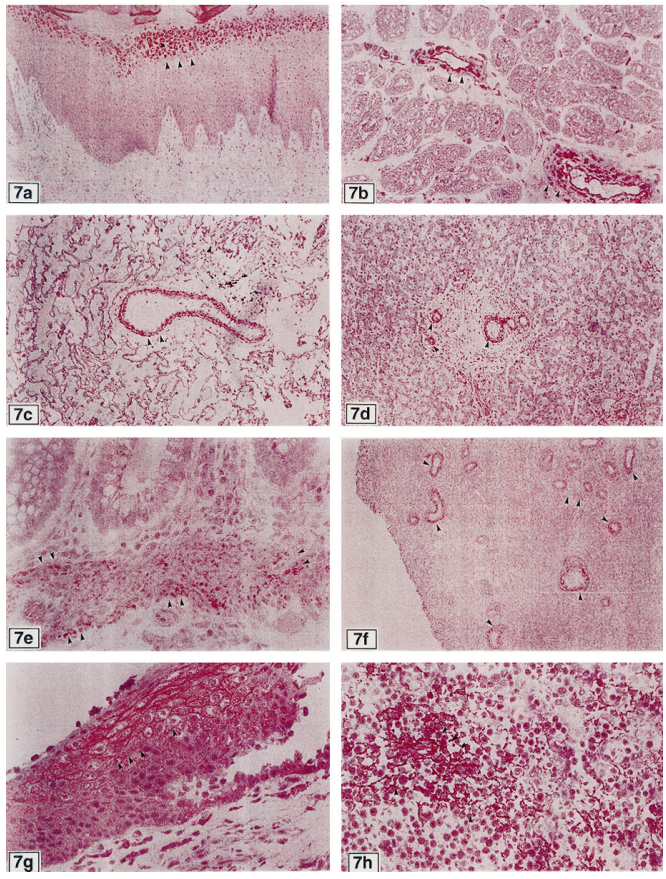

FIG. 7. **Immunolocalization of core-fucosylated glycoconjugates in human tissues.** Frozen sections of human tissues were stained with CAB2 or CAB4 (1–10 μg/ml IgG) as indicated under "Experimental Procedures." In all tissues staining patterns were almost identical for each of the antibodies, and hence the pattern with either of the antibodies is presented for each tissue. *Arrows* indicate areas stained. Magnifications and the primary antibody used in each case are given in parentheses. *a*, skin (20 ×, CAB4); *b*, heart (20 ×, CAB2); *c*, lung (20 ×, CAB4); *d*, liver (10 ×, CAB2); *e*, colon (20 ×, CAB4); *f*, ovary (10 ×, CAB4); *g*, tonsil (20 ×, CAB2); *h*, spleen (20 ×, CAB4).

or *O*-linked glycans (Tables I and II). Binding to core-fucosylated proteins was not inhibited by L-fucose itself (data not shown). This is not uncommon; for example, the binding of anti-HRP, which is directed against core α1,3 fucose and bisecting β1,2 xylose, is not inhibited by the haptenic sugars (59).

The binding of both *L. culinaris* agglutinin and *P. sativum* agglutinin to the trimannosyl core of *N*-linked sugar chains is enhanced by the presence of core Fucα1,6GlcNAc when it is presented in the context of biantennary chains. These lectins only recognize core-fucosylated triantennary complex chains when the branching occurs on the α1,6 core mannose residue but not when it occurs on the α1,3 core mannose residue (45). By contrast CAB antibodies recognize core Fucα1,6GlcNAc in all structures tested including those not recognized by *L. culinaris* agglutinin and *P. sativum* agglutinin (Table III). The smallest structure tested (Man_3GlcNAc(Fucα1,6)GlcNAc-Tyr-*t*-butoxycarbonyl) appears to block binding of CAB4 to pFg just as well as the largest tetraantennary structure (EPO8), suggesting that other sugars beyond the core do not influence antibody binding. Using free oligosaccharides and substituting the Asn with Tyr-*t*-butoxycarbonyl also shows that the amino acid is not required for binding. The difference between the binding specificities of *L. culinaris* agglutinin and *P. sativum* agglutinin and that of CAB antibodies could be due to the fact that, although the lectins essentially bind to the trimannosyl core of *N*-glycans, the antibodies may be directed more against the core fucose residue itself.

The identification of gain-of-function mutants of CHO cells provided an opportunity to explore novel modifications in the core that could potentially interfere with recognition of the core Fucα1,6 GlcNAc. Addition of a β1,4GlcNAc to the β-mannose residue in the core does not change antibody binding (Fig. 5). However, addition of β1,2GlcNAc to the β-mannose or β/α-

GlcNAc residue to the distal GlcNAc in the core as seen in LEC14 and LEC18, respectively, appears to decrease antibody binding in immunoblots. Addition of a GlcNAc residue to the distal GlcNAc does not block the addition of αFuc1,6 to the proximal GlcNAc (48), so decreased binding probably reflects a lower affinity of the antibody. Increasing antibody concentration 2-fold leads to the appearance of bands of normal intensity. As expected, both CAB4 and *L. culinaris* agglutinin binding are also reduced, but not eliminated, in lysates of Lec13 that cannot synthesize GDP-fucose from GDP-mannose. The modest inhibitory effect of core GlcNAc substitutions argues that the chitobiose core may not be required for, but may influence, antibody binding. Also, this differential binding of the antibody to *N*-glycans with modified core renders it useful in identifying glycoproteins with these modifications.

Blots of tissue extracts probed with these antibodies showed that the modification is widespread (Fig. 6*a*), but it has a restricted and specific cell-type distribution (Fig. 7). Both cryostat and paraffin-embedded sections show similar results in all tissues. Keratinocytes above the basal layer of the skin and particularly those in the granular layer of the epidermis are selectively stained (Fig. 7*a*). Proliferation of keratinocytes occurs in the basal layer, and the cells differentiate as they move through the spinous and granular layers to the tissue surface. Our findings suggest that core Fucα1,6GlcNAc may be a marker for terminal differentiation in the epidermis. There are discrepancies in previous studies using *L. culinaris* agglutinin and *P. sativum* agglutinin to study differentiation of human skin. One study found that *L. culinaris* agglutinin stained the cytoplasm and periphery of epidermal cells and also the dermis (23), and another study found that *P. sativum* agglutinin stained the spinous and granular cell membranes in human epidermis but not in the mouse epidermis (24). Interestingly in the latter study, appreciable α-L-fucosidase activity, among other glycosidases, was identified in the cells of the granular layer. Although cultured human skin fibroblasts are rich sources of the core α1,6-fucosyltransferase enzyme (1), we did not see any staining of fibroblasts (not shown). This may be due to rapid secretion of the newly synthesized glycoproteins.

A striking finding was the well defined staining of arteriolar smooth muscle cells in the heart, lung, liver, and ovary and the smooth muscles of the muscularis mucosa in the colon (Fig. 7, *b–f*). Smooth muscle cells present in the tunica media of vasculature are the major sources of elastin, collagen, and proteoglycans in the extracellular matrix. These cells also have multiple glycoprotein receptors for sympathetic and parasympathetic neurotransmitters. For example, mammalian α- and β-adrenergic receptors have complex *N*-linked glycans (60, 61), and the muscarinic acetylcholine receptors can be precipitated by *L. culinaris* agglutinin (62). The cerebellum has a well defined cellular organization, and CAB4 binds to the white matter in both frozen and paraffin-embedded sections (not shown). *N*-Glycosylation is essential for oligodendroglial differentiation (63, 64). Cell surface neuronal and glial glycoproteins isolated from human fetal brains bind to *P. sativum* agglutinin (28). Neural cell adhesion molecule in neuronal and glial cells and in peripheral tissues including skeletal, cardiac, and smooth muscle cells contain core Fucα1,6GlcNAc (65).

In comparison to CAB antibodies, biotinylated *L. culinaris* agglutinin and *P. sativum* agglutinin do not stain specific structures in either the cryostat or the paraffin-embedded sections and often produce heavy background staining. Under the same conditions, *U. europeus* agglutinin I intensely stains vascular endothelial cells of many tissue sections, showing that these results are not due to tissue processing methods or sample variations. CAB4 at 20-fold lower molar concentrations consistently shows better reactivities than *L. culinaris* agglutinin in immunoblots.

By all criteria we have tested, CAB4 appears to recognize core Fucα1,6GlcNAc in most of the known *N*-linked oligosaccharides. Of course we cannot be certain that all possible core fucosylated *N*-linked oligosaccharides will react with the antibody, but its binding specificity is as well characterized as that of any carbohydrate-specific IgG antibody available. This, together with the highly specific tissue localization of core fucosylation, renders a linkage-specific tool to study tissue distribution of oligosaccharides in transgenic expressions or gene ablations of core fucosyl and other glycosyltransferases. The distribution of core fucosylation in human tissues also forms the basis for extended studies of potentially aberrant expression during malignant transformations and other pathological processes.

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