

Novel N-Glycosylation in Eukaryotes: Laminin Contains the Linkage Unit β -Glucosylasparagine

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Abstract. The linkage unit to protein of N-linked carbohydrate in eukaryotic glycoproteins consists of N-acetylglucosamine, coupled to the amido nitrogen of asparagine. Additional N-glycosyl linkage units have been unequivocally proven to exist only in the cell surface glycoproteins of various bacteria. Based on immunological analyses, isolation and chemical characterization, we report that one of these units, namely glucose linked to asparagine, exists in the mammalian protein laminin, an extracellular basement membrane component. This finding and the occurrence of identi-

cal disaccharide structures in archaeobacterial cell surface glycoproteins and mammalian basement membrane protein complexes points towards a conserved and distinct function of these extracellular structural elements.

In addition, a method is described to uncover a masked epitope in fixed tissues by chemical O-deglycosylation. This has allowed to morphologically localize the antigen β -Glc-Asn by immunofluorescence to the basement membranes of kidney glomeruli.

GLYCOPROTEINS represent the major proportion of mammalian proteins. Whereas a variety of O-glycosyl linkage units exist between carbohydrate and the protein in eukaryotes, N-glycosylation seemed to be restricted to one type of linkage unit: N-acetylglucosamine in β linkage to the amido-nitrogen of Asparagine (β -GlcNAcAsn)¹. Although an N-glycosyl linkage unit made of glucose and Asn with the glucose in α -anomeric form (α -GlcAsn) has been suggested to occur in eukaryotes (22), this type of linkage has not been unequivocally proven to exist. On the other hand, the extreme halophilic archaeobacteriae *Halobacterium halobium* and *H. volcanii* were shown to contain a linkage unit β -GlcAsn, as established by its isolation from the cell surface glycoproteins of these prokaryotic organisms (27, 15) and by subsequent chemical characterization.

In our attempt to analyze mammalian proteins for the existence of α - or β -GlcAsn linkages, we chemically synthesized α - and β -glucosylasparagine, coupled these compounds to a carrier protein and obtained polyclonal antibodies against both anomers. These antibodies show no cross-reactivity with the unit β -GlcNAcAsn, and they are specific for their α - or β -anomeric antigen, respectively.

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1. **Abbreviations used in this paper:** α -Glc-Asn, α -anomeric form; β -GlcNAcAsn, N-acetylglucosamine in β linkage to the amido-nitrogen of Asparagine; GLC, gas-liquid chromatography; HF, hydrogen fluoride, HSPG, heparan sulfate proteoglycan; OPA, ortho-phthalaldehyde; succ-BSA, persuccinylated-BSA.

We report here that the N-glycosyl-protein linkage unit β -GlcAsn indeed exists in mammals, specifically in basement membranes, and establish this immunological finding by isolation of the compound from the basement membrane component laminin and by its chemical characterization. Thus, β -GlcAsn is a second type of N-glycosyl linkage unit in eukaryotes.

Materials and Methods

Biological Material

Halobacterium halobium strain M 175 was grown in complex medium and the flagellae were isolated from the medium after concentration to 1% of the original volume by ultrafiltration according to (28). For the preparation of the cell surface glycoprotein, the cells were broken by twice freezing in liquid nitrogen and thawing a cell suspension in basal salts. Thereafter cell envelopes were prepared and delipidated (27). Glomeruli of various kidneys were isolated according to (22), and kindly provided by Dr. E. Schleicher, Munich. Nonenzymatically glucosylated BSA, containing 2.8 moles 1-amino-1-desoxyfructose residues per mol BSA was also a gift from Dr. E. Schleicher. Laminin isolated from an EHS mouse tumor cell line was a gift from Dr. R. Timpl, Martinsried. Heparan sulfate proteoglycan (HSPG), fibronectin, and collagen were purchased from Sigma Chem. Co. (St. Louis, MO).

General Methods

Western blot analysis. SDS-PAGE in gels containing 6 or 10% (wt/vol) acrylamide and 4% (wt/vol) N,N'-methylene bisacrylamide was performed according to (9). Thereafter the proteins were electrophoretically transferred to nitrocellulose sheets (8). We used a buffer system containing two different buffers for the anode (anode I: 0.3 M Tris 20% methanol in water, anode II: 25 mM Tris, 20% methanol in water) and one buffer for the cathode: 25 mM Tris, 20% methanol and 40 mM ϵ -aminocaproic acid. Electrophoretic transfer was carried out at 24 V, 0°C for 2.5 h.

Enzyme-linked Immunosorbent Assay

Characterization of the antisera via enzyme-linked immunosorbent assay (ELISA) was according to (13) when proteins were used as antigens. For peptide-antigens this prescription was modified by binding the antigen to the membrane via glutaraldehyde. To this end, the microtiterplate was preincubated with 50 μ l per well of 0.1 M sodium phosphate buffer, pH 5, 0.2% glutaraldehyde (vol/vol), for 4 h at 37°C. After removal of this solution an incubation followed with the antigen dissolved in 50 μ l PBS-buffer, pH 6.8 for 3 h at 37°C. Thereafter 50 μ l PBS, pH 8.6, 0.1 M Glycine was added. After 45 min at room temperature incubation with the antisera followed as described in (13).

Antibodies

Antigens were synthesized as follows: glucosyl- α (1-N) asparagine according to (16), glucosyl β (1-N) asparagine and all other beta glycosidically linked glycosyl-asparagine derivatives analogous to the synthesis of N-acetyl glucosylamine β (1-N) asparagine described in (26). Purity of the products, specifically the absence of the corresponding anomeric compounds, was determined by ¹H-NMR-spectroscopy. The antigens were coupled to persuccinylated bovine serum albumin (succ-BSA) according to (17). The yield of this step was controlled by amino acid composition analysis of the dialyzed product after total hydrolysis (120°C, 2 h, 6 N HCl[4]). Antigens were injected into New Zealand White Rabbits according to standard procedures (5).

Since all the antibodies investigated in this paper are of the IgM class, a tryptic digestion was performed for the preparation of specific antibody fragments (12). This procedure includes a gel filtration chromatography on Bio Gel P200 (Bio Rad, Munich, column 16 cm \times 1.7 cm in PBS buffer, pH 7.6), followed by tryptic digestion. To this end the sample was diluted to a protein concentration of 1 mg/ml in a 50-mM Tris/HCl buffer pH 8.0, containing 150 mM NaCl and 20 mM CaCl₂. The solution was treated with 0.01 mg TPCK-trypsin/ml, (Sigma Chem. Co.) for 5 h at 37°C. Thereafter mercaptoethanol was added to give final concentration of 10 mM, and the sample was further incubated for 5 min at 37°C. The digestion was stopped by addition of soybean trypsin inhibitor (0.1 mg/ml), and after 5 min iodacetamide (final concentration 60 mM) was added. After each treatment, antigen-binding capacity of the antibodies was monitored by ELISA, using hydrogen fluoride (HF)-treated flagellae (see below) from *H. halobium* as antigen for the anti β -GlcAsn antibodies. To prepare a matrix for affinity purification of the anti β -GlcAsn antibodies, again flagellae from *H. halobium* were O-deglycosylated with HF (see below) and subsequently bound to a membrane (Immobilon) (24). Purification of the anti α -GlcAsn antibodies started with a depletion of antibodies directed against the carrier molecule by incubation of the antisera with Asn-succ-BSA and succ-BSA adsorbed to Immobilon. Thereafter affinity purification was achieved by incubation with α -GlcAsn-succ-BSA bound to Immobilon.

In general, affinity purification of the antibodies was performed by incubation of the matrix with the antisera, washing, and subsequent desorption of the bound antibodies according to (24). Immunological reactions were monitored using peroxidase-linked anti-rabbit IgG-antibodies (13).

Hydrogen Fluoride Treatment

Thoroughly dried protein samples (in polypropylene vials, e.g., "Eppendorf tubes") were dissolved in liquid HF at 0°C for 1 h, as described in (27). The reaction was stopped by gently aspirating the HF into a fume hood with a stream of nitrogen.

Amino acid composition analysis and chromatographic analysis of the various linkage unit compounds after ortho-phthaldialdehyde (OPA) derivatization was performed by high performance liquid chromatography according to (4). Gradients were optimized for the separation of various asparagine monosaccharides as follows:

Buffer A: 12.5 mM NaH₂PO₄/12.5 mM Na₂HPO₄, 2% (vol/vol) tetrahydrofuran. Buffer B: 12.5 mM NaH₂PO₄/12.5 mM Na₂HPO₄, 30% (vol/vol) acetonitrile.

Elution was at 1 ml/min on a Merck RP 18 column (25 mm \times 4 mm) under the following conditions:

0–2 min: 100% buffer A; 2–4 min: 0–5% buffer B; 4–13 min: 5–60% buffer B; 13–17 min: 60–76% buffer B; 17–19 min: 76–100% buffer B. The eluant was analyzed by a flow-through fluorescence detector with excitation at $\lambda = 335$ nm and emission at $\lambda = 455$ nm.

Analysis by gas-liquid chromatography (GLC) was performed on a 5890

HP gas chromatograph combined with a 5970 series mass selective detector, both from Hewlett Packard, Bad Homburg, Germany, equipped with a DB 1701 column, liquid film 0.25 μ m, 30 mm \times 0.265 mm, J + W-Scientific, Folsom, USA.

Separation conditions: injector temp.: 250°C, carrier gas: He with a flow of 1 ml/min and detector temp. 270°C. Temp. gradient: 0–2 min 200°C, 2–37 min 200–270°C.

Isolation of β -Glc-Asn from Laminin

Laminin carboxymethylated according to (18) was subjected to digestion with trypsin (Sigma Chem. Co.) at a substrate to protease ratio 10:1. Reversed phase HPLC of the resulting peptides is shown in Fig. 1. The elution profile of a typical separation is depicted in Fig. 1A (conditions for the separation are shown in Fig. 1B). For the isolation of an aminoacylmonosaccharide the peptide fraction marked in Fig. 1A was digested with pronase E (substrate to pronase E ratio 10:1, 12 h, 37°C, and after another addition of the same amount of pronase E, 12 h at 56°C). A purification of β -GlcAsn was achieved by adsorption of the sample to Dowex AG 50 WH⁺ cation exchange resin in water, and elution of β -GlcAsn with 200 mM formic acid in water. These conditions had been established before with synthetic β -Glc-Asn.

Gas Liquid Chromatography-Analysis of Pronase Derived Peptides from Laminin

The sample isolated from laminin was methylated by addition of diazomethane in diethyl-ether until the yellow color of diazomethane remained, according to (3), dried and trifluoroacetylated with 250 μ l trifluoroacetic anhydride at 100°C for 1 h (23). The volume of the sample was reduced to 10 μ l, and subsequently 5- μ l amounts were subjected to analysis by combined GLC-mass spectrometry.

O-Deglycosylation of Fixed Tissue

Kidneys of female Wistar rats were fixed by perfusion with a solution of 2% paraformaldehyde, 0.75 M lysine in 10 mM phosphate buffer pH 7.4, according to (14, 20). To avoid chemical destruction of the epitope Glc-Asn, no periodate was included during fixation. Anhydrous HF was condensed into a polypropylene tube cooled with liquid nitrogen.

Thereafter the tube was allowed to warm up to room temperature. Immediately after melting of the HF \sim 500 μ l was mixed with 500 μ l of ethanol that was kept at -70°C to compensate for the exothermic mixing reaction. The fixed tissue was cut into cubes of about 2-mm side length and dehydrated in 100% ethanol. Thereafter the dehydrated fixed blocks were incubated in an ice bath for various times with \sim 200 μ l of the ethanol/HF mixture in polypropylene vials (Eppendorf cups). The reaction was stopped by three times changing the solution to 100% ethanol. Thereafter the tissue was rehydrated in PBS with 0.2 M phosphate buffer, pH 7.4.

Quantification of Carbohydrate

After HF-treatment and washing of the tissue blocks with ethanol, the material was dried in vacuo and the weight of the dry material was determined. Carbohydrate was quantified with the Anthrone method (2). To this end the tissue blocks were homogenized and suspended in 2.5 ml H₂O, and 2 ml were mixed with 4 ml of a solution 0.2 g anthrone in 100 ml concentrate sulfuric acid. The samples were kept at 100°C for 4 min (instead of 16 min [2]). This reduction helped to overcome problems with colored products that would result after prolonged exposure of the tissue to sulfuric acid at 100°C. The glucose standard solution was treated identically. The optical density at 625 nm of the samples was read within 1 h.

Morphological Studies on Tissue Sections

Postfixation was achieved by incubation at room temperature of the tissue samples in 2% osmium tetroxide (0.1 M PBS, pH 7.4) for 1 h (19). Tissue sections were dehydrated in ethanol and embedded in Epon (19). Ultrathin sections (stained with lead citrate and uranyl acetate) were taken from these samples and studied in the electron microscope (Philips 301) (19).

Immunofluorescence Microscopy of Semithin Frozen Sections

Semithin frozen sections (0.5–1 μ m) were cut from fixed kidney tissue

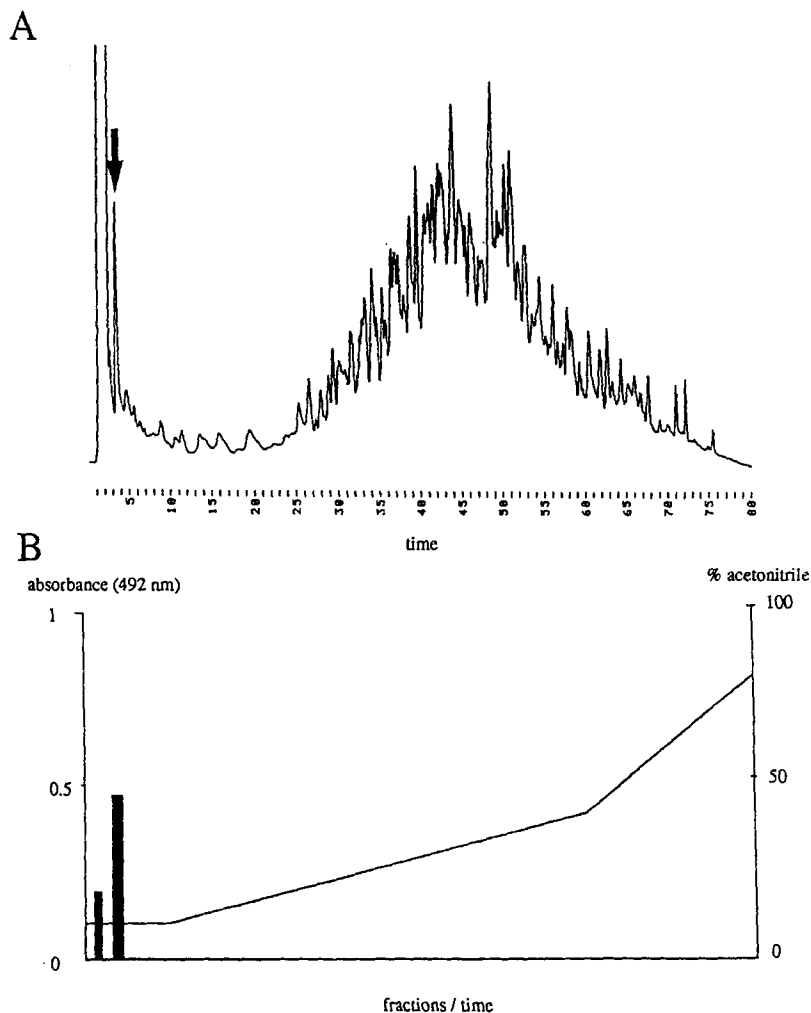


Figure 1. Separation and immunological analysis of tryptic peptides from laminin. (A) RP-HPLC-elution profile of the tryptic peptides ($\lambda = 206$). (B) Profile of the acetonitrile gradient as used for the chromatography in Fig. 1 A (for experimental details see Materials and Methods). Each fraction after HF-treatment was immunologically analyzed for the presence of β -GlcAsn. The corresponding ELISA readings are shown as bars.

blocks at -70°C on a Reichert Ultracut FC-4E apparatus and transferred to glass slides coated with 2% gelatine. The excess of formaldehyde was "neutralized" with 50 mM ammoniumchloride in PBS for 10 min. Blocking of unspecific binding sites was achieved by incubation with a solution of 4% BSA and 1% glycine for 1 h at room temperature. Incubation of these semi-thin sections with the various antibodies was in PBS containing 4% BSA, pH 7.4, at 4°C overnight. Thereafter the slices were washed thoroughly in the above solution and incubated for 20 min with fluorescein- or rhodamine-conjugated sheep anti-rabbit F(ab)₂ (Chapell, diluted 1:1,000 in the above solution); stained sections were mounted in Mowiol. A solution of 10 mg *p*-phenylenediamine in 1 ml Tris/HCl buffer pH 8.8 was diluted 1:10 with a solution containing 20% (wt/vol) Mowiol 4-88 (Polyscience), 50% glycerol in 0.2 M Tris/HCl, pH 8.5 to give the final mounting solution. The sections were examined by epifluorescence illumination on a Leitz Axiovert fluorescence microscope. Photographs were taken with various exposure times (3–120 s) (19, 25).

Results

Characterization of Antisera against α -GlcAsn and β -GlcAsn

α - and β -GlcAsn were synthesized according to (16, 26) and were blocked at their carboxy termini by amidation (26). These compounds were linked to persuccinylated BSA via their NH_2 -termini (17). About forty residues and less were bound per carrier molecule. The conjugates were used to raise rabbit antibodies. Fig. 2 shows their specificity as char-

acterized by ELISA. The anti- α -GlcAsn antiserum was highly specific for the α -GlcAsn-conjugate and did not cross-react with the β -conjugate (Fig. 2 A). Likewise, antibodies obtained after injection of the β -conjugate showed high specificity for the β -anomer (Fig. 2 B). The high specificity of both antisera was also shown by their lack of reactivity with the following N-glycosyl-asparagine derivatives, all bound to succ-BSA: galactosyl $\beta(1\text{-N})$ asparagine (GalAsn), L-rhamnosyl $\beta(1\text{-N})$ asparagine (RhamAsn), N-acetyl glucosaminyl $\beta(1\text{-N})$ asparagine (GlcNAcAsn), and N-acetyl-galactosaminyl $\beta(1\text{-N})$ asparagine (GalNAcAsn). These antigens gave only background readings in the ELISA in the range of concentrations of the antibodies used, and therefore are not included in Fig. 2.

The linkage unit β -GlcAsn had originally been found in the cell surface glycoprotein of the archaebacterium *H. halobium* (27). In this protein, the N-linked glucose bears an additional carbohydrate structure linked to position 4 of the glucose residue. To further characterize the antisera against β -GlcAsn, these additional sugars must be removed from the archaebacterial glycoprotein, thereby "unmasking" the antigenic epitope. This removal is easily achieved under mild conditions by solvolysis in anhydrous HF, a treatment which yields an intact protein core that still contains those sugar monomers that are linked in N-glycosyl units. Reaction of the antibodies with this antigen was analyzed by Western

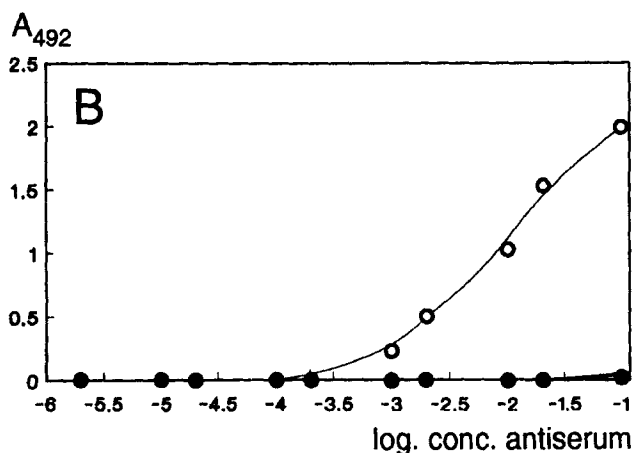
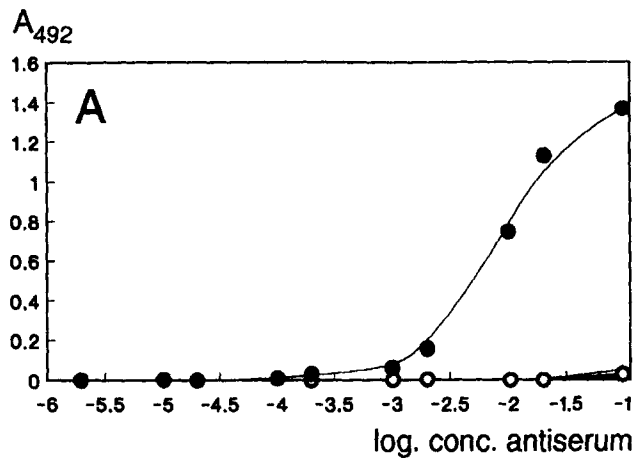


Figure 2. Characterization of the anti- α -GlcAsn and the anti- β -GlcAsn antibodies. (A) Affinity purified anti- α -AsnGlc antibodies were tested by ELISA against their antigen α -GlcAsn-succ-BSA (●) and against β -GlcAsn-succ-BSA (○). In addition, the following antigens were analyzed: β -GalAsn-succ-BSA, β -RhamAsn-succ-BSA, β -GlcNAcAsn-succ-BSA, and β -GalNAcAsn-succ-BSA. (B) The affinity purified anti- β -GlcAsn antibodies were tested against their antigen β -GlcAsn-succ-BSA (○) and against α -GlcAsn-succ-BSA (●), and against the additional antigens listed under A.

blotting of untreated and HF-solvolized halobacterial cell surface glycoprotein (Fig. 3). After solvolysis with HF, the anti- β -GlcAsn antiserum bound to the cell surface glycoprotein (Fig. 3, lane 8). In contrast, anti- β -GlcAsn antibodies did not recognize any antigenic determinants in proteins from halobacteria as long as these were not treated with HF (Fig. 3, lane 7). As an additional control of the specificity of the antibodies, non-enzymatically glucosylated BSA and fetuin were analyzed before and after treatment with HF. Fetuin that contains the common linkage β -GlcNAcAsn unit of N-glycosylated proteins did not react with the anti- β -GlcAsn antiserum, neither before nor after solvolysis (Fig. 3, lanes 9 and 10). The same result was obtained with the non-enzymatically glucosylated BSA (Fig. 3, lane 11). A similar blot (like Fig. 3, lanes 7-11) incubated with anti- α -GlcAsn antiserum was completely blank and therefore is not shown here.

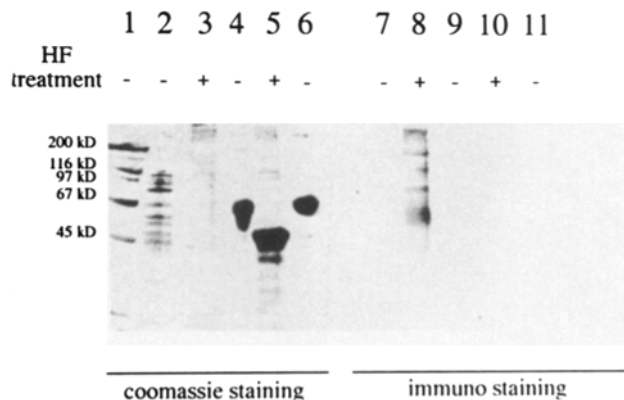


Figure 3. Specificity of the anti- β -GlcAsn antibodies assessed by Western blot analysis. Various glycoproteins were separated by SDS-gel electrophoresis and subsequently electro-transferred to Immobilon sheets. Proteins were visualized by staining with Coomassie blue (lanes 1-6) or by immunoreaction (lanes 7-11) with the anti- β -GlcAsn antibodies. (Lane 1) Molecular weight standard. (lanes 2 and 7) Cell envelope proteins of *Halobacterium halobium*, 30 μ g each; (lane 3) 30 μ g and (lane 8) 5 μ g HF-treated cell envelope proteins of *Halobacterium halobium*. (lanes 4 and 9) fetuin, 30 μ g each; (lanes 5 and 10) HF-treated fetuin, 40 μ g each. (lanes 6 and 11) Non-enzymatically glucosylated BSA, 30 μ g each.

The HF-solvolized cell surface glycoprotein from halobacteria or their HF-solvolized flagellae (28) served as a matrix for immuno affinity purification of the anti- β -GlcAsn antibodies (see Materials and Methods). The resulting immunoaffinity purified antibodies were used for the studies described below. Anti- α -antibodies were purified by neutralizing with succ-BSA and subsequent immunoadsorption to α -GlcAsn-succ-BSA (see Materials and Methods).

Immunological Characterization of Kidney Glomeruli Extracts

Shibata et al. (22) have described a nephritogenic glycopeptide from a rat kidney glomerulus fraction which contained predominantly glucose. They proposed the carbohydrate moiety of this glycopeptide to be attached to an Asn residue in α -anomeric linkage, although this has only been concluded from NMR-spectroscopy of an extended tryptic glycopeptide, without isolation and characterization of the linkage unit. Therefore we have used the anti- α - and anti- β -GlcAsn antisera to screen kidney glomeruli by Western blotting for the occurrence of these epitopes. The result is shown in Fig. 4. Extracts of glomeruli from rat and pig do not at all react with the anti- α -GlcAsn antiserum or the anti- β -GlcAsn-antibodies (Fig. 4, lanes 8 and 10 and 14 and 16). However, after solvolysis with HF, these extracts contained proteins that were stained with the anti- β -GlcAsn antiserum. In both fractions distinct staining was observed in a molecular weight range of close to 200 kD (Fig. 4, lanes 15 and 17). As there is only a small number of proteins in this molecular weight range, we have immunologically analyzed the following isolated basement membrane components of similar size, using the β -GlcAsn antibodies: heparansulfate proteoglycan, collagen, fibronectin, and laminin, each before and after HF-treatment. Tested on the purified proteins, the anti- β -GlcAsn antibodies gave a clearcut positive response only with HF-treated laminin (Fig. 4, lane 19). Again, the anti- α -anti-

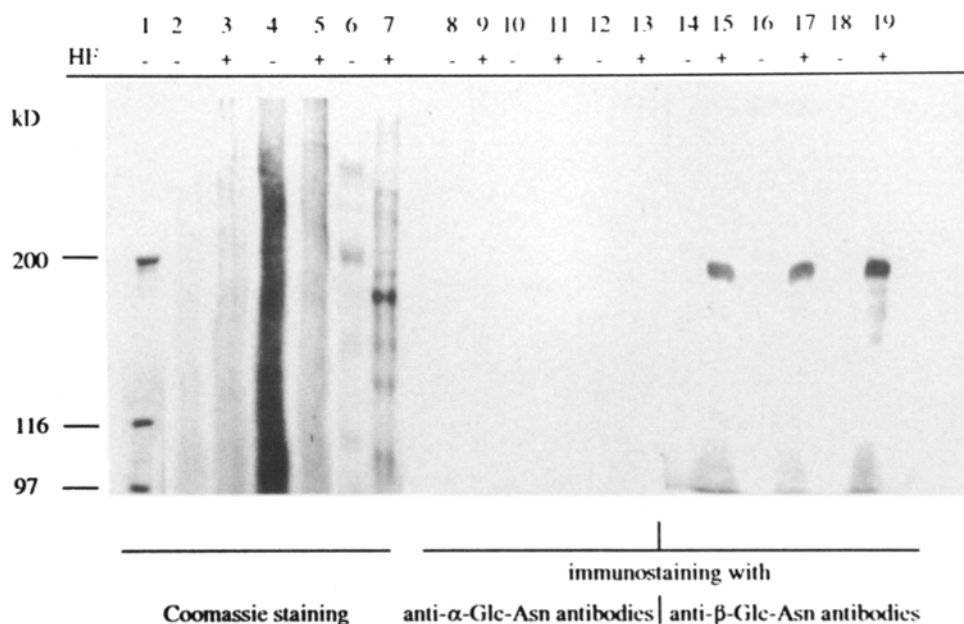


Figure 4. Immunological analysis by Western blotting with anti- α -GlcAsn and anti- β -GlcAsn antibodies of basal membrane proteins. Protein extracts of glomeruli and isolated laminin were separated by SDS-gel electrophoresis and subsequently electrotransferred to Immobilon sheets. Thereafter the proteins were visualized by staining either with Coomassie (lanes 1-7) or by immunoreaction either with anti- α -GlcAsn antibodies (lanes 8-13) or with anti- β -GlcAsn antibodies (lanes 14-19). (Lane 1) Molecular weight standard; (lanes 2, 8, and 14) protein extract from rat kidney glomeruli; (lanes 3, 9, and 15) HF-treated protein extract from rat kidney glomeruli; (lanes 4,

10, and 16) protein extract from porcine kidney glomeruli; (lanes 5, 11, and 17) HF-treated protein extract from porcine kidney glomeruli; (lanes 6, 12, and 18) laminin; (lanes 7, 13, and 19) HF-treated laminin.

bodies did not react (Fig. 4, lane 12 and 13). These immunological results encouraged us to attempt an isolation from laminin of the linkage unit β -GlcAsn and its chemical characterization.

Isolation and Characterization of a Linkage Unit β -GlcAsn from Laminin

Purified mouse laminin (from an EHS cell line) was digested with trypsin and subsequently loaded on a Sep-Pak cartridge. The cartridge was developed with water and subsequently with various concentrations of acetonitrile in water. These fractions were divided, and one half of each was solvolyzed with HF. Thereafter, the samples were digested with pronase E and passed through cation exchange resin under conditions known to elute synthetic β -GlcAsn (Fig. 5).

The resulting eluates were converted to their OPA derivatives and analyzed by HPLC. To this end, OPA derivatives of various synthetic N-glycosyl linkage units were prepared as standard compounds (Fig. 6). The resulting elution profile is shown in Fig. 6 B. β -GlcAsn (peak 3) is clearly separated from the other N-glycosyl-linkage units, e.g., from α -GlcAsn (peak 1), and from β -GlcNAc-Asn (peak 5). In A, separation of an amino acid standard mixture is shown to demonstrate that no natural amino acid-derivatives elute in the region of β -GlcAsn (see arrow). Separation of the OPA-derivatized eluates I(+) and I(-) (Fig. 5) is depicted in C and E, respectively. A peak is observed at the retention time of authentic β -GlcAsn (arrow in C) exclusively in the sample that had been treated with HF before digestion with pronase and cation exchange chromatography (fraction I(+)). Fraction I(-) is devoid of this material. Addition of synthetic β -GlcAsn standard to fraction I(+) and subsequent HPLC yielded the profile shown in Fig. 6 D. No additional peak appeared, but the area of the peak in question is relatively enlarged and it shows no asymmetry. This strongly supports the idea that

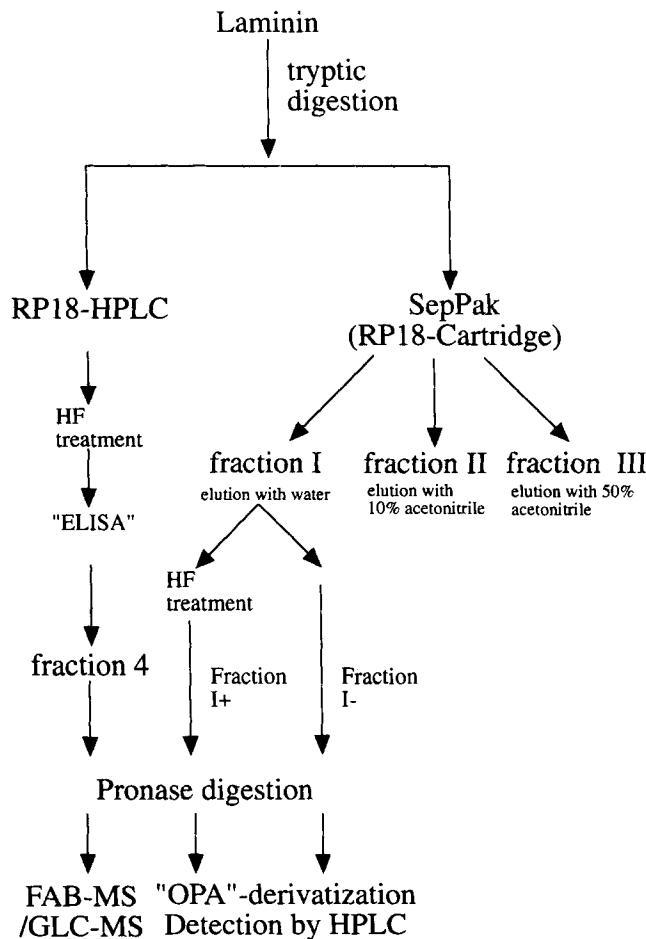


Figure 5. Isolation of β -Glc Asn containing fractions from laminin. For details see Materials and Methods section.

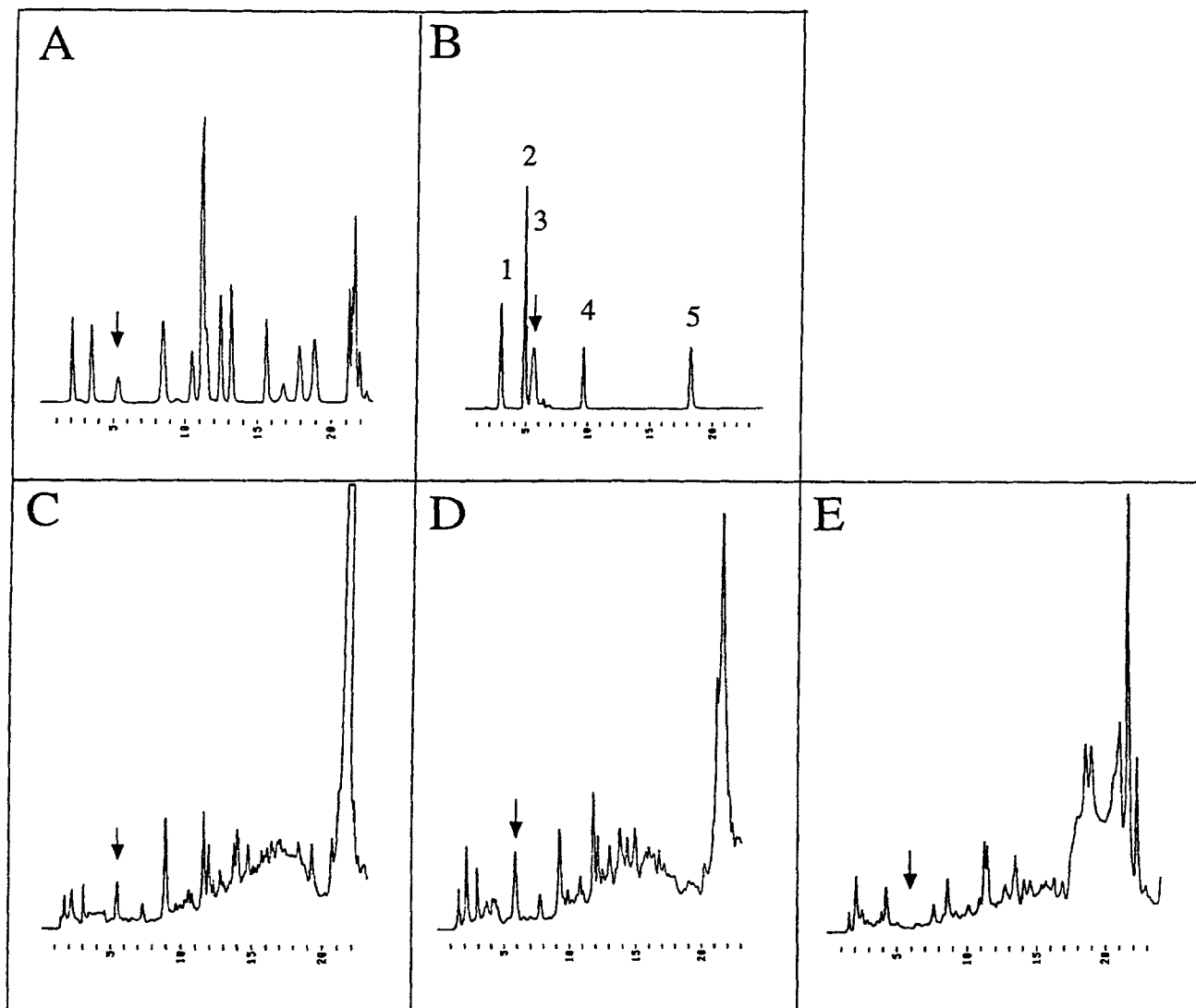


Figure 6. Analysis of fraction I from Fig. 5 by comparison with authentic β -GlcAsn-standard. Sample and standard were reacted with ortho-phthalaldehyde before separation by Reversed-Phase HPLC ($\lambda = 455$ nm). (A) Mixture of an amino acid standard solution and synthetic β -GlcAsn. (B) Mixture of glycosyl-asparagine derivatives: α -GlcAsn (1), β -GalAsn (2), β -GlcAsn (3), β -RhamAsn (4), and β -GlcNAcAsn (5). (C) HF-treated and pronase E derived peptide fraction I+ (Fig. 5) from laminin. (D) Mixture of the fraction shown in C and β -GlcAsn (ratio 1:1). (E) Fraction I- (Fig. 5).

the compound β -GlcAsn actually is contained in fraction I(+). To obtain additional and independent evidence for the occurrence of this compound in laminin, trypsin-derived laminin peptides were partially separated by HPLC, and the resulting fractions were solvolysed by HF and analyzed by ELISA for their content of β -GlcAsn. Two positive fractions were obtained (see Fig. 1 in Material and Methods). The major fraction (arrow in Fig. 1) was digested with pronase E, and the resulting aminoacysaccharide purified by cation exchange chromatography as described above. The eluate was dried and subjected to FAB-mass spectroscopy in the negative mode. The resulting mass spectrum is shown in Fig. 7. In the mass spectrum of the HF treated sample, a signal appears at m/z 293.3 that corresponds to M-1 of asparagine connected to a neutral hexose (Fig. 7 A). In contrast, the untreated sample showed no such signal (Fig. 7 B).

Finally, the retention time of synthetic β -GlcAsn by GLC-MS on a 30-mm-capillary column was determined after meth-

ylation and pertrifluoroacetylation (Fig. 8 A). The HF-treated sample from laminin (as used for FAB-mass spectroscopy) was subjected to the same analysis in the single ion monitoring mode at the ions 401 (Fig. 8 B) and 139 (Fig. 8 C) characteristic for β -GlcAsn. In each case, the double peak characteristic for standard β -GlcAsn was observed at identical retention times (Standard: 20.13 and 20.52 min, ion 401: 20.13 and 20.51 min, ion 139: 20.13 and 20.51 min).

Chemical O-deglycosylation of Fixed Tissue

An epitope that is masked by carbohydrates obviously cannot be analyzed by immunofluorescence microscopy of tissue slices. Therefore we have worked out a method that allows chemical O-deglycosylation of fixed tissue blocks, as described in the Materials section. The method takes advantage of the mild solvolysis of O-glycosidic bonds by HF, as explained above. To minimize the incubation times neces-

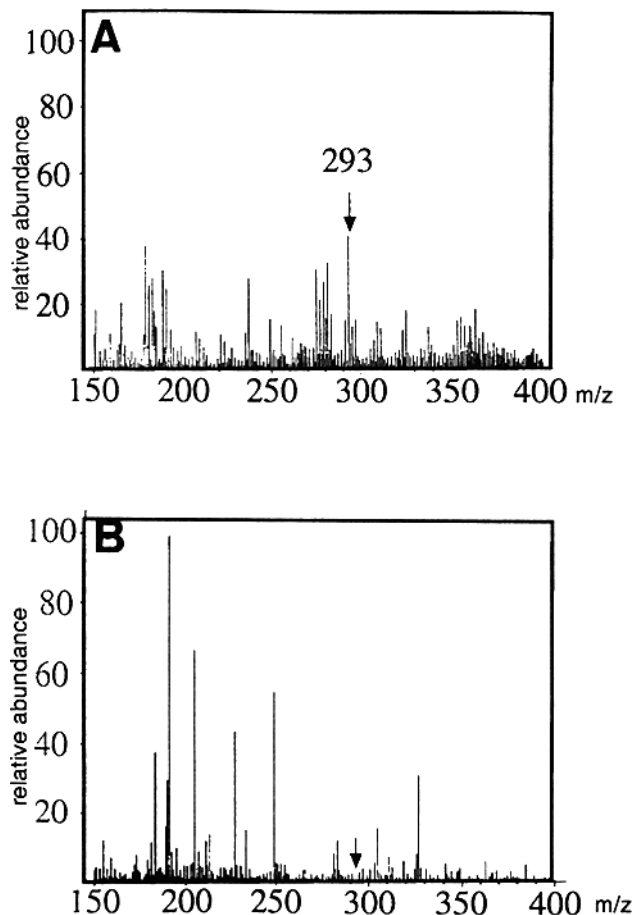


Figure 7. FAB-MS of the pronase E derived peptide fraction from laminin after *A* and before HF treatment *B*. Fraction 4 (Fig. 5) was digested with pronase and purified on a Dowex ion exchanger as described in Materials and Methods, and thereafter treated with HF, and analyzed by FAB-MS run in the negative mode. (*A*) HF-treated sample, (*B*) untreated control sample.

sary for complete O-deglycosylation, we have quantitated the carbohydrate content of tissue blocks after deglycosylation for various times. The result is shown in Fig. 9. Maximal release of carbohydrate is observed after incubation of fixed tissue cubes (size $\sim 2 \text{ mm}^3$) at 0°C in HF/Ethanol (1/1, vol/vol) for 3–4 h.

Therefore, for O-deglycosylation, tissue of similar size were routinely incubated for 4 h under the above mentioned conditions.

The extent of damage to the cellular structures caused by this treatment was assessed by electronmicroscopy. Fig. 10 *A* shows an area of an HF-treated section at low magnification stained with lead citrate and uranyl acetate. A peritubular capillary is depicted located between a distal tubulus and a proximal tubulus. The basement membrane of the tubular epithelium (arrows) appears unfragmented and regular. Fig. 10 *B* shows a control electronmicrograph of an untreated kidney tissue sample at higher magnification. A similar area at the same magnification after HF-treatment is shown in Fig. 10 *C*. Although some alterations can be observed, the overall architecture of the HF-treated sample is still visible. Specifically, the glomerular basement membrane appears again as a regular and continuous linear structure. Due to the loss

of carbohydrate, the overall staining with uranyl acetate and lead citrate is less intensive in the HF-treated sample. This holds specifically for the staining of the nucleus. Some influence can be observed on the structure of the podocytes, (Po) with their foot protrusions. In the control, these structures protrude from the basement membrane and are separated by spaces $\sim 2\text{-nm}$ wide. In the HF-treated sample the foot protrusions are still visible, but the spaces have disappeared. This may be due to the lack of negatively charged carbohydrate (glycocalix) in the treated samples that under physiological conditions by charge repulsion may maintain this spatial separation.

Thus, a fixed tissue block can be deglycosylated without severe destruction of its architecture, and therefore we were interested to analyze the immunological integrity of the deglycosylated samples. This was achieved by immunofluorescence microscopy on "semithin" (0.5–1 mm) cryosections.

In Fig. 11 the immunofluorescence of such slices is shown after incubation with an anti-laminin-antibody directed against Laminin protein epitopes before (Fig. 11 *A*) and after (Fig. 11 *B*) treatment with HF. The untreated sample (Fig. 11 *A*) shows an extended linear fluorescence. The area depicts cross sections through proximal and distal tubuli. In Fig. 11 *B*, the HF-treated sample, no differences can be observed. Thus, laminin epitopes in the basement membrane are intact after HF-treatment.

Localization of the Epitope $\beta\text{-Glc Asn}$ to the Glomerular Basement Membrane

With these results and our finding in mind that anti $\beta\text{-Glc Asn}$ antibodies bind to isolated laminin after HF-solvolysis, we analyzed the localization of the novel type of N-glycosyl linkage by immunofluorescence of deglycosylated semithin kidney slices by reaction with the anti- $\beta\text{-GlcAsn}$ antibodies, as shown in Fig. 12, *C* and *D*. As a control, incubations were performed with the anti- $\alpha\text{-GlcAsn}$ -antibodies (Fig. 12, *A* and *B*).

Exclusively in the HF-treated sample that was incubated with the anti- $\beta\text{-GlcAsn}$ antibodies, an extended linear fluorescence signal was observed (Fig. 12 *D*). The untreated sample showed only background staining (Fig. 12 *C*) (note that exposure for the photograph in Fig. 12 *D* was 3 s and in Fig. 12, *A*, *B* and *C* was 120 s). Likewise no signal was observed with the anti- $\alpha\text{-GlcAsn}$ -antibodies, neither after nor before HF-treatment (Fig. 12, *B* and *A*).

Discussion

By a combination of immunological and chemical analyses we have established that laminin from kidney glomerular basement membranes contains the carbohydrate to protein linkage unit $\beta\text{-GlcAsn}$. A linkage unit $\alpha\text{-GlcAsn}$ that has been proposed in the literature could not be detected with our methods.

The antibodies used in this study were characterized (*a*) by their reactivity against their chemically synthesized antigens; (*b*) by their lack of reactivity against the synthesized anomeric isoforms of their antigens; (*c*) by their lack of reactivity with protein-conjugated synthetic $\beta\text{-GlcNAcAsn}$ as well as natural $\beta\text{-GlcNAcAsn}$ as prepared by solvolysis with HF of conventional N-linked glycoproteins; (*d*) by their lack of reactivity against natural glycoprotein known to contain

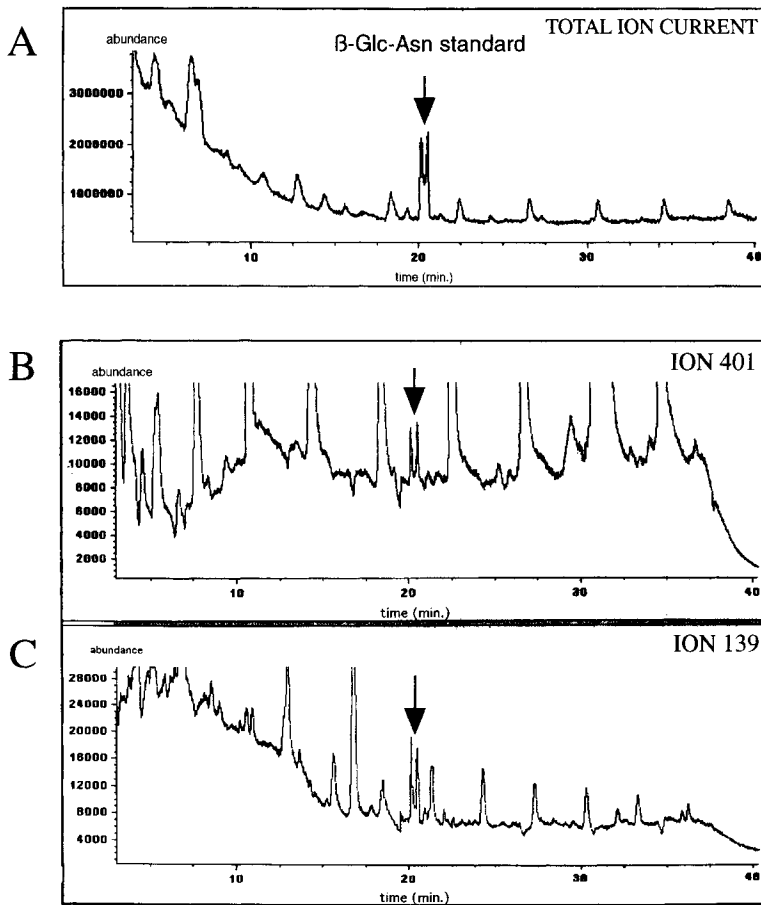


Figure 8. GLC-MS of β -GlcAsn (A) and the pronase derived peptide fraction from laminin after HF treatment (B and C). (A) Total ion current of a GLC run of 20 μ g synthetic standard β -GlcAsn monitored by a mass selective detector. (B and C) Single ion current after GLC run of HF-treated, pronase treated fraction 4, Fig. 5, at single ions set to a mass 401, and 139, respectively. Glc was performed on a Durabond 1701 capillary column in a Hewlett Packard combined gas liquid chromatograph-mass spectrometer. Conditions were as described in the Materials and Methods section.

complex carbohydrates connected to protein by β -GlcAsn linkage, and their reactivity against these glycoproteins after solvolysis with HF, which is known to leave the N-linked monohexosyl units connected to the protein; and (e) by their lack of reactivity with non-enzymatically glycosylated proteins.

These various lines of immunological characterization almost exclude any unspecific reaction of the antibodies. Spe-

cifically, cross-reactions would have been detected with the glycoproteins before solvolysis with HF.

In case of the cell surface glycoprotein of *H. halobium*, the existence of carbohydrates linked to the glucose residue of β -GlcAsn was established, and therefore the need for HF-solvolysis to unmask the linkage unit was expected. Our finding that laminin also had to be treated with HF before its reaction with the anti- β -GlcAsn antibodies indicates that in laminin the glucose involved in this linkage carries additional sugars as well. These sugars have not been identified to date. Alternatively the epitope may be "masked" by neighboring oligosaccharides.

The monohexosyl-amino acid GlcAsn is a very small epitope, and the strong immunoresponse against this antigen may seem remarkable. At first glance the exclusive antibody reaction with the α - or β -anomer, respectively, also seems surprising. However, stringent discrimination of anomeric carbohydrate antigens is well established in the protein-carbohydrate interactions of lectins (6). For a review of carbohydrate-protein interactions see (21).

In addition to its immunological characterization, the linkage unit β -GlcAsn has been isolated from laminin of basement membranes and characterized by cochromatography with the authentic standard substance. Furthermore, the isolated compound yielded an FAB-mass spectrum with a molecular ion mass peak as expected for a monohexosylasparagine and was identical with the spectrum of the synthesized standard substance. In addition, by GLC, identical retention times were observed for the standard compound and the sample isolated from laminin.

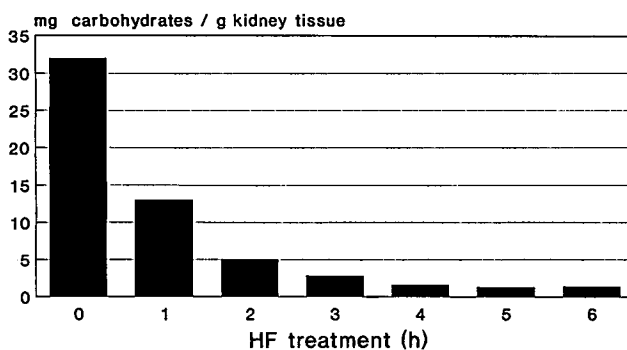


Figure 9. Quantification of the carbohydrate content of tissue blocks after HF treatment. Fixed tissue blocks of \sim 2-mm side length from rat kidney were treated with HF/ethanol for various times as described in the Materials and Methods section. After rehydration the tissue was homogenized and aliquots taken for the colorimetric quantification of carbohydrate with a modified anthrone method as described in Materials and Methods.

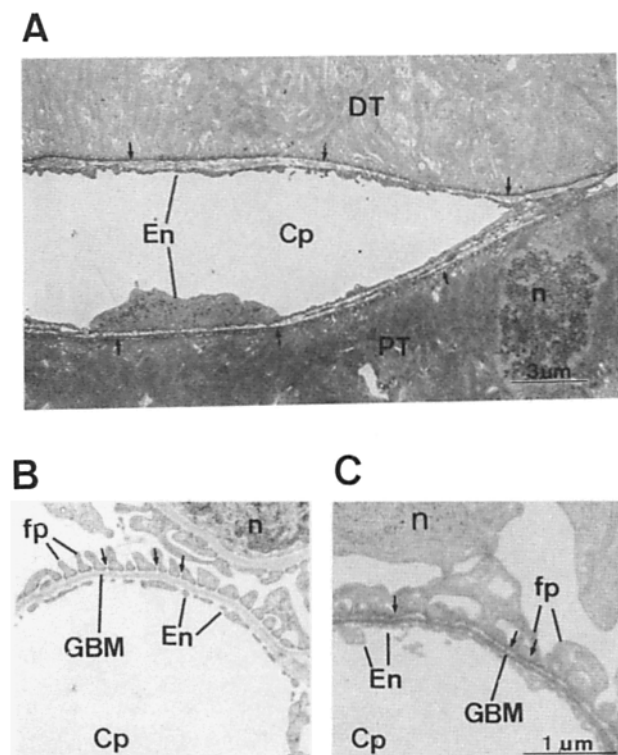


Figure 10. Electromicroscopy of fixed rat kidney tissue after HF treatment. Fixed tissue blocks from rat kidney were solvolyzed with HF/ethanol for 4 h and processed for electromicroscopy as described in Materials and Methods. (A) HF-treated section, magnification 5,400-fold; (B) Untreated section, 18,700-fold; (C) HF-treated section, 18,700-fold. Cp, peritubular capillary; DT, distal tubulus; PT, proximal tubulus; En, fenestrated endothelium; GBM, glomerular basement membrane; fp, foot protrusions; n, nucleus.

These results together are taken as proof that the linkage unit β -GlcAsn exists in laminin from kidney. Laminin is an unusually large and complex glycoprotein (1). In addition to this novel N-glycosyl linkage it contains complex carbohydrates linked via the conventional N-linkage β -GlcNAcAsn

(7). At this time we cannot quantitate the number of linkages of the β -GlcAsn type in the basement membrane protein, but our lowest estimate indicates a stoichiometry of 1 β -GlcAsn to 1 laminin. According to electrophoretic mobility, it seems to be the B2-chain of laminin that contains the novel linkage-type.

We have obtained no immunological or chemical results that would suggest the presence of a linkage unit α -GlcAsn in protein extracts from mammalian kidney glomeruli.

We do not know at this time the mechanism of glycosylation of the extracellular glycoprotein laminin. However, it is possible that formation of the β -GlcAsn linkage, like conventional N-glycosylation, might occur in the lumen of the ER. In the cell surface glycoprotein from halobacteria, like in eukaryotic glycoproteins, all N-glycosyl-sites show the consensus tripeptide structure Asn X Thr/Ser, and we would therefore expect this sequence as the site of β -GlcAsn-formation in laminin as well. It will be of interest to know the precise localization of this novel posttranslational modification activity, and to learn about the structural requirements that allow the N-glycosyltransferases for the conventional and the novel linkage unit to recognize their specific sites of operation.

In addition to its presence in isolated laminin, we have localized the epitope β -GlcAsn to the basement membranes of kidney tissue by immunofluorescence microscopy. Again, no indication was observed for the presence of a glucose residue in α -linkage to asparagine. For immunofluorescence microscopy we have worked out a method to chemically O-deglycosylate fixed tissue blocks. Surprisingly, under optimal deglycosylation conditions, the general architectural characteristics as well as the immunogenic properties of the tissue remain essentially intact. This method may be useful for the detection of masked epitopes in a more general way. Specifically, we would expect that peptide epitopes that are covered with carbohydrate may be efficiently unmasked. Likewise, this method may prove useful to discriminate whether a carbohydrate or a protein epitope is recognized by an antibody.

At present we do not know about a possible specific func-

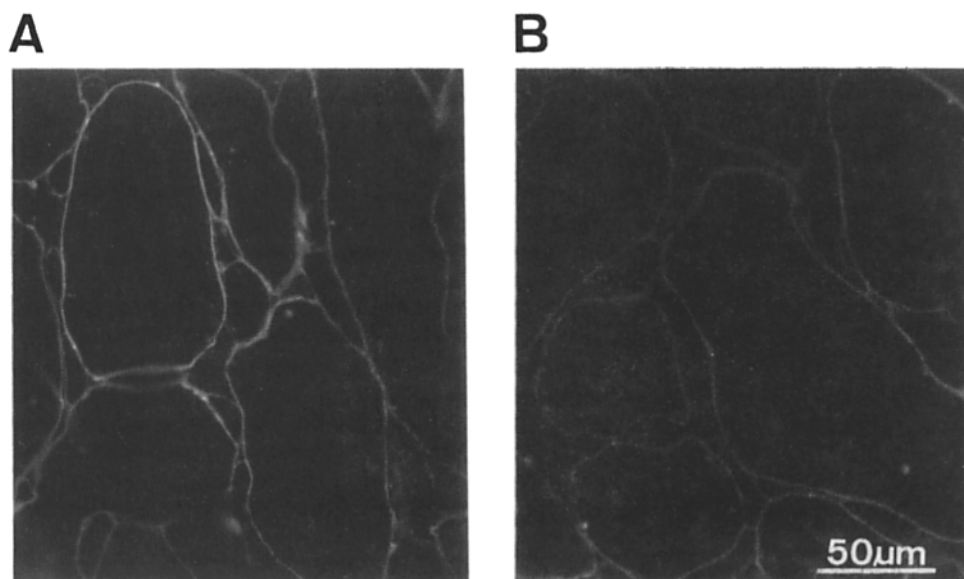


Figure 11. Immunofluorescence microscopy with anti-laminin (protein) antibodies of semithin sections from rat kidney before and after treatment with HF. Semithin frozen sections were taken and processed as described in Materials and Methods. (First antibody) anti-laminin (protein) antibodies; (second antibody) fluorescein-conjugated sheep anti-rabbit IgG F(ab)₂. (A) Untreated tissue; (B) HF-treated tissue.

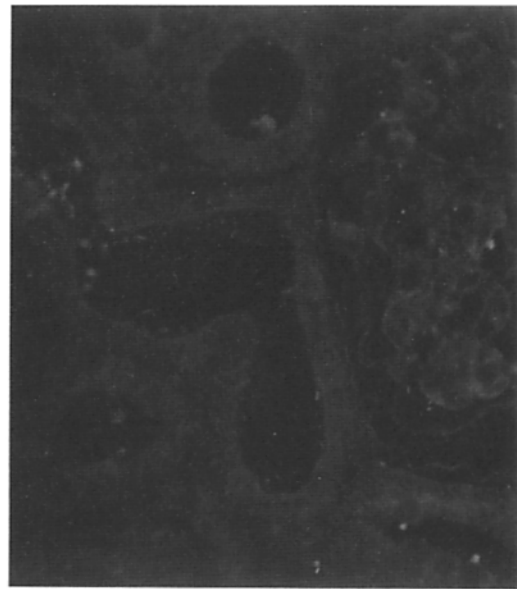
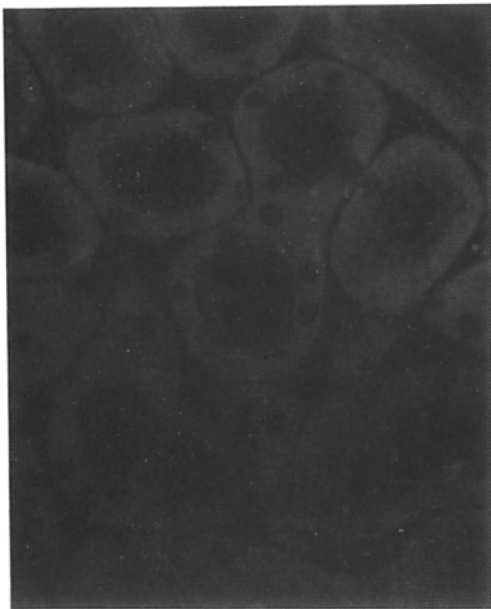
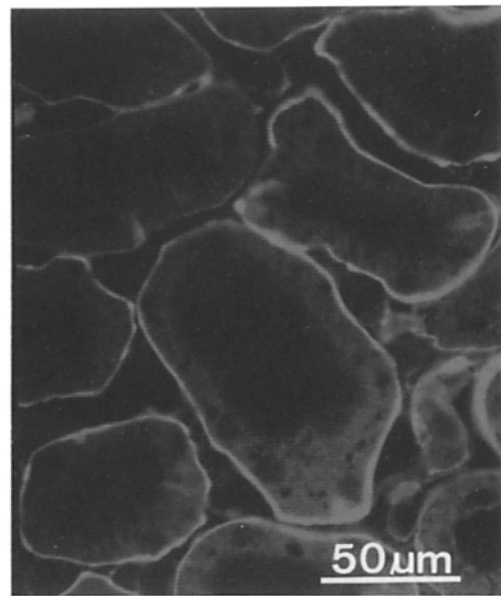
A**B****C****D**

Figure 12. The epitope β -GlcAsn can be visualized in kidney tissue after HF treatment. Immunofluorescence microscopy was performed on frozen semithin sections from fixed kidney tissue and processed for immunofluorescence microscopy as described in Materials and Methods. (A) Untreated (*control*) tissue incubated with anti- α -GlcAsn-antibodies; (B) HF-treated tissue incubated with anti- α -GlcAsn antibodies; (C) Untreated (*control*) tissue incubated with anti- β -GlcAsn antibodies; and (D) HF-treated tissue incubated with anti- β -GlcAsn antibodies.

tion of the glycoconjugate linked to laminin by the novel unit β -GlcAsn. It seems remarkable, however, that the linkage is found on the cell surface of halobacteria as well as on the surface of mammalian cells, and in both cases in combination

with glycosaminoglycans (the repeating unit sulfated saccharide in halobacteria (10), and the heparansulfate in the basement membrane). In addition, in both cell surfaces identical disaccharides of the structure Glc (α 1-2) Gal are present in

O-glycosyl linkage: linked to threonine in the halobacterial cell surface glycoprotein (10) and to hydroxyproline in collagen type IV of the basement membrane (11). These surprising structural identities in two organisms as distant as halobacteria and mammals indicate that the novel structure described here might have an important function to establish or maintain the integrity of cell surface structures.

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