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Description and Application of an Immunological Detection System for Analyzing Glycoproteins on Blots

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By introducing the steroid hapten digoxigenin specifically into sugars, a sensitive detection system for glycoproteins on blots has been developed. Sugars are oxidized to obtain aldehyde groups, which then react with digoxigenin-succinyl-ε-amido caproic acid hydrazide. A high-affinity antibody, conjugated to alkaline phosphatase, is used for the detection of the incorporated digoxigenin.

This system allows the detection of nanogram-amounts of glycoproteins on blots, and it's specificity allows a clear distinction of a glycoprotein from a non-glycoprotein. In combination with endo- and exoglycosidases it is very useful for determining the type of carbohydrate linkage in a glycoprotein, and by varying the oxidation conditions, specific labeling of sialic acids and terminal galactoses can be achieved.

The widespread occurrence of glycoconjugates in biological organisms and their importance have prompted the development of numerous techniques for the analysis of carbohydrates. Of major interest among them are methods for the detection and characterization of glycoproteins on gels and blots. One of the still widely used methods is the periodate-Schiff staining (PAS) of glycoproteins separated on polyacrylamide gels. This method suffers from requiring a rather large amount of sample material (5-100 μg) per analysis [1].

Introduction of fluorescent labels [2] and a sugar specific silver staining method [3] increased the sensitivity somewhat; however, these methods are relatively tedious and susceptible to interference. With the emergence of protein blotting techniques several

Abbreviations: CpY; Carboxypeptidase Y: CHO; Chinese hamster ovary: Dig-hydrazide; digoxigenin-succinyl-ε-caproic acid hydrazide: <Dig>Fab-AP; polyclonal sheep anti-digoxigenin Fab fragments, conjugated with alkaline phosphatase: NBT; nitroblue tetrazolium chloride: SDS-PAGE; sodium dodecylsulfate-polyacrylamide gel electrophoresis: TCA; trichloroacetic acid: X-phosphate; 5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt.

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methods for the analysis of glycans on blots have been developed. Generally they involve introducing labels by hydrazide-derivatization of aldehyde groups generated in sugars by oxidation with periodate or galactose oxidase [4]. Both alkaline phosphatase [5] and horseradish peroxidase [6] have been used as enzymatic labels. So far the best sensitivity has been obtained by using the biotin/(strept)avidin amplification system.In one version biotin-aminocaproyl-hydrazide is used in combination with labeled (strept)avidin conjugates [7], and in another, (strept)avidin is introduced *via* the hydrazide derivative and visualized by biotinylated alkaline phosphatase[8].

Although the biotin/(strept)avidin system increased the sensitivity of carbohydrate detection on blots considerably, it still suffers from the disadvantage that (strept)avidin binds non-specifically to membranes and unglycosylated proteins to a certain extent. Moreover biotin binding sites, which are present in virtually all biological organisms, can also lead to non-specific results. In order to circumvent these problems we investigated a system where a steroid hapten, digoxigenin, is used for the labeling of sugars. The hapten-labeled sugars are immunologically detected using a specific, high affinity antibody. In this paper the basic features of this system for the detection of glycoproteins on blots are described. Applications for a more detailed structural analysis of glycan chains by combining this detection system with the use of various endo- and exoglycosidases are also presented.

Materials and Methods

Materials

Transferrin (human), carboxypeptidase Y (CpY), erythropoietin (recombinant, from CHO cells), *N*-glycosidase F, neuraminidase (*Arthrobacter ureafaciens*), β-galactosidase (bovine testes), endo-α-*N*-acetylgalactosaminidase (*O*-Glycosidase), galactose oxidase, digoxigenin-succinyl-ε-amido caproic acid hydrazide (Dig-hydrazide), polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase(<Dig>Fab-AP), nitroblue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (X-phosphate), blocking reagent (Cat.No. 1096 176; Casein, special quality) and molecular weight markers for SDS-PAGE were from Boehringer Mannheim. Fetuin (fetal calf serum) and α_1-acid glycoprotein (human) were from Sigma (Deisenhofen), and Ponceau S from Serva (Heidelberg).

For blotting, nitrocellulose BA85 from Schleicher and Schüll (Dassel) was used.

Oxidation and Labeling of Glycoproteins with Digoxigenin in Solution

Protein (0.1-10 µg) in 10 µl H_2O are mixed with 10 µl 0.1 M sodium acetate buffer, pH 5.5, and 10 µl 30 mM sodium metaperiodate. The mixture is incubated for 20 min in the dark at room temperature. Then 10 µl 80 mM sodium disulfite is added to destroy the periodate and incubation is continued for 5 min at room temperature. 5 µl 5 mM Dig-hydrazide (in dimethylformamide) is added and incubated for 1 h at room temperature. This mixture is either applied to SDS-PAGE or aliquots are spotted directly on dry nitrocellulose membranes for dot blot analysis.

Oxidation and Labeling of Glycoproteins already Immobilized on Membranes

The membrane with bound proteins is first incubated in 10 mM sodium metaperiodate dissolved in 0.1 M sodium acetate buffer (pH 5.5) for 20 min at room temperature. After washing three times with phosphate buffered saline (PBS: 0.05 M potassium phosphate, pH 7.5, 0.15 M sodium chloride), the membrane is incubated in 1 µM Dig-hydrazide in 0.1 M sodium acetate (pH 5.5) for 1 h at room temperature, and washed three times with Tris buffered saline (TBS; 0.05 M Tris-HCl, pH 7.5, 0.15 M sodium chloride). The immunodetection steps of the labeled glycoproteins are identical for both methods.

SDS-PAGE and Blotting

12% Polyacrylamide gels were used according to the procedure of Laemmli [9]. Gels were run in a Mini-Protean II from Bio-Rad (München) or a Minigel Twin from Biometra (Göttingen). When glycoproteins which had been oxidized and labeled in solution were loaded on gels, 15 µl of four-times concentrated SDS-sample buffer (0.1 M Tris-HCl, pH 6.8; 8% SDS, w/v; 40% glycerol, v/v ; 20% 2-mercaptoethanol, v/v) was added to give a final volume of 60 µl. After boiling for 2 min an aliquot depending upon the protein concentration was loaded into a gel slot. Proteins were transferred electrophoretically in a Mini-Transblot cell from Bio-Rad at 200 V (constant) for 1 h to nitrocellulose membranes as described by Burnette [10].

Protein Staining

Proteins in gels were stained with 0.1% Coomassie blue (Serva Blue G, Serva, Heidelberg) in 50% ethanol, 10% acetic acid for 20 min at 40°C, followed by destaining in 10% acetic acid. Proteins on nitrocellulose were stained by a 5 min incubation at room temperature with 0.2% Ponceau S in 3% TCA, followed by destaining in H_2O until the protein bands were visible. The Ponceau S staining of the protein bands disappears during the subsequent incubation of the membrane in the blocking solution.

Immunodetection

The membrane is first treated for 30 min with 0.5% blocking reagent (w/v) in TBS, pH 7.5. After washing three times with TBS, pH 7.5, the membranes are incubated for 1 h at room temperature with a 1:1000 dilution of <Dig>Fab-AP (750 U/ml) in TBS, pH 7.5. After washing the membrane again three times with TBS, pH 7.5, the alkaline phosphatase reaction is carried out by incubating the filter without shaking in 10 ml of the following solution (freshly prepared): 37.5 µl X-phosphate solution (50 mg/ml, in dimethylformamide) and 50 µl NBT solution (75 mg/ml, in 70% dimethylformamide) in 10 ml 0.1 M Tris-HCl, pH 9.5, 0.05 M $MgCl_2$. The reaction is normally complete within a few minutes, but can be extended for up to overnight, e.g. if very little protein is present.

Digestion with N-Glycosidase F

Glycoprotein (10 µg) dissolved in 10 µl H_2O is denatured by boiling for 2 min in the presence of 1% SDS. *N*-Glycosidase F buffer (90 µl; 20 mM sodium phosphate, 5 mM EDTA, 0.2 mM

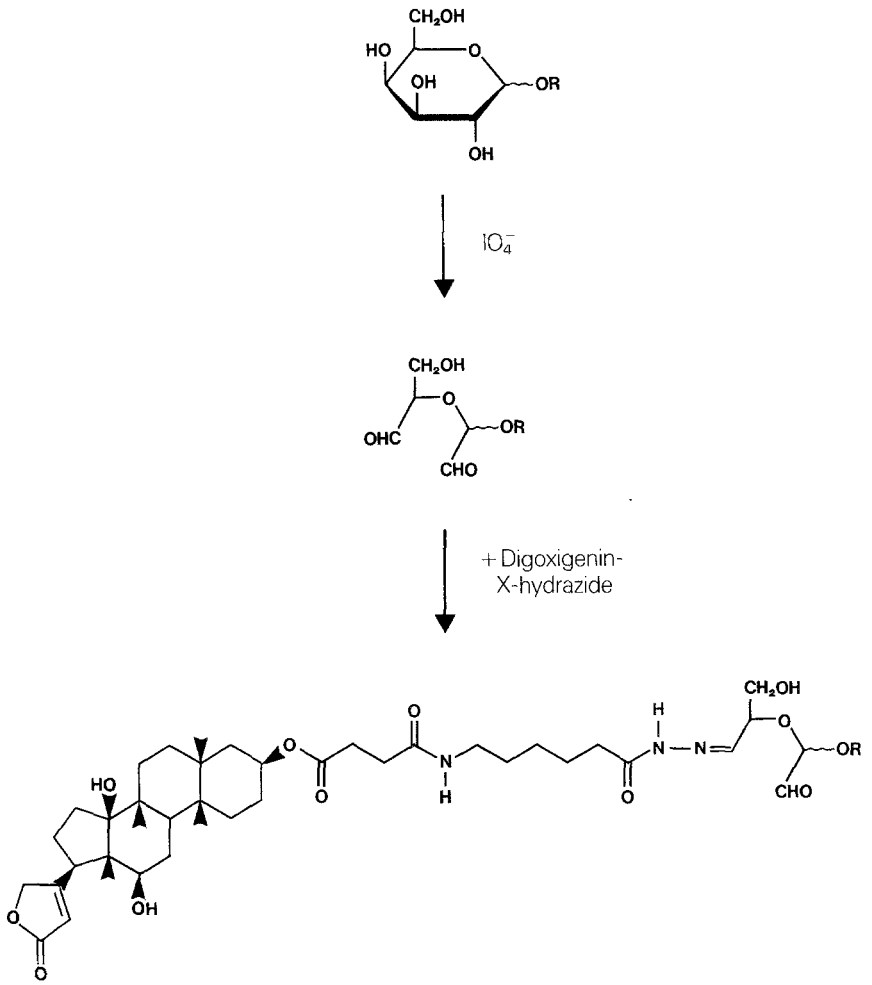

Figure 1. Reaction scheme for oxidizing and labeling glycoproteins in solution, followed by immunodetection on blots. For the sake of simplicity, digoxigenin substitution is shown at one aldehyde group only. The other should react similarly.

sodium azide, pH 7.2) and 0.5% (by vol) Nonidet P-40 (Sigma) is added, and the mixture is again boiled for 2 min. The mixture is cooled to 37°C, 0.4 units *N*-glycosidase F is added, and the reaction is incubated for 18 h at 37°C. A volume of 10 µl (≈1 µg of protein) is usually loaded on one lane of an SDS-gel or used for the oxidation and labeling reactions in solution. For dot blot analysis aliquots are spotted directly on dry nitrocellulose.

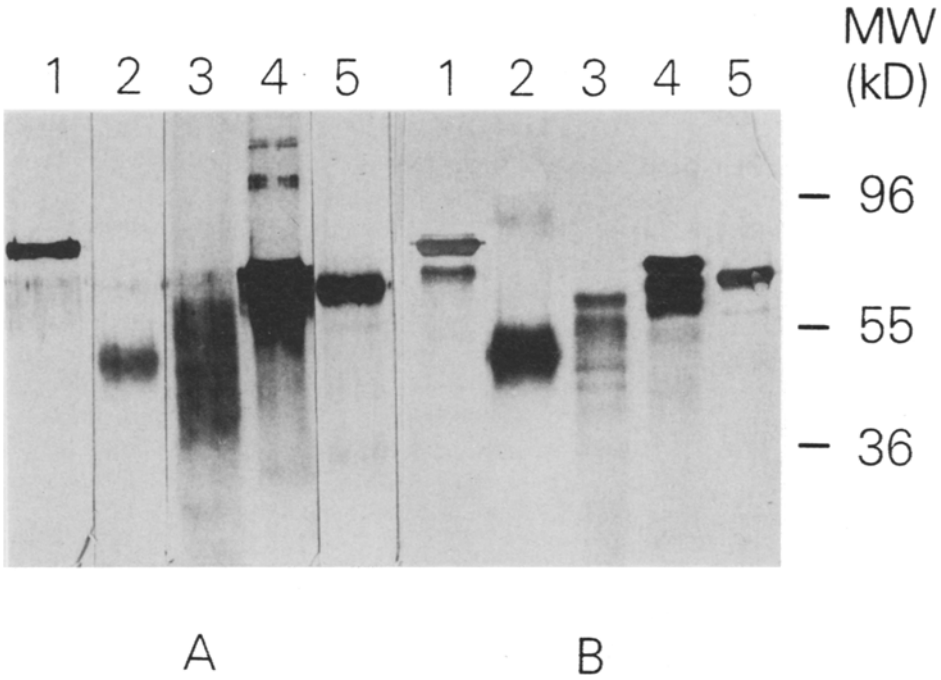

Figure 2. SDS-PAGE. The following glycoproteins (1 µg) were applied: lane 1, transferrin; lane 2, α_1-acid glycoprotein; lane 3, asialofetuin; lane 4, fetuin; lane 5, CpY. In panel A the glycoproteins were oxidized and labeled with digoxigenin in solution, and in B on the nitrocellulose membrane.

Digestion with Neuraminidase and β-Galactosidase

Glycoprotein (20 µg) was incubated with 0.02 U neuraminidase in 100 µl 0.05 M sodium acetate, pH 5.5, or with 5 mU β-galactosidase in 100 µl 10 mM citrate-phosphate buffer, pH 4.5, for 18 h at 37°C. A volume of 10 µl (=2 µg protein) was loaded on one lane of an SDS-gel.

Oxidation with Galactose Oxidase

For oxidizing terminal galactose residues of glycoproteins, the nitrocellulose with immobilized glycoproteins is soaked for 5 min in 0.1 M potassium phosphate buffer, pH 6.0, and then incubated for 15 h at 37°C with 7.5 U galactose oxidase and 2 µl 5 mM Dig-hydrazide (in dimethylformamide) dissolved in 10 ml 0.1 M potassium-phosphate buffer, pH 6.0. After washing the membrane three times with TBS, pH 7.5, blocking and immunodetection were performed as described above.

Results

General Principle

The general principle of the glycan detection system is shown in Fig.1. Mild periodate treatment oxidizes vicinal diol groups of sugars to aldehydes [8] and also the exocyclic carbon atoms of sialic acid by generating an aldehyde group at C-7 [11]. The steroid hapten digoxigenin is covalently linked to these aldehyde groups by derivatization with digoxigenin-succinyl-ε-amidocaproic acid hydrazide. Digoxigenin has been chosen as label for mainly two reasons: (a) A very specific, high affinity antibody was available ,and (b) unlike biotin, digoxigenin is not present in most biological systems. Therefore interferences caused by endogenous substances or binding sites should be negligible. The glycoproteins can be labeled with digoxigenin either in solution (method A), as depicted in Fig.1, or after transfer to suitable membranes (method B). Nitrocellulose and polyvinylidene difluoride (PVDF) membranes perform equally well with both methods. Nylon type membranes generally cause somewhat more background problems and should be avoided if possible. Fig. 2 shows a comparison of the two methods with a set of different glycoproteins.

Both methods generally produce very weak background staining, and both methods have certain advantages. A higher sensitivity for the detection of some glycoproteins can be achieved by performing the labeling in solution with subsequent separation of the digoxigenylated glycoproteins by SDS PAGE (method A). On the other hand the oxidation by periodate and the incorporation of digoxigenin can impair the electrophoretic separation behavior of glycoproteins to a certain extent, e.g. in the case of proteins that produce multiple banding patterns (see fetuin and asialofetuin in Fig. 2A). Moreover, labeling in solution is prone to be influenced by interfering substances which could be present in the glycoprotein solution (e.g. mercaptoethanol; dithiothreitol; glycerol; amino group-containing buffers, like Tris). Therefore, labeling in solution prior to electrophoresis (method A) is somehow more problematic than method B.

General Labeling and Application

By using the standard oxidation conditions (10 mM periodate for 20 min at room temperature), all types of periodate-oxidizable sugars are labeled. This results in the labeling of almost all known *N*- and *O*-type glycan chains, including also the glycan chain linked to the phosphoinositol anchor of proteins.

The detection of the glycoproteins can be performed with electrophoretically separated glycoproteins, or in a dot blot fashion. The latter technique can be particularly useful for the monitoring of glycoproteins or glycopeptides during chromatographic separations. Aliquots of fractions can be spotted on membranes, oxidized, labeled and detected within four hours. Moreover it is very useful for following the deglycosylation of a glycoprotein by *N*-glycosidase F, an enzyme which cleaves all types of *N*-linked glycan chains. The *N*-glycosidase F digested samples can be applied directly to nitrocellulose and analyzed for the presence or absence of carbohydrate. The removal of carbohydrate from a purified *N*-glycoprotein, e.g. with variation of incubation time and enzyme concentration, can be followed conveniently by this method (data not shown).

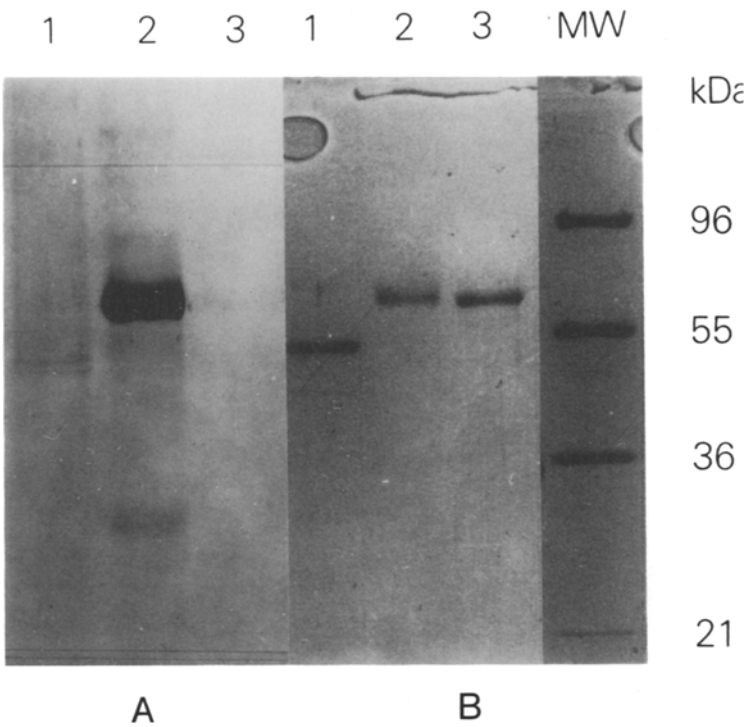

Figure 3. SDS-PAGE. Lane 1 contains 1 µg of CpY, deglycosylated with *N*-glycosidase F as described in the Methods section; oxidation and labeling with digoxigenin was done in solution. Lane 2 contains the same glycoprotein, oxidized and labeled with digoxigenin without prior deglycosylation. Lane 3 contains CpY as in lane 2, except that sodium metaperiodate was replaced by H_2O during the oxidation reaction. Two identical gels were run, one used for blotting and glycan detection (panel A), and one for staining with Coomassie blue (B).

Specificity of Carbohydrate Detection

That this detection system is highly specific for the carbohydrate part of glycoproteins is illustrated in Fig. 3. CpY, a glycoprotein with four *N*-linked oligo-mannose type glycan chains [12] (lane 2), was compared to the same compound which had been deglycosylated by *N*-glycosidase F (lane 1). As a further control glycosylated CpY, which had been digoxigenylated without prior oxidation by periodate, was included (lane 3)

The Coomassie blue stain reveals: a) The incorporation of digoxigenin does not alter the mol. wt. and the electrophoretic behaviour of the CpY significantly (compare lanes 2 and 3); b) The deglycosylation with *N*-glycosidase F leads to the expected decrease in the mol wt. Part A, stained for the presence of carbohydrate, shows clearly that only the glycosylated form is detected after oxidation. There is no non-specific staining of the deglycosylated protein.

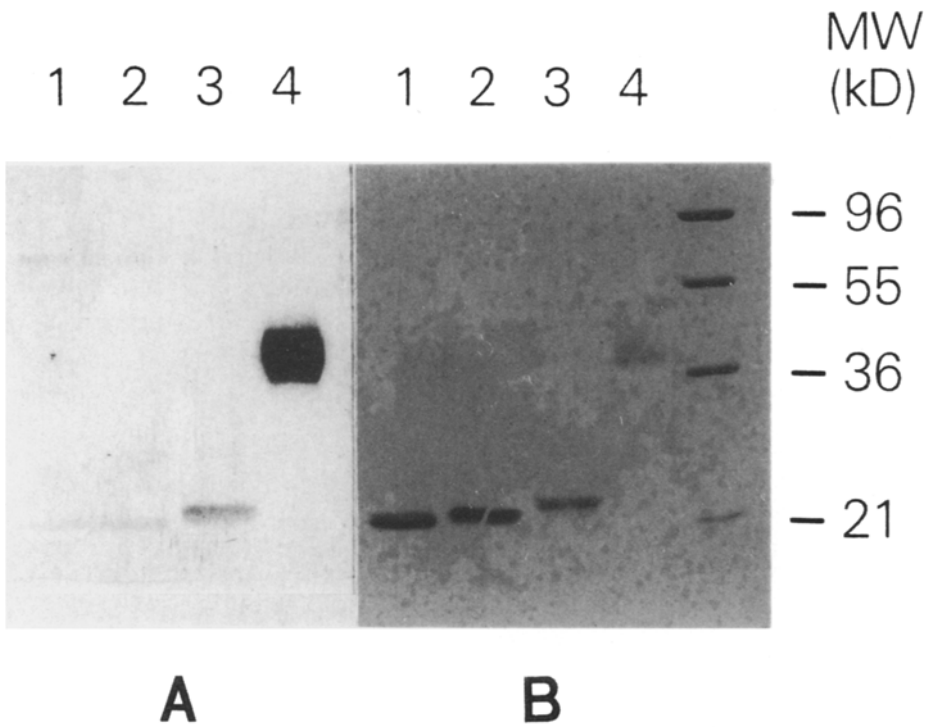

Figure 4. Erythropoietin (EPO; 10 μg) was deglycosylated with *N*-glycosidase F in a volume of 100 μl as described in the Methods section. When indicated, 1 mU neuraminidase and 0.3 mU endo-α-*N*-acetylgalactosaminidase were included in the *N*-glycosidase F digestion assay. Protein (1 μg) was applied per lane to a 15% SDS gel. Lane 1: EPO; deglycosylated with *N*-glycosidase F, neuraminidase and endo-α-*N*-acetylgalactosaminidase. Lane 2: EPO, deglycosylated with *N*-glycosidase F and neuraminidase. Lane 3: EPO, deglycosylated with *N*-glycosidase F. Lane 4: EPO. Two gels were run in parallel, one stained for carbohydrate, performing the oxidation and labeling reaction on the membrane (panel A), and the other for proteins with Coomassie (B).

From this type of analysis it is possible to obtain some information about the nature of the glycan chains (*N*-linked or not), since *N*-chains can be removed entirely by *N*-glycosidase F. Positive reaction of a glycoprotein after this treatment indicates the presence of differently linked carbohydrate, e.g. as *O*-glycan or a phosphatidylinositol glycan structure. Fig. 4 shows the analysis of the glycoprotein erythropoietin by a combination of the glycan staining technique and the use of exo- and endoglycosidases. Treatment with *N*-glycosidase F reduces the mol wt of erythropoietin to 22,000, suggesting the release of three *N*-glycan chains (the *N*-glycan chains were mostly resistant to endo F or endo H; data not shown). Since the 22 kDa band still shows a positive glycan stain it suggests that further glycans are

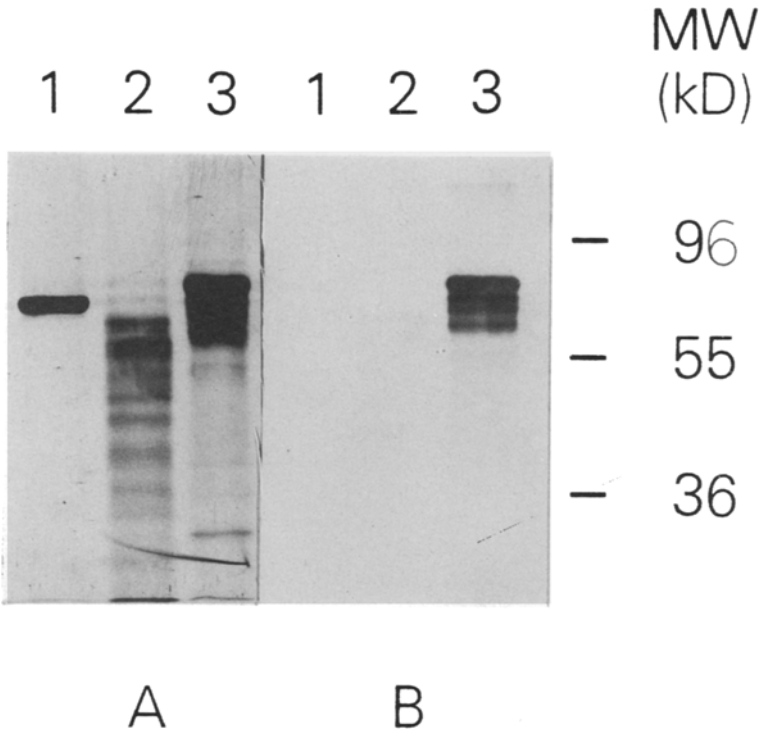

Figure 5. SDS-PAGE. Lane 1: 2 µg CpY. Lane 2: 2 µg asialofetuin. Lane 3: 2 µg fetuin. Two identical gels were run and blotted; one blot was oxidized for 20 min with 1 mM sodium metaperiodate at 0°C (panel B) and one with 10 mM sodium metaperiodate at room temperature (standard condition, panel A).

present. This is corroborated by the fact that neuraminidase treatment lowers this band to a 21 kDa band, which is still glycan positive. Treatment with endo-α-*N*-acetylgalactosaminidase further reduces the mol wt and eliminates the glycan staining. This is in accordance with the fact that erythropoietin has three complex *N*-glycan chains and one *O*-glycan, the latter consisting of a Gal-GalNAc structure carrying one or two sialic acids [13]. Due to incomplete digestion with endo-α-*N*-acetylgalactosaminidase in this particular experiment, a small amount of the form containing Gal-GalNAc is seen as a faint glycan-positive band just above the deglycosylated and glycan-negative band (lane 1 in Fig. 4).

Selective Detection of Sialic Acid

Since sialic acids are oxidized by periodate much more easily than other sugars [7] this labeling procedure can be made sialic acid-specific by selecting the appropriate oxidation conditions. Fig. 5 shows a comparison of the oxidation under standard conditions (part A),

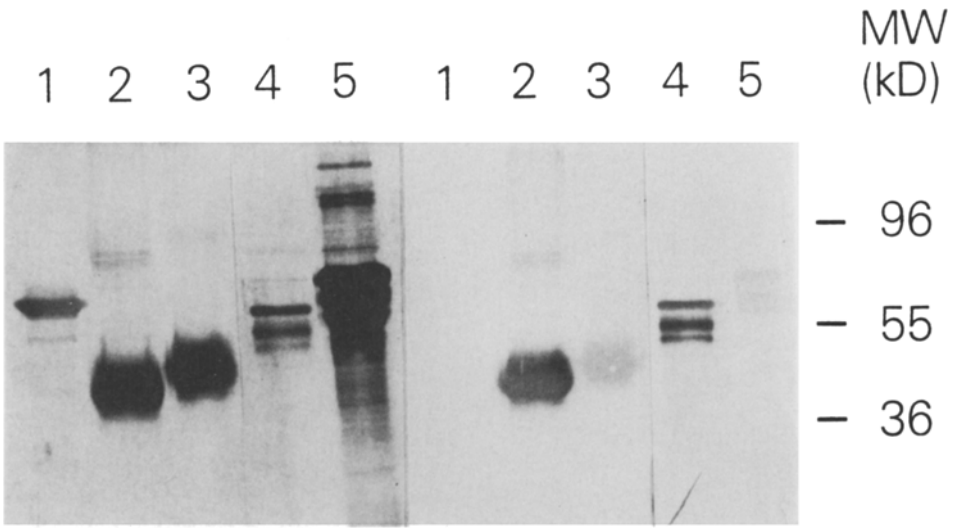

Figure 6. The following proteins (2 µg) were loaded on SDS gels. Lane 1; CpY: lane 2; α_1-acid glycoprotein, digested with neuraminidase: lane 3 α_1-acid glycoprotein: lane 4; fetuin, digested with neuraminidase: lane 5; fetuin. Two identical gels were run and blotted, one blot oxidized chemically according to the standard procedure (panel A), and the other with galactose oxidase as described in the Methods section (panel B).

and the sialic acid specific conditions (1 mM periodate at 0°C for 20 min; part B). From the three different glycoproteins seen in A, only the sialic acid-carrying fetuin is detected under the oxidation conditions used for B. The removal of sialic acid, e.g. by neuraminidase, can be conveniently followed by this type of analysis, preferably again by dot blots (data not shown).

Specific Detection of Terminal Galactose

This general glycan detection system can further be used for the specific detection of terminal galactose. For this purpose the oxidation is performed enzymatically by using galactose oxidase [14, 15]. This enzyme introduces aldehyde groups at the C-6 position of galactose residues, which can then be labeled with digoxigenin. The preferable way to do this is to treat the blotted glycoproteins on the membranes with galactose oxidase and Dig-hydrazide together at 37°C for about 15 h. Fig. 6 illustrates the result of such an experiment. Only fetuin and α_1-acid glycoprotein, which had been previously treated with neuramini-

dase, are stained positive. Further treatment with β-galactosidase renders the glycoproteins negative again (data not shown). Likewise, a strictly oligo-mannose type glycoprotein like CpY does not show any staining. The slight reaction seen with the sialic acid-containing glycoproteins fetuin and α_1-acid glycoprotein is due to some terminal galactose residues, already present in these heterogeneously glycosylated proteins.

These results illustrate, that the method can be used to identify terminal galactose units quite specifically.

Discussion

It has been demonstrated that the carbohydrate part of glycoproteins can be detected on blots by labeling with digoxigenin and detection with a specific, labeled antibody. The detection sensitivity depends on various things, among them the nature of the glycoprotein. With the standard procedure, 1 ng of α_1-acid glycoprotein could be seen on dot blots in overnight incubations, whereas ca.20 ng of CpY were necessary to produce a similar signal. Consequently the appropriate detection conditions have to be determined for the individual glycoproteins under investigation, and should be adjusted according to the experimental needs.

This glycan detection system can be used to establish glycoprotein patterns in various extracts and also for following glycoproteins during protein purification procedures. It also proves to be very useful in combination with exo- and endoglycosidases to obtain evidence on structural characteristics of glycoproteins. Especially important is the combination with *N*-glycosidase F, since this enzyme is able to remove all *N*-glycan chains from glycoproteins under suitable conditions. If the glycoprotein still shows a positive signal after thorough treatment with *N*-glycosidase F, it very likely contains *O*-glycan chains or sugars linked to phosphatidylinositol. Unfortunately, the other endoglycosidases (e.g. D,F and H) used in glycoconjugate research are not as useful in this combination, since they leave after hydrolysis one *N*-acetylglucosamine residue on the protein, which still produces a positive signal upon periodate oxidation. Nevertheless, these enzymes are very valuable in order to distinguish between the different types of *N*-linked carbohydrate chains when combined with SDS-PAGE analysis.

With the additional options to use sialic acid or terminal galactose-specific oxidation conditions, a broad arsenal of methods is now available for the structural analysis of glycoproteins on blots. Still more structural information about glycan chains can be obtained by probing glycoproteins on blots with lectins of known sugar specificity. The use of lectin-digoxigenin conjugates, which can be detected by the same immunological system as described above for the general detection of glycans, is under investigation. So far, the method has only been used with the membranes described in the Results section, but it should be adaptable to other surfaces, e.g. TLC plates.

The combination of all these detection methods with the high resolution power of the widely applied, different electrophoretic techniques should prove to be very valuable for the structural analysis of glycoproteins.

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