Biotinylated Carbohydrate Markers – A Novel Tool for Lectin Research

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One of the key obstacles in lectin research is the lack of specific techniques for their detection. Novel markers, biotin-labeled carbohydrates, could contribute to overcome this problem. Being at least 10 times more sensitive than neoglycoproteins in the membrane-screening assays, they also enable direct detection of lectins in complex mixtures. The markers were synthesized by linking biotin to one, and a carbohydrate (galactose or glucose) to the other amino group of (the amino acid) lysine. After synthesis the markers were chromatographically purified on lectin (RCA for galactose marker, ConA for glucose marker) and avidin affinity columns. The applicability of the markers to detect lectins was demonstrated *e.g.* with sponge extracts (from *Geodia cydonium*). Following incubation with biotin-labeled carbohydrates covalent cross-linking between lectins and markers was induced by UV radiation. After transfer to the blotting membrane, lectins were detected with deglycosylated anti-biotin antibody labeled with alkaline phosphatase. Besides for the cross-linking technique, the biotinylated carbohydrate markers were also used for detection of lectins on the nitrocellulose membrane in gene library screening and slot blotting.

Introduction

In many important biological phenomena like cell migration, organ formation and embryogenesis, molecular recognition plays a key role. Improper functioning of the cell recognition is thought to underlie the uncontrolled cell growth and motility that characterize neoplastic transformation and metastasis (Sharon and Lis, 1989a). Although the nature of molecules involved in cellular recognition is still largely unknown, in recent years much attention has been focused on the glycoproteins and their receptors – lectins, as candidates for this function (Barondes, 1984; Liener *et al.,* 1986; Sharon and Lis, 1989b). With a very few exceptions, in eukaryotic cells, most secreted and membrane-associated proteins are glycosylated (Dwek *et al.,* 1993). The attached carbohydrates enrich the glycoproteins with a huge potential to encode information. Molecules which are physiological receptors for glycoproteins, and thus responsible for decoding their information, are lectins (Bohlool and Schmidt, 1974; Harrison and Chesterton, 1980). Although lectin research had its origins at the turn of the century, animal lectins have started to attract more attention only during the last decade (Barondes, 1981; Drickamer and Taylor, 1993; Sharon, 1993).

One of the key problems in the research of lectins was the lack of specific techniques for their detection. In this paper we introduce novel markers, biotin-labeled carbohydrates, which may help to overcome this problem. In addition to being at least 10 times more sensitive than neoglycoproteins in the membrane screening, biotin-labeled carbohydrate markers allow direct analysis of the carbohydrate-binding proteins in complex mixtures including even whole tissue homogenates.

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Gal, galactose; Glc, glucose; Lys, lysine; Bio, biotin; BCIP, 5-bromo-4-chloro-indolylphosphate; NBT, nitroblue tetrazolium; ConA, concanavalin A; RCA, *Riccinus communis* lectin; Tris, tris-(aminomethyl)-aminomethane; IPTG, isopropyl β-D-thiogalactopyranoside.

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Materials and Methods

Materials

5-Bromo-4-chloro-indolylphosphate (BCIP), nitroblue tetrazolium (NBT), α-D-glucopyrano-

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side-phenylisothiocyanate, β-D-galactopyranoside-phenylisothiocyanate, lactose and biotin-labeled BSA, monomeric avidin-agarose, *Ricinus communis* lectin agarose, concanavalin A and anti-biotin antibody labeled with alkaline phosphatase were purchased from Sigma (St. Louis, MO), Immobilon PVDF membrane from Millipore (Bedford, MA), D-biotin-N-hydroxy-succinimidester from Boehringer Mannheim (Mannheim). Specimens of the marine sponge *Geodia cydonium* were collected in the Adriatic Sea near Rovinj (Croatia) and stored frozen at −20 °C until analyzed.

Deglycosylation of antibodies

Antibodies as supplied by SIGMA (1.5 mg/ml) were diluted 1:1 in the phosphate-buffered saline (PBS, pH = 7.4, and incubated overnight with 1 unit of N-glycosidase F from *Flavobacterium meningosepticum.* After deglycosylation the enzyme remained in the antibody solution. Its presence did not cause any problems in the subsequent detection assays.

Concentration of proteins with deoxycholic acid and trichloroacetic acid

Deoxycholic acid (DHA) was added to protein sample to final concentration of 0.5%, and precipitated with addition of trichloroacetic acid (TCA) to final concentration of 5%. Denatured proteins were pelleted by centrifugation for 5 min on 6000×*g*. Supernatant was discharged. The pellet was resuspended in acetone and centrifuged again. After another round of acetone washing, the pellet was dissolved in a small volume of electrophoresis sample buffer.

Electrophoresis and blotting

Gel electrophoresis of proteins was performed in 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (SDS-PAGE) according to Laemmli (1970). After electrophoresis proteins were transferred to the PVDF Immobilon membrane (Towbin *et al.,* 1979) in a semi-dry blotting system (Pharmacia, Sweden). After overnight blocking with 3% bovine serum albumin membranes were incubated with deglycosylated anti-biotin antibody conjugated to alkaline phosphatase and visualized with BCIP/NBT.

Colony screening

The bacterial cells were seeded onto nitrocellulose filters layered on the top of an agar plate containing 2 mM IPTG and were incubated overnight at 37 °C. Subsequently the filters were removed from the agar plates and exposed to a saturated chloroform atmosphere for 10 min. After chloroform exposure the filters were dried, and incubated in a lysozyme buffer (50 mM Tris/HCl, pH = 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 3% BSA, 400 μg/ml lysozyme, 1 U/ml DNase I) for 60 min with gentle agitation. The filters were then washed and transferred to 3% BSA for overnight blocking. The blocked filters were incubated with Gal–Lys–Bio (biotinylated galactose marker). Marker-lectin complexes were detected with deglycosylated anti-biotin antibody-labeled conjugated to alkaline phosphatase and visualized with BCIP/NBT.

Results and Discussion

Lack of adequate techniques for specific detection of the carbohydrate-binding proteins was the major obstacle in lectin research. Until now, lectins were usually first isolated by affinity chromatography, and then detected with the classical staining methods as silver staining or autoradiography (Jia and Wang, 1983; Schröder *et al.,* 1992). In the case of proteins which are able to refold on a membrane after SDS-PAGE, the neoglycoproteins have also been used for detection. However, if the protein was unstable, then the final proof that it is really a lectin was possible only after cloning. Due to such a complicated procedure only few animal lectins are cloned until now.

To overcome this problem we have designed carbohydrates labeled with biotin, a low molecular weight marker which can specifically detect lectins. The marker consists of a carbohydrate-phenylisothiocyanate attached to one, and biotin-succinimide ester to the other amino group of lysine. Lectins bind specifically to the carbohydrate part of the marker, leaving the biotin label free in the solution. After exposure to UV radiation, or some suitable chemical cross-linking reagent, the phenyl ring in the carbohydrate part opens and cross-links the marker and the lectin, resulting in a lectin with the covalently linked biotin label. Since the average molecular weight of a

monosaccharide marker is only about 1000 daltons, the size difference between the original and the biotin-labeled lectin is only marginal, and does not impose any problem for the analysis on the SDS-PAGE.

Besides the cross-linking application, the markers can be also used for membrane screening. Due to their small size and very hydrophilic nature, they display at least ten times higher sensitivity than neoglycoproteins, and they can be used for direct screening of the gene libraries.

We have synthesized biotin-labeled carbohydrate markers with several different monosaccharides. Since the procedure differs only in final (purification) steps, we shall present only the procedure for synthesis of the galactose marker. Lysine (10 mg/ml) was dissolved in 0.1 M borate buffer (pH = 8.8) and mixed with β-D-galactopyranoside-phenylisothiocyanate and D-biotin-N-hydroxy-succinimidester, both prior dissolved in a small volume of dimethylsulfoxide. The approximate molar ratio of β-D-galactopyranoside-phenylisothiocyanate, D-biotin-N-hydroxy-succinimidester and lysine was 2:2:1. The mixture was incubated overnight on an overhead shaker at +4 °C. The reaction was stopped by addition of 1 M NH_4Cl to final concentration of 50 mM. The pH was adjusted to 7.5 by addition of 0.1 volume of 1 M Tris/HCl (pH = 7.5). As estimated by gel filtration in Bio-Gel P2, about 60% of β-D-galactopyranoside-phenylisothiocyanate were incorporated into molecules with a size corresponding to completely reacted lysine, *i.e.* lysine with labels on both amino groups (Gal–Lys–Gal or Gal–Lys–Bio [Bio: biotin]).

After synthesis the reaction mixture consisted of several different compounds: Gal–Lys–Gal, Gal–Lys–Bio, Bio–Lys–Bio, Lys–Gal, Lys–Bio and the unreacted lysine, β-D-galactopyranoside-phenylisothiocyanate and D-biotin-N-hydroxy-succinimidester. The galactose marker was purified using the following two affinity columns. (i) A lectin column, yielding carbohydrate-containing compounds, and (ii) an avidin column yielding compounds containing both biotin and carbohydrate. Different carbohydrate markers were purified using different lectin columns. For the galactose–lysine–biotin (Gal–Lys–Bio) marker, a galactose-specific *Ricinus communis* lectin (RCA) was used. After an overnight labeling reaction, the reaction mixture (pH adjusted to 7.5) was supplemented with 1 mM $CaCl_2$ and applied onto RCA-agarose column, equilibrated with Tris/HCl buffer (pH = 7.5, 0.5 M NaCl, 1 mM $CaCl_2$). Following the washing with the equilibration buffer, the galactose-containing molecules were eluted with 0.1 M galactose in the same buffer. Approximately 10 nmol of galactose-containing compounds were obtained per ml of packed gel. Eluted fractions were pooled and applied onto a monomeric avidin-agarose column. After washing with Tris/HCl buffer (pH = 7.5, 0.5 M NaCl), the pure Gal–Lys–Bio was eluted with 0.2 mg/ml biotin. The average yield was in the range of 20 nmol Gal–Lys–Bio/ml packed gel.

Glucose–lysine–biotin (Glc–Lys–Bio) complex was purified analogously to Gal–Lys–Bio, but using the mannose/glucose-specific lectin concanavalin A (ConA) instead of RCA lectin. The ConA column was prepared with 5 mg of pure concanavalin A per milliliter of Affi-Prep gel (BioRad) according to the manufacturer's instructions. Glucose-containing molecules were eluted from the column with 0.2 M glucose. The average column capacity for glucose was 10–20 nmol/mg gel. Eluted fractions were pooled and applied onto monomeric avidin-agarose column. Pure Glc–Lys–Bio was eluted with 0.2 mg/ml biotin in TBS.

We suggest several procedures for the analysis of the carbohydrate-binding proteins which utilize biotinylated carbohydrate markers. The novel, most effective technique is the *cross-linking method.* Biotin-labeled carbohydrate markers were incubated with lectins to allow specific recognition and establishment of the lectin-marker complexes. The formed complexes were subsequently cross-linked using UV radiation. The result is a lectin with attached biotin label. After SDS-PAGE separation and blotting lectins were detected on the membrane with deglycosylated anti-biotin antibody conjugated to alkaline phosphatase. Antibodies were deglycosylated as described in "Materials and Methods". Deglycosylation was necessary to eliminate non-specific binding of lectins to the carbohydrate parts of antibodies. The result of one application of this method is presented here for detection of the lactose/galactose-specific lectins from the marine sponge *Geodia cydonium* (Fig. 1).

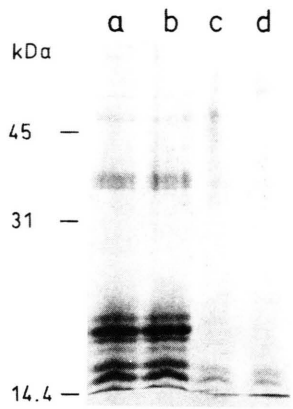

Fig. 1. SDS-PAGE of lectins from the marine sponge *Geodia cydonium* UV cross-linked with galactose-lysine-biotin, transferred to the PVDF membrane and detected with deglycosylated anti-biotin antibody conjugated to alkaline phosphatase using 5-bromo-4-chloro-indolyl-phosphate + nitroblue tetrazolium as a substrate. (a) 100 μg of total *Geodia* proteins, (b) 10 μg of purified *Geodia* lectin, (c) 10 μg of purified *Geodia* lectin + 0.1 M lactose, (d) 10 μg of purified *Geodia* lectin cross-linked with Glc–Lys–Bio instead of Gal–Lys–Bio.

1 μl of β-D-galactopyranosidephenyl–Lysine–Biotin (Gal–Lys–Bio) was incubated for 15 min with 10 μl of sponge extracts (Müller and Zahn, 1973) to allow binding of markers to the lectins. After binding, samples were diluted with 1 ml of bidistilled H_2O and exposed to UV radiation in the "Stratagene cross-linker 1800" for 10 min on ice. Following cross-linking samples were concentrated with DHA/TCA as described in "Materials and Methods", separated on SDS-PAGE and transferred to an PVDF membrane. Characteristic lactose/galactose-specific *Geodia* lectins (Pfeifer *et al.*, 1993) are clearly visible in the Fig. 1. Since the approximate molecular weight of the galactose marker is only about 1000 daltons, the apparent size of labeled lectins differed only slightly from the previously reported values. Fig. 1, lane "a" shows the detection of lectins from the crude *Geodia* extract. In lane "b" a sample of the purified *Geodia* lectin was detected as a control.

Ten minutes were found to be the optimal cross-linking time for this system. A shorter exposure produced a lower signal, while a longer cross-linking resulted in an increased proportion of the non-specific cross-linking and a higher background. Another important point for diminishing the background was cross-linking at low protein concentrations. Sufficiently low protein concentrations were obtained by diluting samples with approximately hundred volumes of bidistilled water prior to the UV exposure. Samples were concentrated after cross-linking. This approach resulted in a markedly lower background, thus providing an increased sensitivity of the method. The third important factor for the reduction of background was the temperature. Cross-linking at 0 °C proved to be much more effective than at room temperature, probably due to lower non-specific cross-linking because of a decreased diffusion rate.

The specificity of the method has been tested in two ways: (i) by competing the galactose marker with 0.1 mol lactose, and (ii) by cross-linking with Glc–Lys–Bio, the glucose marker instead of the galactose marker. Since the *Geodia* lectins have higher affinity for lactose than for galactose (Müller *et al.*, 1983; Diehl-Seifert *et al.*, 1985), and lactose was added in great surplus, a significant reduction in the intensity of labeling was to be expected. Indeed, almost complete disappearance of lectin bands is clearly visible in Fig. 1 c. The similar effect was also observed if Glc–Lys–Bio, glucose instead of a galactose marker, was used (Fig. 1 d).

The second possible application for biotinylated carbohydrate markers is the detection of lectins on a nitrocellulose membrane. Although a similar detection is possible with neoglycoproteins, detection with biotinylated carbohydrate markers is at least ten times more sensitive. A possible explanation for the enhancement in the sensitivity could be the small size of our markers. They are allowed to enter many carbohydrate-binding sites on the lectins which are inaccessible for neoglycoproteins because of steric hindrance or an inappropriate orientation of lectins on the membrane. Another advantage of our markers is their hydrophilic nature which drastically reduces their affinity for PVDF membranes. This permits a significantly shorter time for membrane blocking, thus making procedures less time consuming.

An example of this detection method is displayed on Fig. 2. Partly purified lectins from the marine sponge *Geodia cydonium* were loaded on a PVDF membrane in a slot blot apparatus. Lane "b" was detected using Gal–Lys–Bio, a galactose marker, and lane "a" with lactose–BSA–biotin, a neoglycoprotein. Our galactose marker detected

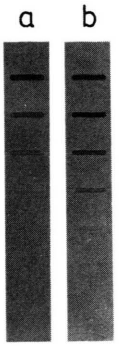

Fig. 2. Detection of *Geodia* lectins with biotinylated galactose marker on a slot blot. Partly purified lectins were loaded on the PVDF membrane (Millipore) in a slot-blot apparatus. Each slot from the top to the bottom contains five times less proteins than the previous one, beginning with 10 μg of total protein. Lane a: detection with BSA labeled with lactose and biotin (SIGMA); lane b: detection with Gal–Lys–Bio, a biotinylated galactose marker. Biotin labels were detected with deglycosylated anti-biotin antibody conjugated to alkaline phosphatase and developed with 5-bromo-4-chloro-indolylphosphate and nitroblue tetrazolium.

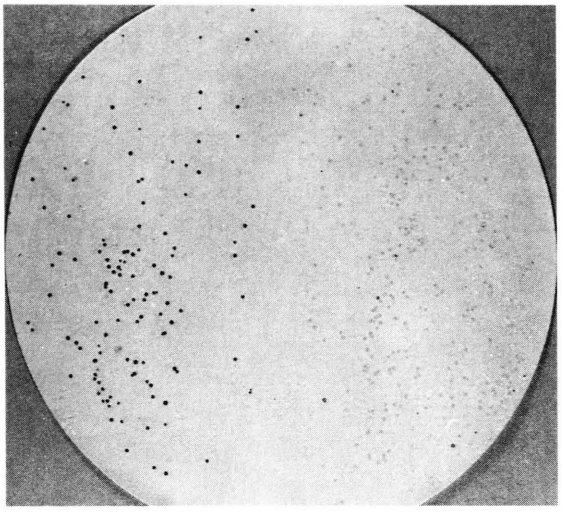

Fig. 3. Applicability of the biotinylated carbohydrate markers for screening of lectin-producing clones is demonstrated with the *E. coli* clone producing lectin I from the marine sponge *Geodia cydonium* (Pfeifer *et al.*, 1993). Bacterial cells producing the lectin were seeded on the left side of the filter. On a right side cells lacking the lectin-coding plasmid were seeded as a control. The filters were detected using Gal–Lys–Bio and deglycosylated anti-biotin antibody conjugated to alkaline phosphatase. The positive clones were visualized with 5-bromo-4-chloro-indolylphosphate and nitroblue tetrazolium.

at least ten times lower amount of lectins than neoglycoprotein.

A third important application for the biotinylated carbohydrate markers is in cloning of carbohydrate-binding protein genes. This method permits omittion of the monoclonal antibodies production and enables a direct screening of the gene library. Since the production of monoclonal antibodies is time consuming, and in the case of animal lectins, due to their low intracellular concentrations often a limiting step, the introduction of our method opens new perspectives in lectin research. The detection of all carbohydrate-binding protein-producing clones might seem to be a problem for screening, but since the number of different lectins in an animal cell is usually not very large, SDS-PAGE analysis of positive clones usually reduces the number of clones to a reasonable figure.

Demonstration of this screening method is presented in Fig. 3. Two different clones of *XL1-blue E. coli* (Pfeifer *et al.*, 1993) were seeded onto nitrocellulose filters. The clone producing *Geodia* lectin I (Diehl-Seifert *et al.*, 1985) on the left half, and the control clone (lacking the plasmid) on the right half. After induction with IPTG (isopropyl β-D-thiogalactopyranoside), filters were screened using Gal–Lys–Bio, the galactose marker. Positive (lectin producing) clones were detected with deglycosylated anti-biotin antibody conjugated to alkaline phosphatase and visualized with BCIP/NBT. Fig. 3 clearly shows that all control clones (right half) are negative, while most clones on the left side are positive. Several negative colonies on the right side of the filter could be attributed to clones which have ceased to produce lectins, either due to mutations, or some other reason.

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