Glycoconjugate Journal (1994) **11**:541–549

Identification and purification of a stress associated nuclear carbohydrate binding protein (M_r 33 000) from rat liver by application of a new photoreactive carbohydrate probe

GORDAN LAUC[1,2], MIRNA FLÖGEL[1], BARBEL DIEHL-SEIFERT[2], HEINZ C. SCHRÖDER[2] and WERNER E. G. MÜLLER[2]*

[1] *Department of Medical Biochemistry, Faculty of Pharmacy and Biochemistry, University of Zagreb, Domagojeva 2, Zagreb, Croatia*

[2] *Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität, Duesbergweg 6, 55099 Mainz, Germany*

Received 20 April 1994, revised 25 July 1994

A photoreactive α-D-glucose probe has been designed for the specific detection of carbohydrate binding proteins (CBPs). The probe consists of four parts: (i) an α-D-glucose moiety; (ii) the digoxigenin tag; (iii) the photoreactive cross-linker; and (iv) the lysyl-lysine backbone. After incubation with lectins in the dark, the probe is activated and cross-linked to the CBPs after being treated by several flashes.

Using this method we have identified a new α-D-glucose CBP of M_r = 33 000, termed CBP33, in the nuclei of rats exposed to transient immobilization stress. Monoclonal antibodies were raised against the partially purified protein and subsequently used to enrich CBP33. It was purified (>2400-fold) to apparent homogeneity from a 0.6 M nuclear salt extract by two subsequent affinity chromatography steps (antibody-affinity as well as α-D-glucose affinity column).

Keywords: carbohydrate binding proteins; lectins; stress; rat liver; CBP33.

Abbreviations: BSA; bovine serum albumin; CBP; carbohydrate binding protein; DIG; digoxigenin; Gal; galactose; Glc, glucose; Lys; lysine; PAGE; polyacrylamide gel electrophoresis; SDS; sodium dodecyl sulphate.

Introduction

Animal carbohydrate-binding proteins [CBPs] (lectins) are subdivided into two large groups; the calcium-dependent (C-type) CBPs and the metal-independent β-galactoside-binding CBPs [1, 2]. Both types are provided with a characteristic carbohydrate-recognition domain [1]. The C-type CBPs are either free or membrane-bound proteins. Some of them like the selectin cell adhesion molecules, or the macrophage mannose receptor are involved in cell adhesion [3]. The metal-independent β-galactoside-binding CBPs, namely electrolectin, galaptin, S-Lac lectins, occasionnally require thiol groups for binding [1, 2]. Until recently they were described only from vertebrates; they have now also been cloned from the nematode *Caenorhabditis elegans* and the lowest multicellular eukaroytes, the marine sponges [*Geodia cydonium*] [1, 4–6].

Glycoproteins occur not only extracellularly or at the cell surface, but are present also intracellularly [7]. Several novel types of intracellular glycoconjugates have been described; e.g. *N*-acetylglucosamine residues on proteins in the nucleus or cytoplasm, *O*-linked mannose-containing proteoglycans in adult rat brain and glycosyl residues attached to the 'primer' for glycogen synthesis [7, 8].

Several CBPs have been identified in the nuclei of mammalian cells [9–15]. Their preferential location is the extranucleolar region, the site of transcriptional and post-transcriptional events [16]. Their expression and intracellular distribution apparently depend on the proliferation and differentiation state of the cell [17]. Two nuclear CBPs, CBP35 (also known as Mac2, L29, or IgE-binding protein) [18, 2] and CBP67, have been characterized in more detail [12, 19–21]. Previously we have described the purification of the CBP67, an α-D-glucose specific nuclear CBP with a molecular mass of 67 kDa from rat liver nuclei [20]. We demonstrated that CBP67 exists in the nuclear

* To whom correspondence should be addressed.

ribonucleoprotein complex (RNP) and is absent in the polysomal RNP. This protein was isolated from nuclear RNP complexes by extraction in the presence of 0.6 M NaCl. The binding of CBP67 to glucose-containing glyconjugates depends on the presence of Ca^{2+} and Mg^{2+}. Therefore, CBP67 is considered to be a C-type CBP [1]. With respect to this property it is distinct from CBP35 which is classified as a metal-independent β-galactoside-binding CBP, because it does not require metal ions for binding to galactose. More recently, a CBP with an $M_r = 70000$ (CBP70) has been isolated in association with the CBP35 from nuclei of the human tumoural cell line HL60 [22]. Although both CBP67 and CBP70 have similar specificities they are probably distinct proteins [22].

Affinity matrix purification is one of the most widely applied techniques to isolate CBPs [23, 24]. Interactions with neoglycoproteins [25, 15] have often been used as a method to detect carbohydrate binding activity, but this method requires either pure lectin, or the lectin must retain its activity during SDS/polyacrylamide gel electrophoresis or refold on the membrane after Western blotting.

In spite of intense research the exact function of the nuclear CBPs is not yet clear. In this paper we report on the identification and purification of a new glucose-specific nuclear CBP, termed CBP33, which is induced in the rat liver after exposure to transient immobilization stress. The CBP was identified using a newly developed photoreactive carbohydrate probe. This method allows specific detection of CBPs in complex mixtures.

Materials and methods

Materials

^{125}I-sodium iodine (carrier free) was purchased from Amersham International PLC (Buckinghamshire, England); 5-bromo-4-chloro-indolylphosphate (BCIP), nitro blue tetrazolium (NBT), 4-azidobenzoic acid-*N*-hydroxysuccinimide ester, lysyl-lysine, α-D-glucopyranoside-phenylisothiocyanate and other carbohydrates used from Sigma (St Louis, MO); Immobilon PVDF membrane from Millipore (Bedford, MA), digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester and anti-digoxigenin Fab fragments labelled with alkaline phosphatase from Boehringer Mannheim (Mannheim), Bio-Gel P2, Affi-Prep 10 and Affi-Gel 102 from Bio-Rad Laboratories (Hercules, CA).

Preparation of the glucose probe

(i) *Glc-Lys-Lys.* In the first step the α-D-glucose (Glc) tag was introduced into lysyl-lysine by reacting α-D-glucopyranoside-phenylisothiocyanate with one of the amino groups of lysyl-lysine. α-D-glucopyranoside-phenylisothiocyanate (1 mg) was dissolved in 15 µl DMSO and slowly transferred to 0.5 ml lysyl-lysine solution under constant shaking. To ascertain that only one molecule of glucose was introduced per one lysine molecule, the molar ratio of α-D-glucopyranoside-phenylisothiocyanate to lysyl-lysine was adjusted to 1:5. Reaction was performed for 2 h at room temperature in 0.1 M borate buffer (pH 8.8). Non-reacted α-D-glucopyranoside-phenylisothiocyanate and lysyl-lysine molecules were separated from the reaction product (Glc-Lys-Lys) by gel filtration through a Bio-Gel P2 column (0.6 × 50 cm), equilibrated in 0.1 M borate buffer (pH 7.4). Eluted fractions were analysed for the presence of glucose with the resorcinol sulfuric acid micromethod [26], and for the presence of NH_2 groups with ninhydrin. Two well separated peaks were obtained ($V_e V_o = 1.44$, and 1.78). The first peak contained both the glucose and the amino groups (corresponding to Glc-Lys-Lys), while the second peak contained only amino groups (corresponding to unlabelled Lys-Lys). There was no cross-contamination among the two peaks. More than 95% of the glucose was incorporated into the Glc-Lys-Lys complex and was eluted in the first peak ($V_e/V_o = 1.44$). Nonreacted lysyl-lysine and α-D-glucopyranoside-phenylisothiocyanate co-eluted in the retarded fraction ($V_e/V_o = 1.78$).

(ii) *Glc-Lys-Lys-Dig.* In the second step the digoxigenin label was added to the Glc-Lys-Lys complex. Digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester (0.3 mg) was dissolved in 10 µl of DMSO and added to the Glc-Lys-Lys solution. The labelling reaction was performed for 2 h at room temperature.

The reaction was performed at pH 7.4 to avoid introduction of more than one digoxigenin label, and a consequent blocking of the remaining amino group in the Glc-Lys-Lys-Dig complex. At this pH the ω-amino groups of lysyl-lysine are almost completely protonated and only the α-amino group reacts with the digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester. Since more than 50% of the α-amino groups were already occupied with the glucose label in the previous step, the molar ratio of digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester to Glc-Lys-Lys complex was set to 1:3. The reaction product (Glc-Lys-Lys-Dig complex) was separated from the nonreacted molecules by a second gel filtration procedure applying the same column size as mentioned above. For equilibration and elution a 0.1 M borate buffer (pH 8.8) was used. Based on absorbance measurements more than 90% of the added digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester was incorporated into the Glc-Lys-Lys-Dig complex. The identity of Glc-Lys-Lys-DIG, which as the largest molecule eluted in the first peak, was confirmed using ELISA with plates precoated with Concanavalin A, a mannose/glucose specific lectin. After incubation with the probe, molecules containing both galactose and digoxigenin were detected with

anti-digoxigenin antibodies. The second peak, containing Glc-Lys-Lys, gave a negative result in the same assay.

(iii) *The photoreactive α-D-glucose probe.* The final step in synthesis of the photoreactive glucose probe was the introduction of azidobenzoic acid, a photoreactive cross-linking reagent. The 4-azidobenzoic acid-*N*-hydroxysuccinimide ester (0.3 mg), dissolved in 5 µl DMSO, was added to the Glc-Lys-Lys-Dig complex (molar ratio 2:1). The reaction was performed for 2 h at room temperature in 0.1 M borate buffer, pH 8.8. Nonreacted *N*-hydroxysuccinimide ester was inactivated by addition of excess NH_4Cl. After inactivation free 4-azidobenzoic acid-*N*-hydroxysuccinimide ester became monovalent and lost the cross-linking ability. It remained together with the glucose probe, but did not cause any problem in subsequent application of the probe.

Concanavalin A – ELISA

The Pro-Bind plates (Falcon) were precoated with concanavalin A by 4 h incubation with 0.1 mg per well concanavalin A. After overnight blocking with 3% BSA in PBS the wells were incubated with samples (fractions from the gel filtration during purification of the photoreactive glucose probe) for 2 h. After washing, the wells were screened with anti-DIG Fab fragments labelled with horseradish peroxidase, and developed with 0.04 mg ml^{-1} o-phenylenediamine in 0.05 M Na citrate, 0.15 M Na phosphate, 0.01% H_2O_2, pH 5.0 as a substrate.

Thin-layer chromatography

Purity of the α-D-glucose probe was analysed by thin-layer chromatography (HPTLC Pre-coated plates, 0.2 mm thick silica gel, 10 × 10 cm; Merck, Darmstadt) using the chloroform:methanol:40 mM KCl (50:42:11) solvent system. The sample was applied, and the plate developed in the dark room under a red light. After development the plate was cut into two halves. One half was stained for glucose-containing compounds, and the other for the amino groups. For detection of glucose containing compounds the plates were sprayed with a resorcinol/HCl reagent (0.2% resorcinol; 0.25 mM $CuSO_4$ in 80% HCl). For detection of NH_2-containing compounds the plates were sprayed with 0.25% ninhydrin in 96% ethanol. Immediately after spraying, the plates were layered on another glass plate pre-heated to 100 °C, fastened with clips and maintained on 100 °C until the colour developed.

Cross-linking assay

Protein samples (8 µl) were supplemented with 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The NaCl concentration was adjusted to 250 mM. The 0.5 µM α-D-glucose probe (2 µl) was added to the samples (final volume = 10 µl) and incubated for 30 min in the dark at room temperature. Samples were cross-linked by illumination with five to 10 flashes of a camera flashlight [Minolta 5200i] from a distance of 25 cm. The exact number of flashes and the subsequent procedures are given in the respective figure legends.

CBP33 antibody

Monoclonal antibodies were raised in Balb/c mice against partially purified CBP33 [28]. Antigen was introduced by intra-spleenal implantation. Two implantations were performed in 3 week intervals. Prior to implantation, antigen (5 µg) was immobilized on a PVDF membrane. After 6 weeks the animal was boosted by an i.p. injection of 5 µg of the antigen. The monoclonal antibody was selected by ELISA and Western blot screenings and the positive clone was named 4B11.

Antibodies were enriched by gel filtration on a Sephadex G-200 column (120 × 2 cm), equilibrated with 0.1 M borate buffer (pH 8.8). With samples of up to 20 ml, complete separation of antibodies from other proteins was achieved. Prior to gel filtration antibodies were concentrated by ammonium sulphate precipitation (60% saturation). Up to 50 mg of pure antibody was obtained from the 500 ml cell culture supernatant. To avoid contamination of the affinity column with nonspecific antibodies originating from the fetal calf serum, hybridoma cells were grown in serum-free medium (high protein serum free hybridoma medium, Gibco – Life Technologies, European Division).

Preparation of antibody column

Affinity column was prepared by coupling purified 4B11 antibody to Affi-Prep 10 gel [27]. Purified antibody (2.5 mg) was added per ml of Affi-Prep gel according to the instructions of the manufacturer. After standing overnight at +4 °C more than 80% of antibodies were determined to be linked to the gel material. Remaining active esters were inactivated by addition of 0.4 ml of 1 M ethanolamine/ HCl (pH 8.0).

Preparation of glucose-affinity column

α-D-glucose was coupled to Affi-Gel 102 according to the instruction of the manufacturer. Four ml Affi-Gel 102 was resuspended in 0.1 M carbonate buffer (pH 9.5), containing 0.3 M NaCl. α-D-glucopyranoside-phenylisothiocyanate (80 mg) was added to the gel and incubated for 24 h on an overhead shaker at room temperature. Remaining active esters were inactivated by addition of 0.4 ml of 1 M ethanolamine/HCl (pH 8.0).

Animals

Male Wistar rats (mature rats: 6–8-month-old) were used. Rats were exposed to immobilization stress for 2 h. The livers from either non-stressed animals or from animals exposed to immobilization stress were used. After the stress experience phase the rats were left undisturbed for 24 h.

Preparation of salt-extracts from rat liver nuclei

Nuclei (approximately 2×10^9) were isolated and purified from the livers of rats essentially as described [28]. In brief, tissue was homogenized in sucrose-free buffer (50 mM Tris/HCl, 25 mM KCl, 15 mM $MgCl_2$; pH 7.5). The homogenate was filtered through cheesecloth (six layers) and then centrifuged at 1000 × **g** for 10 min. After washing in 20 mM Tris/HCl buffer (pH 8.0; 10 mM KCl, 3 mM $CaCl_2$, 1 mM EDTA, 1 mM PMSF), the nuclei were depleted from membranes by treatment with Triton X-100 (final concentration 0.5% [v/v]), followed by centrifugation at 1000 × **g** for 10 min. After two additional washing procedures in 50 mM Tris/HCl buffer (pH 8.0; 20 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM spermidine), the membrane-depleted nuclei were extracted for 60 min with 0.6 M NaCl in the same buffer (10 ml per liver), dialysed against Ca^{2+}, Mg^{2+}-free phosphate buffered saline (PBS) and centrifuged at 5000 × **g** for 30 min. The supernatant was termed 'salt-extract'.

Purification of CBP33

Dialysed nuclear salt-extract from 10 rat livers (100 ml; 3.12 mg of protein per ml) was mixed with 40 ml of the 4B11 antibody-resin and incubated under shaking at +4 °C overnight. Then the slurry was transferred to a column (3 × 10 cm) and washed with 30 mM phosphate buffer (pH 7.4; 300 mM NaCl) until the absorbance at 280 nm reached the base level. The column was eluted with a linear 200 ml NaCl gradient (0.3–2 M). Five-ml fractions were collected. An aliquot from each fraction was tested for carbohydrate binding activity on a slot blot after labelling with the α-D-glucose probe. More than 90% of the CBP activity was detected in the fractions corresponding to a NaCl concentration of 0.8 to 1.2 M. CBP containing fractions were pooled (total volume, 20 ml; 0.14 mg of protein per ml), diluted with four volumes of 30 mM Na-phosphate buffer (pH 7.4; containing 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$). The pooled fractions, which eluted from the 4B11-antibody column and contained the CBP activity, were subsequently applied onto the 4 ml α-D-glucose affinity column, equilibrated with PBS (containing 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$). After washing with the same buffer, pure CBP33 was eluted from the column with 1 M NaCl in 50 mM Na-phosphate buffer (pH 7.4).

Assay for sugar-binding activity

The sugar-binding activity of CBP was determined using a nitrocellulose filter binding assay, essentially as described [20, 22]. Protein samples were adsorbed on nitrocellulose (Millipore, 0.45 μm pore size). The filters were then saturated with 1% bovine serum albumin (BSA) in PBS (containing 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$). Incubation of the filters in the presence of ^{125}I-Glc-BSA or ^{125}I-BSA (12 μg ml^{-1} each) was performed for 1 h at 4 °C. After washing with PBS (containing 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$), the filters were counted for radioactivity. The sugar-binding activity is expressed in arbitrary units; one arbitrary unit is equal to 1×10^3 cpm of ^{125}I-Glc-BSA bound to nitrocellulose filter under standard assay conditions (after subtraction of cpm bound in control assay with ^{125}I-BSA).

Radioiodination of BSA and of the neoglycoproteins with ^{125}I-sodium iodide was carried out according to the chloramine T procedure [29]. The radiolabelled proteins were separated from free iodide by gel filtration on Sephadex G-25 (Pharmacia) column. The specific radioactivities were 6–7 μCi per μg of protein.

Electrophoresis and blotting

Gel electrophoresis of proteins was performed on 12% polyacrylamide slab gels containing 0.1% sodium dodecylsulfate [SDS] (SDS-PAGE) [30]. After electrophoresis proteins were transferred to the PVDF Immobilon membrane [31] in a semi-dry blotting system (Pharmacia, Sweden). After overnight blocking with 3% BSA membranes were incubated with antibodies and visualized with alkaline phosphatase using BCIP/NBT, according to the instructions of the manufacturer (Boehringer).

Protein concentrations were determined spectrophotometrically at 230 and 260 nm [32].

Results and discussion

The photoreactive carbohydrate probe

To detect carbohydrate binding proteins we synthesized a photoreactive carbohydrate probe. The probe is composed of four building blocks: (i) the carbohydrate moiety; (ii) the digoxigenin tag; (iii) the photoreactive cross-linker; and (iv) the lysyl-lysine backbone. The structure of the α-D-glucose probe is shown in Fig. 1. In this study we have described only the preparation and use of the α-D-glucose probe (see Materials and methods), but we have successfully applied analogous procedures for the synthesis of several other carbohydrate probes. The photoreactive glucose carbohydrate probe was prepared by the three subsequent labelling steps using commercially available reagents (lysyl-lysine, α-D-glucopyranoside-phenylisothiocyanate and digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester, 4-azidobenzoic acid-*N*-hydroxysuccinimide ester). The lysyl-lysine dipeptide was selected as the backbone molecule because it contains three spatially distant amino groups prone to chemical modifications. α-D-glucopyranoside-phenylisothiocyanate was selected for the introduction of a carbohydrate group into the lysyl-lysine backbone. We have previously demonstrated that the phenyl spacer does not interfere with the binding of the α-D-glucose to carbohydrate binding proteins [20]. Digoxigenin, and not biotin, was selected as the reporter

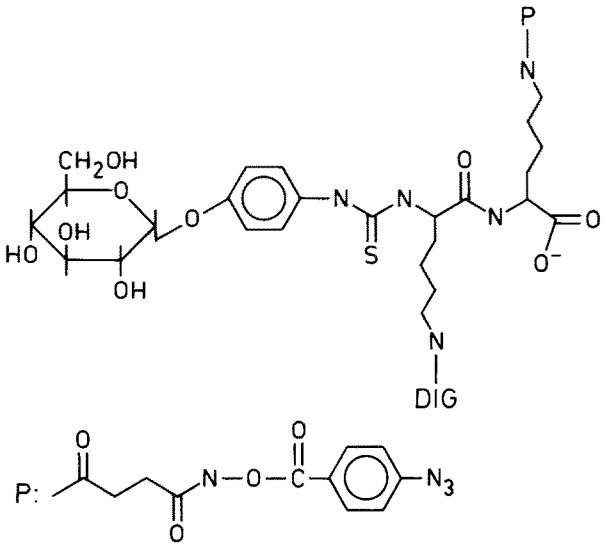

Figure 1. Structure of the α-D-glucose marker. One of the two possible structures is shown. The α-D-glucose is almost exclusively bound to the α-amino group of the lysyl-lysine backbone. The digoxigenin tag (DIG) and the photoreactive cross-linker (P) are randomly distributed over the two ω-amino groups.

tag, because biotin is present in many tissues, especially liver. In addition, it is frequently observed that avidin or streptavidin as well as anti-biotin monoclonal antibodies recognize several nuclear proteins in Western blots especially if crude extracts from rat liver are used. In contrast, the anti-digoxigenin Fab fragments did not recognize any protein in the same extracts. 4-Azidobenzoic acid, a photoreactive cross-linking reagent, was used to create a covalent linkage between the probe and the carbohydrate binding protein. The 4-azidobenzoic acid is completely inert when incubated in the dark. However, if illuminated with UV light, the azide group is converted to a very reactive nitrene group. The nitrene group easily reacts with a variety of chemical bonds including N—O, O—H, C—H and C=C [33]; N—H groups appear to be the primary targets for formation of covalent linkage [34, 35].

The purity of the photoreactive glucose probe was analysed by thin layer chromatography as described in Materials and methods. After development, the plate was cut in two halves and stained for sugars (with resorcinol), and for amino groups (with ninhydrin). Both staining revealed the single band with $r_e/r_f = 0.4$ (Fig. 2) which confirmed the assumed homogeneity of the probe.

A flow scheme for the detection of CBPs using the photoreactive carbohydrate probe is shown in Fig. 3. (i) The probe is first incubated in the dark with the carbohydrate binding protein containing samples; (ii) after the formation of CBP-probe complexes, the samples are illuminated with five to 10 camera flashes to activate the photoreactive cross-linking group; which in turn (iii) covalently cross-links the probe to the CBP. The half-life of the activated azidobenzoic group is extremely short (in the range of m s)

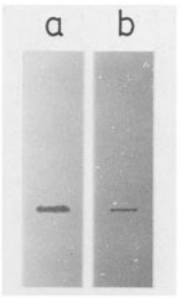

Figure 2. Thin-layer chromatography of the photoreactive α-D-glucose probe. Five μg of the photoreactive α-D-glucose were analysed by thin layer chromatography on HPTLC Pre-coated plates, 0.2 mm thick silica gel, 10 × 10 cm (Merck, Darmstadt) using chloroform:methanol:40 mM KCl (50:42:11) solvent system. The samples were applied, and the plate developed in the dark room under red light. Lane a, staining of sugar-containing compounds with resorcinol/HCl reagent; lane b, staining of NH_2 groups with ninhydrin.

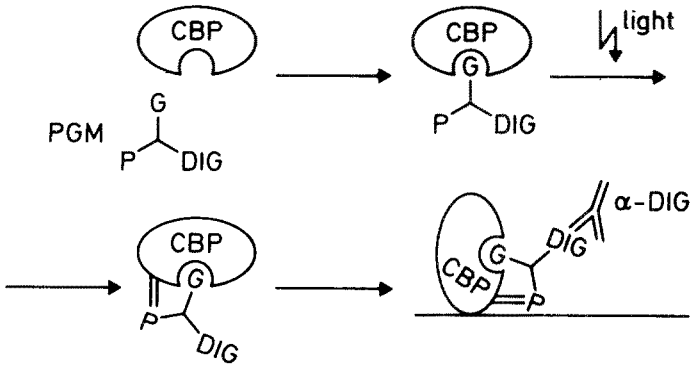

Figure 3. Strategy for application of the photoreactive carbohydrate probe. For specific labelling, the carbohydrate binding proteins were incubated together with the carbohydrate probe. For detailed explanation see Materials and methods. G, glucose; DIG, digoxigenin tag; P, photoreactive cross-linker; CBP, carbohydrate binding protein.

and it reacts highly non-selectively with neighbouring groups [33, 35]. These two characteristics allow efficient labelling of CBPs. Using optimal concentrations of the probe, an almost specific affinity labelling can be accomplished. The product of the labelling reaction, a CBP with a covalently incorporated digoxigenin label, can subsequently be detected easily by anti-digoxigenin antibodies. Due to the small molecular weight of the probe (<1.5 kDa) labelled CBPs can be easily identified by the Western blotting technique, following electrophoretical separation of the proteins.

Figure 4 demonstrates the specificity and sensitivity of the α-D-glucose probe. The mannose/glucose specific lectin Concanavalin A (ConA) was detected with the α-D-glucose probe in the presence of excess BSA. Optimal concentrations of probe have to be chosen to obtain specific and sensitive signals (Fig. 4A). If the probe is used at exceedingly high concentrations (>5 μM) unspecific labelling of protein

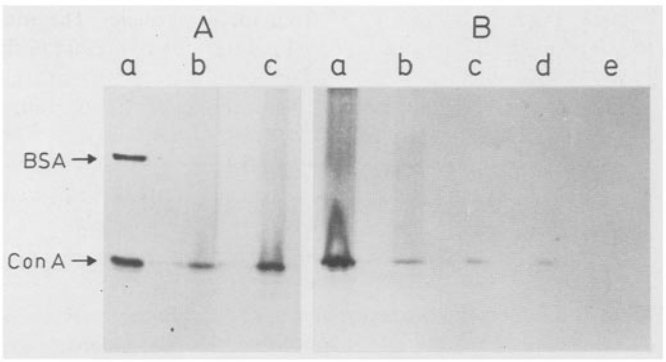

Figure 4. Application of photoreactive α-D-glucose probe. α-D-glucose probe (final concentration, 0.1 μM) was incubated with protein samples (final volume, 10 μl) for 30 min at 37 °C in the dark and then illuminated with seven camera flashes at a distance of 25 cm. Samples were subsequently separated on 12% SDS PAGE, transferred to Immobilon PVDF membrane and detected with anti-DIG-AP-conjugate. A: All samples contained 0.1 μg ConA and 1 μg BSA. The final concentration of the α-D-glucose probe was 5 μM (lane a); 0.01 μM (lane b) and 0.1 μM (lane c). B: The final concentration of the α-D-glucose probe was 0.1 μM. (Lane a) 10 μg ConA plus 100 μg BSA; (lane b) 1 μg ConA plus 10 μg BSA; (lane c) 100 ng ConA plus 1 μg BSA; (lane d) 10 ng ConA plus 100 ng BSA and (lane e) 1 ng ConA plus 10 ng BSA.

occurs; as an example, BSA and ConA are almost equally stained by the α-D-glucose probe when the probe is used at a final concentration of 5 μM (Fig. 4A). Obviously, if the concentrations chosen are too low, the probe will label only a portion of the active available sites resulting in a decreased sensitivity of detection. At optimal probe concentration, the method is very sensitive (Fig. 4B). Less than 0.1 pmol of the probe, corresponding to 1–10 ng of carbohydrate binding proteins, can easily be detected.

We applied this probe as a tool to specifically detect a carbohydrate binding activity present in nuclei from rat liver. Recently, we reported the appearance of an additional protein band at $M_r \approx 35$ kDa when α-D-glucose binding proteins from the livers of rats, that had been exposed to transient immobilization stress, were analysed by SDS PAGE [36]. In higher resolution gels, this band resolved into a doublet of a major 33 kDa polypeptide band and a minor 35 kDa polypeptide band. Due to its reactivity to antibody against CBP35 in Western blots, we concluded that the $M_r \approx 35$ kDa band seen in stressed rats is identical with CBP35, one of the best characterized carbohydrate binding proteins [14]. This conclusion based also on the finding that the expression and intracellular distribution of CBP35 is dependent on the proliferation and differentiation state of the cell [17]; hence the involvement of rat liver CBP35 in stress response seems to be an adequate explanation. Since CBP35 does not display any affinity for α-D-glucose [14], we explained its retardation on the α-D-glucose column as copurification with the α-D-glucose-specific CBP67 [20] through protein-protein interactions.

Purification of CBP33

We have now analysed the carbohydrate binding activity of the stress-induced $M_r \approx 35$ kDa doublet protein band using the photoreactive α-D-glucose probe. Surprisingly, we found that the major 33 kDa polypeptide band within the doublet has a very strong affinity for α-D-glucose (Fig. 5, Table 1). While binding of the glucose probe to this protein could be nearly completely inhibited by the addition of 1 mM α-D-methylglucose to the reaction mixture, lactose showed no significant inhibitory effect (Table 1). On the

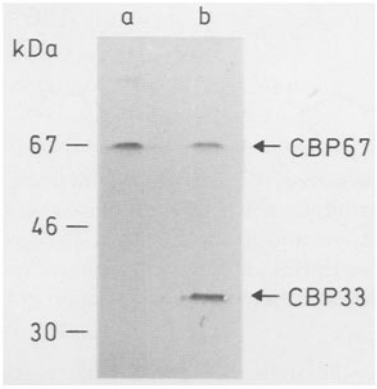

Figure 5. Detection of the carbohydrate binding activity by the cross-linker. Glucose binding protein CBP33 was partially purified from rat liver salt extracts as described under Materials and methods and detected using the photoreactive α-D-glucose marker. Ten μl protein samples (0.1 μg of total protein) were incubated with the α-D-glucose probe at a final concentration of 0.1 μM at 37 °C for 30 min in the dark and then cross-linked by illumination with 10 camera flashes from a 25 cm distance. Subsequently, proteins were separated on 10% SDS PAGE, transferred to Immobilon PVDF membrane and detected with α-DIG-AP-conjugate. (Lane a) α-D-glucose binding proteins from the normal rat; (lane b) α-D-glucose binding proteins from rat exposed to transient immobilization stress.

Table 1. Inhibition of cross-linking of the photoreactive α-D-glucose probe with purified CBP33 by different carbohydrates. Ten ng of purified CBP33 was incubated with 5 pmol of α-D-glucose probe in the dark together with different concentrations of the indicated carbohydrates (total volume = 10 μl). Then the samples were exposed to camera flashes and processed as indicated under Materials and methods. Concentrations of the competing carbohydrates which cause >95% abolition of cross-linking of the α-D-glucose probe to CBP33 are indicated in the Table.

Carbohydrate	*95% inhibition of cross-linking at:*
α-D-methylglucose	1 mM
α-L-methylglucose	10 mM
α-D-methylgalactose	>200 mM
lactose	>200 mM

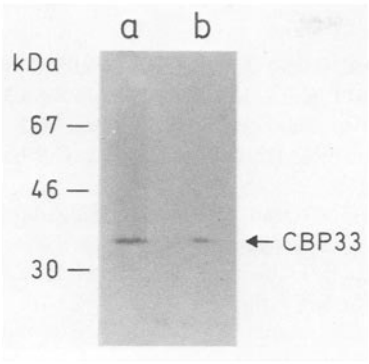

Figure 6. Detection of CBP33 using 4B11 antibody. Protein samples were separated on SDS PAGE, transferred to the Immobilon PVDF membrane and detected with the 4B11 antibody (cell culture supernatant diluted 1:1 with TBS). Lane a, nuclear salt extract from rat liver (5 μg of total protein); Lane b, 2 μg glucose-binding proteins from the liver of rats exposed to immobilization stress enriched by affinity chromatography on α-D-glucose column as described [20].

basis of these results we conclude that the $M_r \approx 35$ kDa protein band we have observed in rats exposed to transient immobilization stress, is composed of two components: (i) the CBP35; and (ii) the novel 33 kDa protein, which has been purified in the present study.

Next we attempted to raise monoclonal antibodies against CBP33. The partially purified CBP33 used for immunization had been obtained by a procedure described previously [20, 36] starting with livers from rats transiently exposed to immobilization stress. Until now, we have obtained only one positive clone (termed 4B11). Although monospecific for CBP33 in the protein mixture eluted from glucose-affinity column (Fig. 6b), the 4B11 antibody recognizes several other, apparently unrelated proteins in nuclear salt extracts (Fig. 6a). Therefore, this antibody could not be applied in screening assays using ELISA- or Western-blotting procedures, or for immunocytochemical studies. Fortunately, the 4B11 antibody also proved to have relatively low affinity; its binding to CBP33 could be completely inhibited by addition of 1 M NaCl to the incubation buffer. These relatively mild conditions for dissociation of the antibody-antigen complex allowed an enrichment of functionally active CBP33 using a 4B11 antibody affinity column. A 40 ml column, containing a total of 100 mg of 4B11 antibody was prepared, and applied for partial purification of CBP33, as described under Materials and methods. The enrichment during this affinity chromatography step was 24-fold. The specific glucose-binding activity was 300 U per mg protein (Table 2).

The final purification of CBP33 was achieved by a second affinity chromatography step using an α-D-glucose affinity resin as described under Materials and methods. Since CBP33 was the only glucose-binding protein retained on the 4B11-antibody column, an ≈100-fold purification and

Table 2. Purification of CBP33 from rat liver nuclei. The total amounts of protein and the glucose binding activity present in the eluate of the antibody (Ab)- and glucose affinity columns are given. The binding activity was measured applying the nitrocellulose binding assay (to ^{125}I-Glc-BSA).

Step	*Total protein (mg)*	*Total activity (U)*	*Yield (%)*	*Purification-fold*
Salt extract	312	3790	100	–
Ab-affinity	2.8	840	22	24
Glc-affinity	0.02	585	15	2413

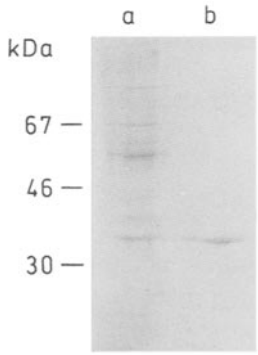

Figure 7. Purification of CBP33. Protein samples were separated on a 12% polyacrylamide gel and stained with silver. (Lane a) Proteins eluted from 4B11-antibody affinity column (10 μg of protein); (lane b) purified CBP33 (1 μg of protein) after the α-D-glucose-affinity column purification step.

a virtually pure CBP33 preparation was achieved (Fig. 7). Two ml (10 μg of protein per ml) were obtained and the specific activity of this fraction was determined to be 29 250 units per mg protein (Table 2). The lectin activity of the purified CBP33 was confirmed using the photoreactive glucose probe. 0.1 μg of pure CBP33 was incubated with the photoreactive glucose probe and cross-linked with 10 camera flashes (Fig. 8a). The specificity of this recognition was confirmed by the inhibition with 0.1 M glucose (Fig. 8b). Although numerically relatively low, the 2400-fold enrichment is high, since it is calculated on the basis of activity in the nuclear salt extract. In an analogy to our earlier procedure for purification of CBP67, referred to crude liver homogenate, the purification factor is assumed to be more than 10 000-fold [20, 36]. It is also important to note that the glucose-binding activity measured in nuclear salt extract (prior to the 4B11-antibody affinity purification step) is not only due to CBP33 but is due to at least another carbohydrate binding protein, CBP67. This explains the apparent low yield of CBP33, measured after antibody-affinity column.

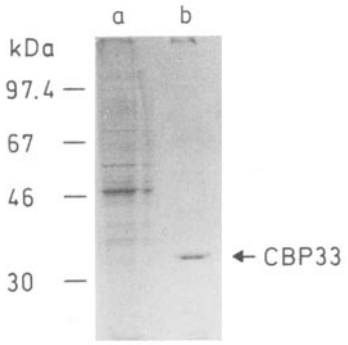

Figure 8. Detection of pure CBP33 with the photoreactive glucose probe. Pure CBP33 (0.1 μg) was incubated with the α-D-glucose probe at a final concentration of 0.1 μM (30 min at 37 °C in the dark) in the presence (lane b) and absence of 0.1 M glucose (lane a). The photoreactive glucose probe was activated by illumination with 10 camera flashes from a 25 cm distance. Subsequently, proteins were separated on SDS PAGE, transferred to Immobilon PVDF membrane and detected with α-DIG-AP-conjugate.

Conclusion

In this study we have introduced a new method for the detection of carbohydrate binding proteins using a photoreactive carbohydrate probe. The procedure turned out to be specific and sensitive and it is applicable for the identification of distinct lectins present in complex mixtures. Applying this method we demonstrated that carbohydrate binding proteins induced in rat liver after immobilization stress do not only consist of CBP35, as previously reported, but also of a novel, α-D-glucose-specific carbohydrate binding protein, termed CBP33. CBP33 could be purified to apparent homogeneity using two affinity purification steps (enrichment >2400).

Cellular stress response is one of the mostly conserved mechanisms for the protection of individual cells or organisms [37, 38]. At present little is known about the role of nuclear CBPs, and their possible functions in stress response are not understood. The psychological nature of immobilization stress argues against the activation of a classical heat shock response. It appears to be more likely that the CBP33 represents a part of the so-called 'acute phase response', a complex reaction to environmental stress which is characteristic for multicellular eukaryotes [39]. Detailed studies are now in progress to elucidate the role of CBP33 within the stress response chain.

Acknowledgements

This work was supported by grants from the Deutscher Akademischer Austauschdienst, the Bundesministerium für Forschung und Technologie (German–Croatian cooperation program, coordinated by Internationales Büro, KFA-Jülich) and from the Deutsche Forschungsgemeinschaft (Mu 348/10-2)

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