Detection of the Membrane Protein Recognized by the Kidney-Specific Alkylglucoside Vector

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Purpose. Previously, we suggested that alkylglucoside can be an effective vector for renal-specific drug delivery (Suzuki *et al., J. Pharmacol. Exp. Ther.,* **288**:57–61, 1999). The purpose of the present study is to characterize the membrane protein which is recognized by this alkylglucoside.

Methods. The binding of [^{125}I] tyrosine conjugated with a octylthioglucoside (Glc-S-C8-[^{125}I]Tyr) Glc-S-C8-[^{125}I]Tyr to crude membrane fractions of kidney was determined. In addition, the membrane was cross-linked with this alkylglucoside and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Results. Glc-S-C8-[^{125}I]Tyr was shown to have a specific binding site on the kidney membrane (K_d = 931 nM and B_{max} = 987 pmol/mg protein). Cross-linking of the membrane with Glc-S-C8-[^{125}I]Tyr resulted in the detection of a protein (M_r = 62,000), which was unaffected by reducing agents. The results of this cross-linking study were consistent with previous information on its localization and binding characteristics.

Conclusions. The kidney membrane protein, to which alkylglucoside binds in a specific manner, has a molecular weight of 62,000. Cross-linking is a useful tool for detecting this novel membrane protein in kidney.

KEY WORDS: kidney; sugar; glucoside; cross-linking; membrane protein.

INTRODUCTION

Previously, we showed that kidney targeting can be achieved by using a modified sugar (1–3). Arginine-vasopressin (AVP), conjugated with a glucose molecule via an octamethylene group (Glc-O-C8-AVP), is specifically taken up by rat kidney after i.v. injection (2). Although liver-specific delivery has been successful using the asialo-glycoprotein receptor (4), this was the first report demonstrating that the renal delivery of peptides is possible. Detailed investigation of the kidney specificity of a series of alkylglycoside derivatives revealed that (i) alkylglycoside structures are essential for kidney recognition, since octylglucoside as well as an AVP derivative are taken up by the kidney, (ii) affinity for kidney membranes depends on the structure of the sugars, the length and hydrophobicity of the alkyl chains and the atoms which link sugars and alkyl chains, (iii) specific binding sites on kidney membranes are involved in its specific uptake, (iv) these sugar derivatives are taken up by the kidney from the blood side, since the *in vivo* data can not be accounted for by only glomeruler filtration, and (v) uptake sites are located predominantly at the proximal tubules of the renal cortex (1–3). Although there are many membrane proteins such as transporters for organic anions and cations, peptides (5–7), and glucose (8), so far, none of them have the identified binding characteristics of alkylglucoside, as far as substrate specificity and localization in renal cells are concerned. The purpose of the present study is to characterize the molecular nature of this kidney-specific binding protein using affinity cross-linking.

MATERIALS AND METHODS

Materials

AVP conjugated with a octylthioglucoside (Glc-S-C8-AVP), tyrosine conjugated with a octylthioglucoside (Glc-S-C8-Tyr) and octyl β-D-thiogalactoside (Gal-S-C7-Me) were kindly donated by Meiji Seika Kaisha, Ltd. All other chemicals were commercially available reagents. Male Sprague-Dawley (SD) rats (230–270 g, Charles River Japan Inc., Kanagawa, Japan) were used. This study was carried out in accordance with the "Principles of Laboratory Animal Care" as adopted and promulgated by the National Institutes of Health.

Preparation of Glc-S-C8-[^{125}I]Tyr

Glc-S-C8-Tyr was iodinated by the chloramine-T method (9). Briefly, 20 μl Glc-S-C8-Tyr (50 nM) solution was added to 50 μl 0.5 M phosphate buffer (pH 7.5) After addition of 1 μl Na[^{125}I] (100 μCi/μl) (Amersham, Buckinghamshire, UK) and 10 μl chloramine-T (2 mg/ml dissolved in 0.5 M phosphate buffer pH 7.5), the reaction mixture was stirred for 30 sec. The reaction was terminated by the addition of 50 μl $Na_2S_2O_5$ (2.5 mg/ml in 0.5 M phosphate buffer) and 10 μl Kl (100 mg/ml in 0.5 M phosphate buffer). To purify the labeled compounds, the reaction mixture was subjected to reverse phase chromatography (C18 Sep-pak light; Waters, Milford, MA), and washed subsequently with 0.1% trifluoroacetic acid (TFA)/water, and 15% acetonitrile in 0.1% TFA/water. Then, the column was eluted with 60% of acetonitrile in 0.1% TFA/water. After evaporation, specimens were kept at 4°C and used within a week. The relative activity of the isotope was 80,000–160,000 cpm/pmol.

Membrane Preparation

Crude membranes of rat kidney and liver were prepared by centrifugation (10). Rat kidney basolateral membranes (BLM) were prepared by density gradient centrifugation on Percoll (Pharmacia, Uppsala, Sweden) gradients as described previously (11). Brush border membranes (BBM) were prepared by Ca^{2+} precipitation and differential centrifugation,

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ABBREVIATIONS: AVP, arginine vasopressin; TFA, trifluoroacetic acid; BLM; basolateral membrane, BBM, brush border membrane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; EGS, [ethylene glycol bis (succinimidylsuccinate)]; DSG, (disuccinimidyl glutarate); DMP, (dimethyl pimelimidate · 2HCl); BS^3, [bis (sulfosuccinimidyl) suberate].

essentially according to published methods (12). Briefly, the renal cortex of male SD rats (230–270 g) was excised, minced and homogenized in Buffer A (10 mM mannitol, 2 mM Hepes-Tris, pH 7.1) [1:9 (w/v)] in a Waring Blender at 1,800 rpm for 2 min. $CaCl_2$ was added to the homogenate to produce a final concentration of 10 mM. After 15 min, the specimen was centrifuged at 500g for 12 min. The resulting supernatant was centrifuged at 15,000g for 12 min and the pellet was resuspended in Buffer A using a Teflon Potter Homogenizer (10 strokes at 1,000 rpm). $CaCl_2$ was added to produce a final concentration of 10 mM, and, after standing for 15 min, centrifugation was performed at 750g for 12 min. The supernatant was then centrifuged at 30,000g for 12 min and the pellet was suspended in Buffer B (100 mM mannitol, 20 mM Hepes-Tris, pH 7.5) using a Teflon Potter Homogenizer. The suspension was centrifuged at 30,000g for 12 min. The pellet was suspended in Buffer B using a Teflon Potter Homogenizer and centrifuged at 48,000g for 20 min. The pellet was resuspended in Buffer B and centrifuged at 2,000g for 5 min. The supernatant was centrifuged again at 48,000g for 20 min to obtain the BBM fraction.

Binding of Glc-S-C8-[^{125}I]Tyr to the Membrane Fraction

Glc-S-C8-[^{125}I]Tyr, with or without unlabeled Glc-S-C8-Tyr, was incubated with the kidney membrane fractions (1 mg protein/ml) in phosphate-buffered saline (pH 7.4) on ice for 10 min. After centrifugation (12,000g for 10 min at 4°C), the supernatant was removed and the radioactivity associated with the supernatant and pellet were determined in a γ-counter. All experiments were performed in triplicate. The binding parameters were estimated from the following equation:

$$B = B_{max} \cdot F/(K_d + F) + \alpha \cdot F \quad (1)$$

where B and F represent the bound and unbound concentration of ligand, respectively, K_d and α represent the dissociation and nonspecific binding constants, respectively. The data were fitted to Eq. (1) by a nonlinear least-squares method using the MULTI program (13) to obtain estimates of the kinetic parameters. The input data were weighted as the reciprocals of the squares of the observed values, and the Damping Gauss Newton algorithm was used for the fitting.

Inhibition of Binding of Glc-S-C8-[^{125}I]Tyr to the Rat Kidney Membrane Fraction

Glc-S-C8-[^{125}I]Tyr (2 nM) with or without unlabeled Gal-S-C7-Me was incubated with kidney membranes (1 mg protein/ml) in phosphate-buffered saline (pH 7.4) on ice for 10 min. The Ki value was estimated from the following equation:

$$B/F = B_{max1}/K_d \cdot (1 + I/K_{i1}) + B_{max2}/K_d \cdot (1 + I/K_{i2}) + \beta \quad (2)$$

where I, K_i and β represent inhibitor concentration, inhibtion constant and nonspecific binding constant, respectively. The K_d value obtained from Eq. (1) is used in Eq. (2). Fitting was performed as described above.

Membrane Solubilization

The membranes were suspended in phosphate-buffered saline (pH 7.4) to give a final concentration of ~ 10 mg protein/ml, and solubilized by stirring with 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfate (CHAPS) and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min on ice. After centrifugation at 100,000g for 10 min, the clear supernatant, containing the soluble membrane fraction, was recovered.

Cross-Linking

Solubilized membranes (1 mg protein/ml) in 250 μl phosphate-buffered saline (pH 7.4) were incubated with 60 or 120 nM Glc-S-C8-[^{125}I]Tyr for 10 min on ice. The cross-linking reagents were dissolved in dimethyl sulfoxide for EGS [ethylene glycol bis (succinimidylsuccinate)] and DSG (disuccinimidyl glutarate), and in phosphate-buffered saline for DMP (dimethyl pimelimidate · 2HCl) and BS^3 [bis (sulfo-succinimidyl) suberate] immediately prior to use. The cross-linkers were added to give a final concentration of 1 mM. The membrane fractions were incubated with the cross-linkers for 2 hr at 4°C. The cross-linking reaction was terminated by adding ice-cold Tris-HCl, pH 7.5, to give a final concentration of 20 mM, and the specimens were incubated on ice for 15 min. The specimens were then subjected to SDS-polyacrylamide gel electrophoresis.

For the cross-linking of membrane fractions without solubilization, the incubation of membrane with Glc-S-C8-[^{125}I]Tyr was terminated by adding 1 ml ice-cold phosphate-buffered saline, and the samples were centrifuged at 12,000g for 10 min at 4°C. The resulting pellets were washed and resuspended in 250 μl phosphate-buffered saline (pH 7.4). The cross-linking reaction was performed as described above. After termination of the reaction, the incubation mixture was centrifuged to obtain the membrane fraction as a pellet. The pellet was washed with 1 ml phosphate-buffered saline before carrying out SDS-polyacrylamide gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis

The specimens were suspended in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v)SDS, 10% glycerol and 0.01% phenol red. The specimens were heated at 100°C for 3 min in the presence or absence of 40 mM dithiothreitol. Electrophoresis was performed using 1.5-mm thick slab gels containing 10% acrylamide. After electrophoresis, the gels were fixed in 10% trichloroacetic acid, then exposed to the imaging plates (Fuji Film, Tokyo, Japan), and the density of radioactivity in the autoradiographic images was measured using an image analyzer, BAS 2000 (Fuji Film, Tokyo, Japan).

RESULTS

Binding Assay

Binding of Glc-S-C8-[^{125}I]Tyr to the kidney membrane fraction revealed the presence of a specific binding site. From Scatchard plot analysis (Fig. 1), the K_d and B_{max} values were estimated to be 931 ± 63 nM and 987 ± 63 pmol/mg protein, respectively, and α was estimated to be 0.142 ± 0.005 (ml/mg protein). The inhibitory pattern of Glc-S-C8-AVP on Glc-S-C8-[^{125}I]Tyr binding was accounted for by assuming the presence of two binding sites. (Fig. 2). The K_i and B_{max} values for high and low affinity sites are 26.7 ± 17.2 nM and 12.9

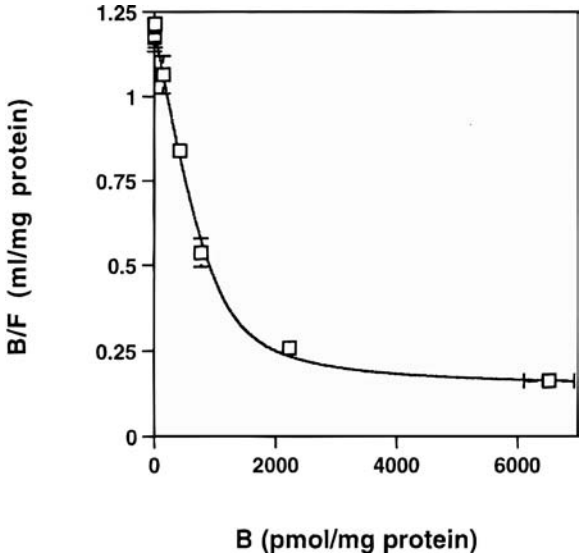

Fig. 1. Scatchard plots of the binding of Glc-S-C8-[^{125}I]Tyr to crude rat kidney membrane. The solid line represents the calculated line obtained for Eq. (1) as described under Methods. Values represent mean ± S.D. of triplicate assays at each concentration.

± 8.8 μM, and 413 ± 27 pmol/mg protein and 333 ± 22 pmol/mg protein, respectively.

Cross-Linking of Kidney-Specific Membrane Protein

In order to characterize the membrane protein to which Glc-S-C8-Tyr binds, cross-linking was performed. In the present study, bifunctional reagents were used to covalently link Glc-S-C8-[^{125}l]Tyr to the membrane protein. To check the effect of membrane solubilization on cross-linking, membrane specimens were cross-linked before and after solubilization of the membrane. A membrane protein with a molecular weight of 62,000 was detected after cross-linking using EGS (Fig. 3). The extent of cross-linking was reduced when 1 μM unlabeled Glc-S-C8-Tyr was added (Fig. 3). Membrane solubilization did not affect the labeling pattern (Fig. 3), nor did 40 mM dithiothreitol (data not shown). Although additional bands at M_r = 50,000 and M_r = 35,000 were observed when cross-linking has performed after solubilization of the membrane, these bands were abolished when PMSF, a widely used inhibitor of serine proteases, was added during solubilization. To find the most efficient cross-linker, specimens were exposed to four kinds of cross-linkers; BS^3, DMP, DSG and EGS. The most efficient cross-linking was observed with BS^3 and EGS (Fig. 4).

Localization of the Membrane Protein Specific for Alkylglucoside

To examine the localization of the membrane protein specific for alkylglucoside, cross-linking was performed using crude liver membrane, and kidney BLM and BBM. In the case of the liver membrane, a labeled band around M_r = 47,500 was observed, although it was not reduced in the presence of 1 μM unlabeled Glc-S-C8-Tyr. No bands were observed with kidney BBM, whereas a band (M_r = 62,000), which was decreased in the presence of unlabeled Glc-S-C8-Tyr, was detectable with kidney BLM (Fig. 5).

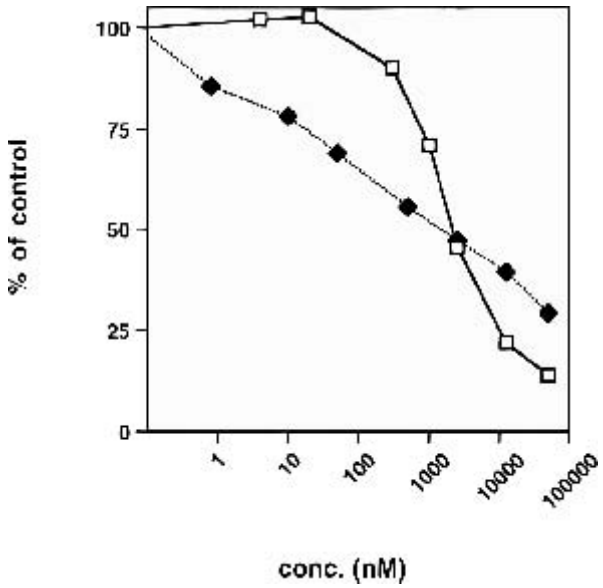

Fig. 2. Inhibitory effect of Glc-S-C8-AVP on the binding of Glc-S-C8-[^{125}I]Tyr to crude rat kidney membrane. Binding of Glc-S-C8-[^{125}I]Tyr (2 nM) to membrane (1 mg protein/ml) was measured in the presence of Glc-S-C8-AVP (♦). For comparison, data for saturation (□) were taken from Fig. 1. Results are given as % of control binding, which was determined in the absence of unlabeled substrates. Values represent mean ± S.D. of triplicate assays at each concentration.

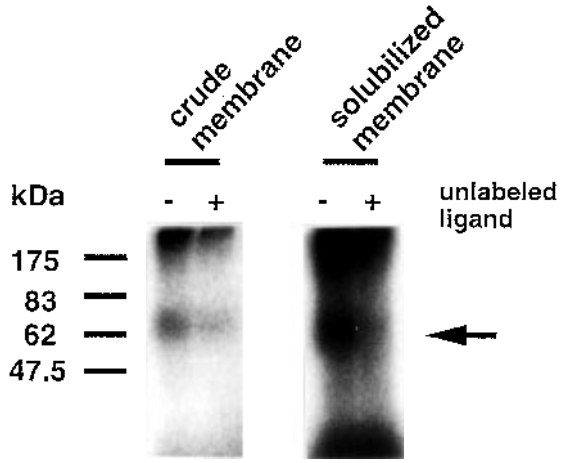

Fig. 3. Cross-linking of crude rat kidney membrane with Glc-S-C8-[^{125}I]Tyr. Left panel: Membrane (1 mg protein/ml) was incubated for 10 min on ice with 120 nM Glc-S-C8-[^{125}I]Tyr in the presence and absence of 1 μM unlabeled Glc-S-C8-Tyr, and then cross-linked using EGS. The membrane was solubilized before applying it to SDS/polyacrylamide gel. Right panel: Membrane (10 mg protein/ml) was solubilized with 1% CHAPS in the presence of 1 mM PMSF in PBS. Solubilized membrane (1 mg protein/ml) was incubated for 10 min on ice with 120 nM Glc-S-C8-[^{125}I]Tyr in the presence and absence of 1 μM unlabeled Glc-S-C8-Tyr. After the cross-linking reaction with EGS, the membrane was applied to SDS/polyacrylamide gel. Positions of marker proteins are indicated.

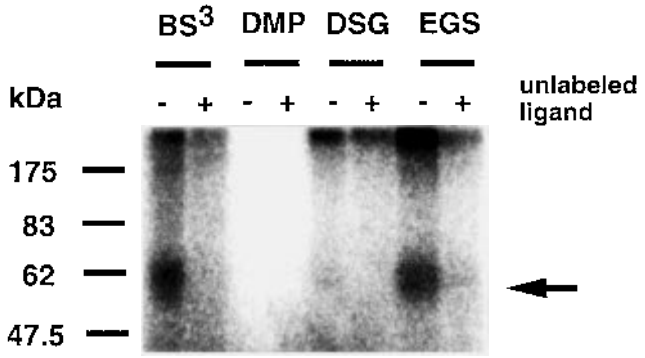

Fig. 4. Comparison of the efficiency of cross-linking reagents on the labeling of crude rat kidney membrane with Glc-S-C8-[^{125}I]Tyr. Membrane (10 mg protein/ml) was solubilized with 1% CHAPS in the presence of 1 mM PMSF in PBS. Solubilized membrane (1 mg protein/ml) was incubated for 10 min on ice with 120 nM Glc-S-C8-[^{125}I]Tyr in the presence and absence of 1 μM unlabeled Glc-S-C8-Tyr. Four kinds of cross-linkers (BS^3, DMP, DSG, EGS) were then added to produce a final concentration of 1 mM followed by electrophoresis.

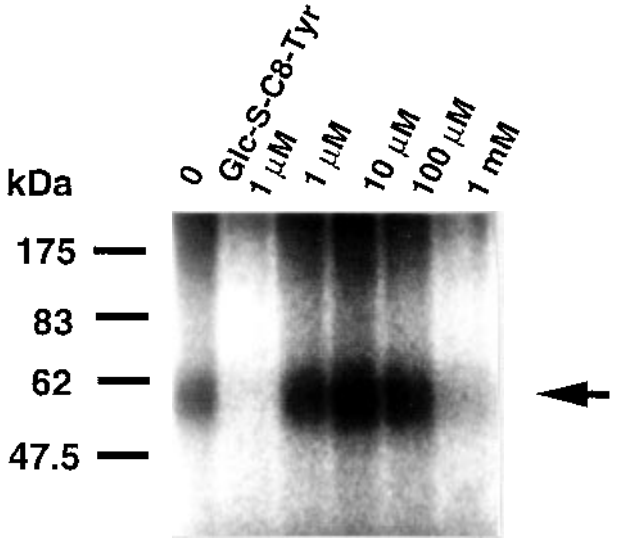

Fig. 6. Effect of Gal-S-C7-Me on the cross-linking of crude rat kidney membrane with Glc-S-C8-[^{125}I]Tyr. Rat kidney membrane (10 mg protein/ml) was solubilized with 1% CHAPS in the presence of 1 mM PMSF in PBS, and then cross-linked with Glc-S-C8-[^{125}I]Tyr using EGS in the presence of several concentrations of unlabeled Gal-S-C7-Me. For comparison, the inhibitory effect of 1μM Glc-S-C8-Tyr is also shown.

Inhibition Experiments

To characterize the nature of the binding of Glc-S-C8-[^{125}l]Tyr, the inhibitory effect of Gal-S-C7-Me was examined. No significant inhibition was observed with Gal-S-C7-Me even at a concentration of 100 μM. The labeled band was reduced only at a concentration of 1 mM (Fig. 6).

DISCUSSION

Previously, we reported that [^{3}H]Glc-O-C8-AVP, an alkyl-glucoside, targets the kidney. Using a series of structurally related compounds, it was shown that the sugar, alkyl chain or aromatic ring, and peptide moieties are important for binding to the kidney membrane fraction (2). Moreover, it was found that a hydrophobic group (alkyl chain or aromatic ring) needs to be introduced to a sugar (D-glucose, D-mannose or 2-deoxy-D-glucose) via a β-glycoside binding atom. Also, S-glucoside exhibited higher affinity than O-glucoside (1–3). Based on these characteristics, Glc-S-C8-Tyr, a possible substrate which can be used to label the binding protein via cross-linking reagents, is expected to exhibit affinity. Indeed, from Scatchard plot analysis of the binding to the kidney membrane fraction (Fig. 1), the presence of a specific binding site was indicated (K_d = 931 nM; B_{max} = 987 pmol/mg protein). Glc-S-C8-Tyr has a lower affinity and higher capacity compared with other previously characterized ligands. For example, [^{3}H]Glc-S-C7-Me has a K_d and B_{max} of 16.4 nM and 24.4 pmol/mg protein, respectively (3). To examine whether Glc-S-C8-Tyr and a series of compounds previously reported (1–3) bind to the kidney membrane fraction via the same binding site, we studied the inhibitory effect of Glc-S-C8-AVP on Glc-S-C8-[^{125}l]Tyr (Fig. 2). The inhibition curve fitted a model with two binding sites best. The K_i value for the high affinity site was estimated to be 26.7 nM, which is comparable with the K_d value of Glc-S-C8-[^{125}l]AVP (28.7 nM) (Shirota et al., in preparation). The K_i value for the low affinity site was estimated to be 12.9 μM, and the B_{max} values for high and low affinity sites were 413 and 333 pmol/mg protein, respectively. This result suggests that at least two binding sites are present on the membrane fraction. Together with the previous finding that the binding of [^{3}H] Glc-S-C8-AVP to rat kidney membrane fraction can be described by considering one binding site (K_d = 8.63 nM (3)), these data can be accounted for by assuming that Glc-S-C8-Tyr binds to two binding sites with almost equal affinity. On the other hand, Glc-S-C8-AVP binds to one site with high affinity (K_i = 26.7 nM, Fig. 2) but does not significantly bind to the other, although it inhibits the binding of Glc-S-C8-Tyr to both sites. Since the K_i value for the high affinity site (26.7 nM) is comparable with

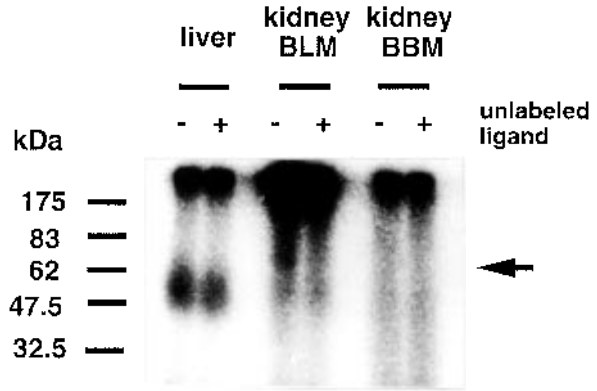

Fig. 5. Cross-linking of crude liver membrane, kidney BLM and BBM. Crude liver membrane, kidney BBM and BLM (10 mg protein/ml) were solubilized with 1% CHAPS in the presence of 1 mM PMSF in PBS. Solubilized membranes (1 mg protein/ml) were incubated for 10 min on ice with 120 nM Glc-S-C8-[^{125}I]Tyr in the presence and absence of 1 μM unlabeled Glc-S-C8-Tyr, and cross-linked using EGS.

the K_d value of Glc-S-C8-[^{125}I]AVP (28.7 nM, Shirota *et al.*, in preparation), Glc-S-C8-Tyr may share a binding site with Glc-S-C8-AVP. Thus Glc-S-C8-Tyr can be used to detect kidney-specific binding proteins since it has an accessible α-amino group on the N-terminal of the peptides which reacts with cross-linkers.

Characterization of this novel binding protein is a prerequisite for understanding the mechanisms of ligand-protein interactions. In the present investigation, cross-linking reagents were used as tools to identify cell surface binding protein by affinity labeling. An M_r = 62,000 protein was labeled, and its binding activity was unaffected by solubilization with CHAPS (Fig. 3). Since dithiothreitol did not affect the affinity labeled bands, it appears that S-S bonds are not involved in the assembly of the binding protein unit. While the NHS-esters; BS^3, DSG and EGS all labeled M_r = 62,000 protein, regardless of the spacer arm length, the imidoester cross-linking reagent DMP failed to label any protein (Fig. 4). The difference in the activity of cross-linkers may be accounted for by considering the mechanism of their reaction. Although accessible α-amino groups present on the N-termini of peptides and proteins are principal targets for both NHS-esters and imidoesters, NHS-esters, but not imidoesters, can also react significantly with ϵ-amine of lysine. In addition, NHS-esters, but not imidoesters, has some cross-reactivity toward nucleophilic groups other than primary amines in proteins. Collectively, the binding protein may not contain any free amino groups in the ligand binding region that can be reached by DMP.

Several pieces of evidence indicate that the M_r = 62,000 band obtained from cross-linking is the kidney-specific novel binding protein reported earlier (1–3). Firstly, all the cross-linking reagents used resulted in labeling of the same M_r = 62,000 protein band (Fig. 4). Furthermore, the kidney basolateral specific localization of the binding protein, suggested from the *in vivo* tissue uptake study (3) and the *in vitro* binding study (Shirota *et al.*, in preparation), were consistent with the results of the present cross-linking studies; i.e., M_r = 62,000 affinity-labeled bands were observed in kidney BLM, but not in crude liver or kidney BBM (Fig. 5). Since liver uptake of alkylglucoside was not observed *in vivo* (2), a non-specific M_r = 47,500 band observed in liver is not identified. In addition, increasing the concentration of unlabeled Gal-S-C7-Me resulted in a reduction in the intensity of the cross-linked band only at higher concentrations (Fig. 6), which is consistent with low affinity binding to the crude kidney membrane fraction (1). This result suggests that the cross-linked band has the same tendency toward recognition of sugar moiety as the previously clarifed kidney specific binding protein.

Some reports suggest that Na^+/glucose cotransporters interact with alkylglucosides (14,15), and that the alkylglucoside structural requirements are quite similar to the binding protein (15). However, both low and high affinity Na^+/glucose cotransporters (16–19) are located specifically in the BBM. In addition, the affinity for alkylglucosides is much lower (10 ∼ 100 μM) (14,15,20). These findings suggest that Na^+/glucose cotransporters cannot explain the sugar recognition molecules in the present investigation. The cross-linking method can be used to purify the kidney-specific alkylglycoside binding protein in future studies.

In conclusion, the results of the present study suggest that the alkylglucoside specific binding protein (M_r = 62,000) is located on the BLM of the renal cortex.

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