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Target size analysis of the peptide/H^+-symporter in kidney brush-border membranes

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Abstract

The apparent functional molecular mass of the kidney peptide/H^+-symporter was determined by radiation inactivation in brush-border membrane vesicles (BBMV) of rat kidney cortex. Purified BBMV were irradiated at low temperatures with high energy electrons generated by a 10-MeV linear accelerator at doses from 0 to 30 megarads. Uptake studies were performed with [^{3}H]cefadroxil, a β-lactam antibiotic which serves as a substrate for the kidney peptide/H^+-symporter. Inhibition of influx of [^{3}H]cefadroxil into BBMV was used to determine the functional molecular mass of the transporter. Additionally, direct photoaffinity labeling of the transport- and/or binding proteins for [^{3}H]cefadroxil in control and irradiated BBMV was performed to determine the molecular mass of the putative transporter by SDS-polyacrylamide gel electrophoresis. Initial rates of pH-gradient dependent uptake of [^{3}H]cefadroxil decreased progressively as a function of radiation dose. The apparent radiation inactivation size (RIS) of the transport function was found to be 414 ± 16 kDa. Direct photoaffinity labeling yielded labeled membrane proteins with apparent molecular masses of 130 kDa and 105 kDa, respectively. The proteins displayed different labeling characteristics with respect to incubation time, specificity and the response to irradiation. It appears that only a 105 kDa protein is directly involved in transport function since (a) only it showed a specific pH gradient dependent labeling pattern and (b) the covalent incorporation of [^{3}H]cefadroxil into this protein decreased parallel to the loss of transport function in irradiated BBMV. The peptide/H^+-symporter in kidney brush-border membranes therefore appears to have a monomer mass of 105 kDa and may function in an oligomeric arrangement.

Keywords: Brush-border membrane; Peptide/proton symporter; Functional molecular mass; (Rat kidney)

1. Introduction

A function of the kidney brush-border membrane is the uptake of dipeptides and tripeptides from the tubular fluids [1–3]. Transmembrane transport of di- and tripeptides is largely mediated by an electrogenic peptide/H^+ symporter [4–9]. Beside di- and tripeptides also amino β-lactam antibitotics like cefadroxil serve as substrates for the transporter [10]. Although the transport system has been characterized with respect to its driving force [5,8,9] and its substrate specificity [10,11], no information is available regarding the structure and size of the transport protein.

Radiation inactivation has been a useful tool in determining the molecular features of soluble enzymes and membrane bound proteins [12–14]. The major advantage of the technique is, that it allows the functional molar mass of membrane proteins to be measured without the need of purification and solubilization. In the present studies we applied radiation inactivation in kidney brush-border membrane vesicles to determine the functional molecular mass of the transport protein based on the inhibition of [^{3}H]cefadroxil uptake into BBMV as a function of radiation dose. Direct photoaffinity labeling of membrane proteins with [^{3}H]cefadroxil in control and irradiated BBMV was used (a) to identify the putative transport protein in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and (b) to correlate the loss of transport function in BBMV with the decline of incorporation of photolabel into the membrane proteins.

Abbreviations: BBMV, brush-border membrane vesicles; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; diSC$_3$(5), 3,3′-dipropylthiadicarbocyanine iodide; EGTA, (ethylenebis(oxyethylenenitrilo))tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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2. Experimental procedures

2.1. Materials

Adult male Wistar rats with a body weight of 240 ± 15 g were purchased from Winkelmann, Borchen, Germany. Custom-synthesized [^{3}H]cefadroxil (39 Ci/mmol) was obtained from Amersham Buchler, Braunschweig, Germany. Enzymes, enzyme substrates and all other chemicals in the highest purity were purchased from Sigma (Deisenhofen, Germany) and reagents for protein-dye-binding were obtained from Bio-Rad Laboratories (München, Germany). The potential sensitive fluorescent dye 3,3'-dipropylthiadicarbocyanine iodide (diSC$_3$(5)) was obtained from Molecular Probes (Eugene, OR, USA).

2.2. Methods

Preparation of rat kidney BBMV

BBMV were prepared from rat kidney cortex using a Mg^{2+}/EGTA precipitation technique as previously described [8,10]. BBMV were washed and resuspended in preloading buffer (50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4, pH 8.3). Determination of the specific activity of the membrane marker enzymes (8) revealed no enrichment of K^+-stimulated ouabain-sensitive *p*-nitrophenolphosphatase, but a 21-fold enrichment for γ-glutamyltransferase and 25-fold enrichment for alkaline phosphatase. Protein concentration in the BBMV preparations was adjusted to 7.5 mg/ml and BBMV were stored in liquid N_2.

Transport assay

Uptake studies were performed at 24°C by using a rapid filtration technique with membrane filters (type ME 2500, 0.45 μm pore size, Schleicher and Schüll, Dasseln, Germany) as described previously [8,11]. Uptake of radiolabeled cefadroxil was initiated by rapidly mixing 10 μl of membrane suspension (75 μg of protein) preloaded with buffer (pH 8.3) with 40 μl of medium consisting of 50 mM Hepes, 50 mM Mes, 25 mM Tris, 300 mM mannitol (pH 6.0) or 50 mM Hepes, 75 mM Tris and 300 mM mannitol (pH 8.3) and containing usually 5.0 μCi/ml (0.14 μM) of [^{3}H]cefadroxil. Mixing of vesicles loaded with buffer pH 8.3 with incubation buffer pH 6.0 (1:5, v/v) resulted in a final pH during the uptake studies of 6.7 (pH_{in} 8.3/pH_{out} 6.7). Incubation of vesicles was terminated by a 50-fold dilution with an ice-cold stop solution (2 mM Hepes/Tris, 210 mM KCl, pH 7.5), followed by filtration. The radioactivity associated with the filters was counted in 5 ml Rotisszint 2200 (Roth, Karlsruhe, Germany) in a β-scintillation counter (LSC 6000, Beckmann, München, Germany). Unspecific binding of the labeled compound to the filter (usually less than 1% of uptake) was substracted from the transport data.

Studies with the potential sensitive fluorescent dye diSC$_3$(5)

To determine whether irradiated BBMV are able to maintain a transmembrane pH gradient across the brush-border membrane, the transmembrane electrical potential was measured in BBMV using the potential sensitive fluorescent dye diSC$_3$(5). The measurements were carried out at 25°C in a spectrofluorophotometer (Shimadzu RF-500) with an excitation wavelength of 622 nm and emission wavelength of 669 nm. 10 μl of BBMV (7.5 mg protein/ml) preloaded with buffer pH 8.3 were mixed with 730 μl of incubation buffer pH 6.0 (50 mM Hepes, 25 mM Tris, 300 mM mannitol) and 10 μl of a 0.3 mM diSC$_3$(5) stock solution in ethanol to allow the fluorescent dye to equilibrate between the intra- and extravesicular space. After 1 min of constant stirring in a quartz glass cuvette, 10 μl of a stock solution of FCCP (final concentration 75 μM) were injected into the cuvette through a small opening at the top of the sample compartment. Magnetic stirring of the incubation medium in the cuvette was performed throughout the recordings. Changes in diSC$_3$(5) fluorescence caused by the collapse of the pH gradient in the presence of FCCP were compared between control BBMV and vesicles irradiated at 10 Mrad.

Determination of enzyme activities

Activities of brush-border membrane enzymes and enzymes added to the BBMV were used as standards for the determination of the accuracy of target size analysis. We determined the activities of the intrinsic enzymes alkaline phosphatase (EC 3.1.3.1), dipeptidylpeptidase IV (EC 3.4.14.5) γ-glutamyltransferase (EC 2.3.2.2) and aminopeptidase M (EC 3.4.11.2). Additionally alcohol dehydrogenase (EC 1.1.1.1) and β-galactosidase (EC 3.2.1.23) were added to BBMV prior to irradiation and remaining activities of these enzymes were measured after radiation inactivation.

Photolabeling

BBMV (750 μg of protein) preloaded with buffer pH 8.3 (50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4) were mixed with 400 μl medium of either pH 6.0 or pH 8.3 containing 10 μCi/ml of [^{3}H]cefadroxil. After 15 s, 10 min or 30 min of incubation samples were irradiated in quartz glass tubes with one high energy UV light flash (capacity 8000 μF, 630 V, 952 W s). BBMV then were washed free of label three times and pelleted at 12000 × *g*. The final pellet was suspended in 100 μl of a buffer containing 2 mM Hepes/Tris, 210 mM KCl, pH 7.5 and membrane proteins were extracted with methanol/chloroform according to Wessel and Flügge [15]. Extracted proteins were freeze-dried, dissolved in 60 μl of 5% SDS, boiled and applied onto a 10% SDS polyacrylamide gel. Electrophoresis was carried out at 10 mA. Proteins were stained with Coomassie blue R250. Quantitation of radioactive cefadroxil incorporated into the proteins was performed by slicing the gels (2 mm slices) and counting

the radioactivity. Determination of apparent molecular sizes of the labeled proteins was performed by densitometry of the Coomassie stained gels (scanner GS 300, Hoefer, San Francisco, CA, USA) based on the calibration with molecular weight marker proteins (Sigma, Deisenhofen, Germany).

The specificity of photolabeling of membrane proteins was determined by the following experiment: BBMV (750 μg protein) preloaded with buffer pH 8.3 were flashed after 15 s in the presence of a pH gradient and in the presence of 0.5 μM of unlabeled cefadroxil. In control BBMV 0.5 μM cefadroxil was added after photoirradiation. BBMV then were washed with loading buffer pH 8.3, pelleted and resuspended in loading buffer. After 16 h of equilibration at 4°C uptake of [^{3}H]cefadroxil (0.25 μM) into control BBMV and BBMV flashed in the presence of unlabeled cefadroxil was measured as a function of time in the presence of a transmembrane pH gradient as described above.

To determine whether covalent incorporation of cefadroxil into membrane proteins is dependent on a transmembrane pH gradient, BBMV were flashed with UV light after 15 s of incubation both in the absence and the presence of a pH gradient as described in the uptake experiments. In an additional series of experiments the specific H^+-ionophore FCCP (75 μM) was used to abolish the protonmotive force during photolabeling of the membranes.

Since all alterations of pH during photolabeling also affected non-specific incorporation of label (baseline), data shown represent the amount of radioactivity incorporated after normalization by a factor derived from the total areas under the curves (AUC) between individual gels.

Radiation procedure

BBMV preloaded with 50 mM Hepes, 75 mM Tris, 100 mM K_2SO_4 (pH 8.3) stored in polypropylene vials were kept under liquid N_2 before and during the radiation procedure. Irradiation of the samples was performed in the electron beam of a 10-MeV linear accelerator at the radiation laboratories, Justus-Liebig-University, Giessen, Germany. Samples were fixed in a particular geometry with respect to the electron beam in an aluminium rack which also allowed to keep the temperature constant between −90 and −120°C. The dose distribution along the samples was determined by ferrous sulfate dosimetry and during irradiation by use of radiation sensitive films (Far West Technology, CA, USA).

Calculations and statistics

Uptake rates and enzyme activities for individual inactivation curves were plotted semilogarithmically against radiation dose and rate constants for inactivation (D_{37}-values) were derived by linear regression analysis. D_{37} is the radiation dose that is necessary to obtain 37% residual biological activity. The functional molecular mass of the transporter was calculated by the following empirical equation:

$$\text{Molecular weight} = 6.4 \cdot 10^{11} \cdot f/D_{37}(\text{rad})$$

derived by Kepner and Macey [16]. Because experiments were performed at −90 to −120°C the empirical temperature correction factors of Kempner and Haigler [17] ($f = 10^{0.85 - 0.0028 \times C}$ where C = −90 to −120°C) were used to correct inactivation data to 25°C at which temperature the dosimetry was done.

The validity of this approach was proven by the determination of the apparent functional molecular masses of six different enzymes with known molecular weights.

All calculations were performed by using INPLOT (GraphPAD, Los Angeles, CA). The experiments were generally carried out in triplicate with at least three preparations and results are presented as the mean ± S.E. Significance of differences between the uptake rates and constants derived were determined by a non-paired t-test.

3. Results

3.1. Effect of irradiation on uptake of [^{3}H]cefadroxil into BBMV

As shown previously [5,8,10], the presence of an inwardly directed pH gradient is necessary for the transient accumulation of peptides and amino β-lactam antibiotics in kidney BBMV. Uphill transport of [^{3}H]cefadroxil (0.14 μm) is evident by a 5-fold overshoot in the presence of a pH gradient (Fig. 1). Irradiation of BBMV caused a progressive decrease in [^{3}H]cefadroxil uptake in the presence of a pH gradient (Fig. 1). Uptake in the absence of a pH gradient as well as under equilibrium conditions was not

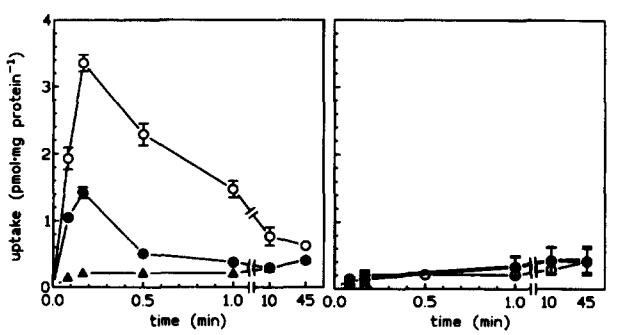

Fig. 1. Uptake of [^{3}H]cefadroxil as a function of time in control and irradiated BBMV. Uptake of 0.25 μM [^{3}H]cefadroxil as a function of time in control-BBMV (open symbols) and BBMV irradiated with 3.8 Mrad (filled circles) or 10.5 Mrad (filled triangles). BBMV (75 μg) preloaded with 50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4, pH 8.3 were incubated in either a buffer pH 6.0 containing 50 mM Hepes, 50 mM Mes, 25 mM Tris and 300 mM mannitol (left side) or pH 8.3 containing 50 mM Hepes, 75 mM Tris, and 300 mM mannitol (right side) for the times indicated. Data represent the mean ± S.E. of three membrane preparations.

significantly affected by irradiation (Fig. 1). Initial rate uptake of cefadroxil in the presence of a pH gradient as a function of radiation dose is shown in Fig. 2. A semi-logarithmic plot was used to fit the data to a single exponential dependence on radiation dose. The apparent radiation inactivation size (RIS) of the transport function was found to be 414 ± 16 kDa (D_{37} value 3.4) with a coefficient of correlation (r^2) of 0.977.

It needs to be addressed here, that this assumption neglects the possibility of multiple carrier systems involved in cefadroxil transport. Although two transport systems have been shown to mediate uptake of peptides in kidney BBMV [2,8,18] the use of low substrate concentrations (< 10 μM) ensures that cefadroxil uptake is mediated almost entirely ($> 95\%$) by the high affinity peptide/H^+-symporter [8]. Thus, radiation inactivation data shown here represent inhibition of cefadroxil uptake by the high affinity peptide/H^+-symporter.

That the inactivation of transport function by irradiation is not a consequence of impaired vesicle integrity which could cause a rapid dissipation of the protonmotive force was investigated by measuring changes in the transmembrane electrical potential difference using the fluorescent probe $diSC_3(5)$. The fluorescence of $diSC_3(5)$ increases with decreasing inside-negative membrane potentials by a proton influx in the presence of FCCP. $diSC_3(5)$ fluorescence in control BBMV and BBMV irradiated at 10 Mrad was determined after addition of the protonophore FCCP in the presence of a transmembrane pH gradient (pH_{in} 8.3/pH_{out} 6.0). Addition of the protonophore immediately increased the fluorescence of the dye in both vesicle preparations by 8.2 ± 2.1 (control) and 7.8 ± 2.5 units (irradiated BBMV). The identical changes in fluorescence indicate that irradiated BBMV are still able to maintain a pH gradient of the same magnitude as control BBMV. The inhibition of transport function by irradiation therefore is not caused by a decrease in the driving force but by inactivation of the transport protein.

In contrast to pH gradient dependent cefadroxil influx, measured at 5 s of incubation, which declined dramatically as a function of radiation dose, the corresponding equilibrium values (45 min) remained almost unaffected by radiation (Fig. 2).

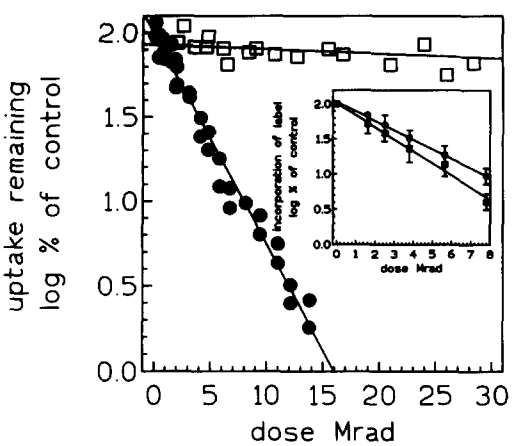

Fig. 2. Molecular size determination of the transport system for [^{3}H]cefadroxil transporter. BBMV (75 μg) preloaded with 50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4, pH 8.3 were irradiated at -100 to -120°C and doses of 0 to 30 Mrad as described in the method section and uptake of 0.25 μM [^{3}H]cefadroxil was determined at 5 s (filled circles) or 45 min of incubation (open squares) in buffer pH 6.0 (50 mM Hepes, 50 mM Mes, 25 mM Tris, 300 mM mannitol). The results are expressed as log of the percentage of transport activity remaining as a function of radiation dose. The apparent functional molecular size was calculated as 414 ± 16 kDa. Each value represents the mean $\pm$ S.E. of three membrane preparations each done in triplicate. (Inset) Decline of incorporation of [^{3}H]cefadroxil into the two major brush-border membrane proteins identified by direct photolabeling in irradiated BBMV after separation by SDS-PAGE. BBMV irradiated at various doses were photolabeled in the presence of a pH gradient (pH_{in} 8.3/pH_{out} 6.7) with 0.5 μM of [^{3}H]cefadroxil 15 s after the start of incubation. Covalent incorporation into the 130 kDa (filled squares) and 105 kDa (filled circles) proteins was measured after SDS-PAGE separation and incorporation is expressed as log % of label detectable as a function of radiation dose.

3.2. *Effect of irradiation on brush-border membrane enzyme activities*

The inactivation curves of the brush-border enzymes as well as the enzymes added to BBMV prior to irradiation were used to calculate the corresponding D_{37} values and to verify the accuracy of the calculations of the functional molecular weights. We determined apparent functional sizes of 118.2 ± 5.9 for γ-glutamyltransferase, 94.9 ± 3.7 for alkaline phosphatase, 119.9 ± 2.1 for aminopeptidase M, 111.9 ± 3.3 for dipeptidylpeptidase IV, 71.5 ± 4.7 kDa for alcohol dehydrogenase and 114.4 ± 4.0 for β-galactosidase. Molecular weights reported for either the corresponding purified enzymes or determined by radiation inactivation experiments in rat kidney brush-border membranes are 93–112 for γ-glutamyltransferase [19], 104.8 for alkaline phosphatase [14], 110 for aminopeptidase M [20], 100 for dipeptidylpeptidase IV [21], 80 kDa for alcohol dehydrogenase [22] and 116.3 for β-galactosidase [23]. It should be emphazised here that β-galactosidase was chosen as a high molecular marker enzyme considered to be inactivated with a functional molar mass of 478 kDa [28]. However, β-galactosidase was inactivated, as more frequently seen [23], in its monomer size with approx. 120 kDa. In general, comparing the functional molecular sizes determined here, reveals a good agreement with data published by others.

3.3. *Photolabeling of membrane proteins with [^{3}H]cefadroxil*

In a first series of experiments photolabeling of BBMV with [^{3}H]cefadroxil was carried out as described by Kramer et al. [24,25] for labeling of the putative peptide transporter in BBMV of kidney and intestine with benzylpenicillin as

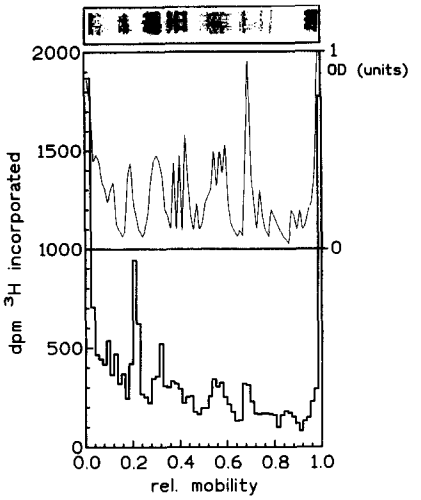

Fig. 3. Covalent incorporation of [^{3}H]cefadroxil into brush-border membrane proteins by direct photoaffinity labeling. BBMV (750 μg of protein) preloaded with buffer pH 8.3 (50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4) were mixed with 400 μl medium pH 6.0 (pH_{in} 8.3/pH_{out} 6.7) and 10 μCi/ml of [^{3}H]cefadroxil. After 10 min of incubation samples were irradiated, washed free of label and membrane proteins were separated by SDS-PAGE. Distribution of [^{3}H]cefadroxil labeled proteins is shown as a function of their relative mobility within the gel. The upper section shows the corresponding gel and the distribution of proteins as measured by densitometry of the Coomassie stained gels. Mean of three membrane preparations.

a substrate. BBMV were preincubated with [^{3}H]cefadroxil for 10 min prior to UV exposure. This resulted in the incorporation of [^{3}H]cefadroxil predominantly into a membrane protein with an apparent molecular weight of 130 kDa (Figs. 3 and 4b). In contrast, when BBMV were flashed with UV light 15 s after addition of the probe, membrane proteins with apparent sizes of 130 kDa and 105 kDa were labeled (Fig. 4a). When photolabeling was performed under equilibrium conditions (30 min of preincubation) again only a 130 kDa protein could be identified (Fig. 4c).

To establish that photoirradiation of BBMV with [^{3}H]cefadroxil not only labels membrane proteins but also functionally inactivates the transporter, BBMV were flashed in the absence and the presence of 0.5 μM of non-radioactive cefadroxil followed by the determination of BBMV transport function. UV irradiation of BBMV in the presence but not in the absence of non-radioactive cefadroxil significantly reduced ($P < 0.001$ at 5 s and 10 s) the transport activity of the peptide/H^+-symporter when uptake was assayed in vesicles equilibrated with loading buffer (Fig. 5). This indicates that the covalent incorporation of cefadroxil into one of the membrane proteins indeed inhibits the function of the pH gradient dependent transport system.

Since uptake of cefadroxil into BBMV is pH gradient dependent we investigated whether photolabeling of membrane proteins also displays a distinct dependence on a pH gradient during UV-irradiation of the membranes. As shown in Fig. 6, at 15 s of incubation two proteins of apparent molecular masses of 130 kDa and 105 kDa were labeled when UV irradiation was performed in the presence of a pH gradient (pH_{in} 8.3/pH_{out} 6.7). When labeling was carried out in the absence of a pH gradient (pH_{in} 8.3/pH_{out} 8.3) or the presence of a pH gradient and the presence of the protonophore FCCP, incorporation of label into the 105 kDa protein was reduced to a larger extent than incorporation into the 130 kDa protein. This indicates that the labeling pattern of the 105 kDa protein more closely resembles the pH gradient dependence of uptake than does the 130 kDa protein.

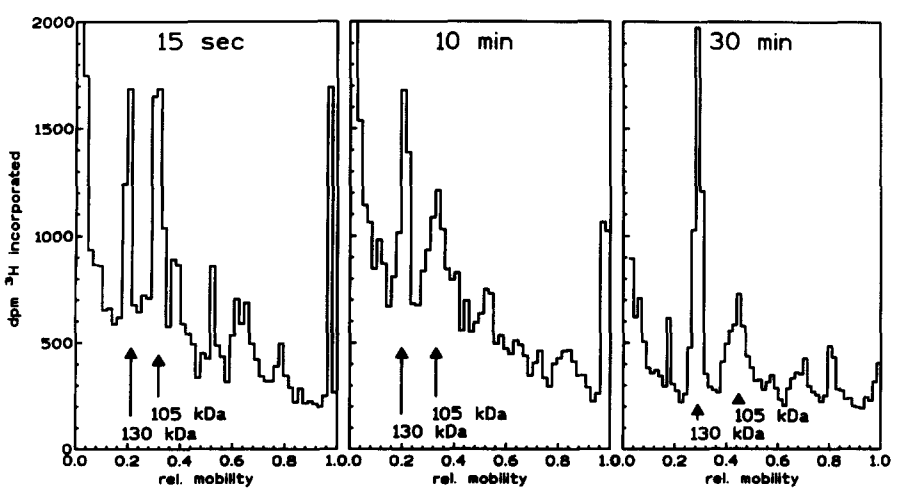

Fig. 4. Effect of incubation time on covalent incorporation of [^{3}H]cefadroxil into brush-border membrane proteins by direct photoaffinity labeling. BBMV (750 μg of protein) preloaded with buffer pH 8.3 (50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4) were mixed with 400 μl medium pH 6.0 (pH_{in} 8.3/pH_{out} 6.7) and 10 μCi/ml of [^{3}H]cefadroxil. After 15 s, 10 min and 30 min of incubation samples were irradiated, washed free of label and membrane proteins were separated by SDS-PAGE. Distribution of [^{3}H]cefadroxil labeled proteins is shown as a function of their relative mobility within the gel (mean of three membrane preparations).

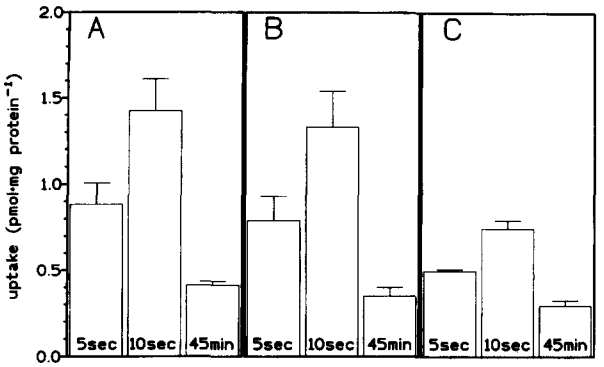

Fig. 5. Inactivation of transport function by direct photoaffinity labeling of BBMV in the presence of 0.5 μM of non-radioactive cefadroxil. BBMV (75 μg) preloaded with 50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4, pH 8.3 were suspended in incubation in buffer pH 6.0 (50 mM Hepes, 50 mM Mes, 25 mM Tris, 300 mM mannitol) and irradiated at 15 s of incubation with UV light in the absence (A) or the presence of 0.5 μM of unlabeled cefadroxil (C). As a control (B) 0.5 μM of unlabeled cefadroxil was added to the incubation mixture after UV irradiation. BBMV then were washed twice, pelleted at 40000× g and equilibrated for 14 h with 50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4, pH 8.3. Uptake of 0.18 μM of [^{3}H]cefadroxil was measured for 5 s, 10 s and 45 min in the presence of a pH gradient (pH_{in} 8.3/pH_{out} 6.7) as described in Experimental procedures. Each value represents the mean ± S.E. of three membrane preparations each done in triplicate.

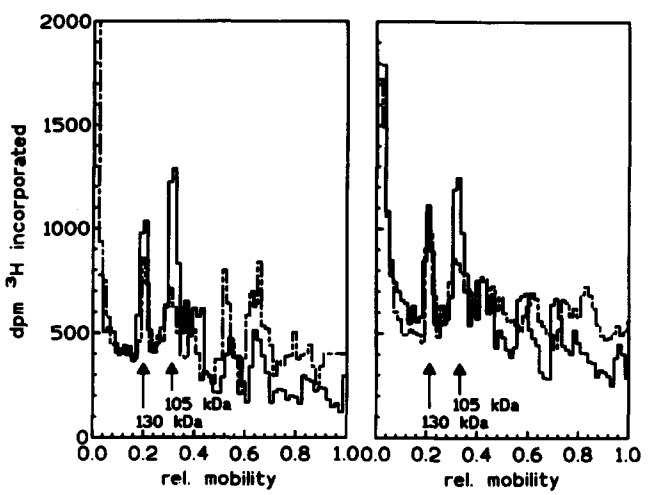

Fig. 6. Effect of pH gradient and FCCP on covalent incorporation of [^{3}H]cefadroxil into brush-border membrane proteins by direct photoaffinity labeling. BBMV (750 μg of protein) preloaded with buffer pH 8.3 (50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4) were mixed with 10 μCi/ml of [^{3}H]cefadroxil and (a) 400 μl medium pH 6.0 (pH_{in} 8.3/pH_{out} 6.7) containing 2% ethanol (control) or (b) 400 μl medium pH 6.0 (pH_{in} 8.3/pH_{out} 6.7) containing 75 μM of FCCP in ethanol or (c) 400 μl medium pH 8.3 (pH_{in} 8.3/pH_{out} 8.3). After 15 s of incubation samples were irradiated and membrane proteins were separated by SDS-PAGE. Distribution of [^{3}H]cefadroxil labeled proteins is shown as a function of their relative mobility within the gel. The left side shows the incorporation of [^{3}H]cefadroxil in the presence of FCCP (dashed line) in comparison to the controls (solid line). The right side demonstrates the incorporation of [^{3}H]cefadroxil in the presence of a pH gradient (solid line) and the absence of a pH gradient (mean of three membrane preparations).

When photolabeling with [^{3}H]cefadroxil was performed in BBMV irradiated at doses between 1 and 8 Mrad incorporation of [^{3}H]cefadroxil into the 130 kDa and 105 kDa protein fractions declined progressively with increasing doses (Fig. 2, inset). A correlation of the fractional inhibition of uptake (log % of control uptake) at different radiation doses and the amounts of [^{3}H]cefadroxil incorporated into the 105 kDa protein in the same BBMV preparations (log % of control) revealed a significant linear correlation between loss of transport function and incorporation of label into the 105 kDa protein (r^2: 0.997) and the 130 kDa protein (r^2: 0.988). The slope for the 105 kDa protein was -0.135 ± 0.094 and the corresponding D_{37} value was 3.4. For the 130 kDa protein a slope of -0.172 ± 0.103 and a D_{37} value of 2.6 was determined. Since the D_{37} value for inactivation of transport function was found to be 3.4, only the 105 kDa protein therefore displayed a significant parallel decrease in photoincorporation of [^{3}H]cefadroxil and inactivation of transport after irradiation.

4. Discussion

Irradiation of kidney BBMV with high energy electrons resulted in a dose dependent loss of transport activity of the peptide/H^+-symporter. Uptake of [^{3}H]cefadroxil into irradiated BBMV as a function of time was inhibited in the presence but not the absence of a transmembrane pH gradient. We assume that this alteration in transport function is due to a specific inactivation of the peptide/H^+-symporter and not to unspecific effects on vesicle integrity. Previous studies have shown that irradiation of rat kidney BBMV with high energy electrons does not alter vesicle size as judged by electron microscopy, nor vesicle integrity and intravesicular volume [14]. When in the present study 45 min uptake rates of [^{3}H]cefadroxil were plotted as a function of radiation dose (Fig. 2), equilibrium uptake and therefore intravesicular space and vesicle integrity also remained unaffected. Possible artifacts arising from alterations of the driving forces caused by irradiation were eliminated on the basis of transport measurements performed in initial rate conditions. Studies with the fluorescent probe $diSC_3(5)$ in addition revealed that irradiation does not impair the ability of the membrane vesicles to maintain a transmembrane pH gradient. The irradiation effects were thus not caused by a decrease in the driving force, but resulted from the inactivation of the carrier. Based on initial uptake rates for the pH gradient dependent transport of [^{3}H]cefadroxil as a function of radiation dose the apparent functional molecular size of the transporter was determined to be 414 ± 16 kDa.

Direct photolabeling with [^{3}H]cefadroxil caused the covalent incorporation of label into one or two membrane proteins depending on the time of preincubation of BBMV with the substrate prior to UV exposure. After 10 min or

30 min of preincubation of BBMV with [^{3}H]cefadroxil a protein with an apparent molecular size of 130 kDa was labeled. A protein of this size has been identified previously as the putative dipeptide transporter in intestinal and kidney brush-border membranes by photoaffinity labeling with [^{3}H]benzylpenicillin as a probe [24–27]. However, as we have shown recently, benzylpenicillin does not serve as a substrate for the kidney peptide/H^+-symporter [10]. In contrast to a large variety of di- and tripeptides and aminocephalosporins which generally display affinities in the range of 10 μM to 500 μM, cephalosporins and penicillins lacking an α-aminogroup failed to interact with the substrate binding site of the transporter [10]. Assuming identity of the 130 kDa proteins labeled in kidney BBMV with either benzylpenicillin [25] or cefadroxil, the question arises, how the same protein is labeled to the same extent by two compounds from which one does not serve as a substrate for the transporter ($K_i > 10$ mM) whereas the other one displays a very high affinity (K_i 60 μM) for the peptide/H^+-symporter [10]. Although we can not exclude that a 130 kDa protein is directly involved in cefadroxil transport it rather appears to be a brush-border binding protein for β-lactam antibiotics. This assumption is supported by the observation that a 130 kDa protein is labeled by [^{3}H]cefadroxil in the absence of any driving force, when no transport is observed, as well as under equilibrium conditions. If binding of cefadroxil to the 130 kDa protein would contribute to cefadroxil transport into BBMV, irradiation effects on the 130 kDa protein as seen in the photolabeling experiments will not be detectable in flux studies, since cefadroxil binding to BBMV accounts for only 20% of overall cefadroxil transport. Although the binding of cefadroxil to the 130 kDa protein might not be necessary for transport function, we can not exclude that the 130 kDa protein represents a subunit of the oligomeric transport protein.

When photolabeling was performed under overshoot-conditions (at 15 s) in the presence of a pH gradient as a driving force, a second protein with an apparent molecular size of 105 kDa was identified. This protein could not be labeled efficiently (a) when the pH gradient was abolished by use of the protonophore FCCP, (b) when labeling was performed in the absence of a pH gradient (pH_{in} 8.3/pH_{out} 8.3) or (c) under equilibrium conditions (at 30 min). Covalent incorporation of [^{3}H]cefadroxil into the 105 kDa protein therefore was strongly pH gradient dependent and occurred only under overshoot conditions.

The most plausible explanation for this finding is, that incorporation of label into the 105 kDa is dependent on the transient accumulation of [^{3}H]cefadroxil within the BBMV. This implies, that the protein is labeled from the internal surface of the BBMV, in which at 15 s of incubation the concentration of radiolabeled cefadroxil exceeds its external concentration at least four to five times. Alternatively, the protein may only be labeled during its transmembrane transport when a pH gradient provides the driving force. That the cotransport of protons with cefadroxil is not only a necessary prerequisite for transport but also for photolabeling of a 105 kDa protein becomes evident by the decrease of incorporation of label when the pH gradient is dissipated by use of the H^+-ionophore FCCP. This is an important observation, since FCCP abolishes the pH gradient without affecting the extravesicular pH due to high external buffering capacity. If labeling of the 105 kDa protein would simply be the consequence of a low extravesicular pH of 6.7, than FCCP should not have reduced the incorporation of [^{3}H]cefadroxil. Previous studies on transport function of the peptide/H^+-symporter in kidney BBMV revealed that a low extravesicular pH per se does not accelerate peptide uptake by increasing the affinity for substrate binding [8]. The inwardly directed pH gradient as the protonmotive force is obviously necessary for both, efficient transport function as well as efficient photoincorporation of [^{3}H]cefadroxil into a 105 kDa protein.

Recently the first mammalian proton coupled peptide transporter has been cloned from the rabbit small intestine [29]. Intestinal and renal peptide/H^+ cotransport systems appear to have similar but not identical characteristics. Although both transport systems operate by an electrogenic substrate/proton cotransport, there are functional differences including substrate affinity and specificity. For example, affinity constants for selected substrates are generally in the millimolar range in the intestine and in the micromolar range in the kidney [5,6,10,11]. With respect to specificity, differences are found for substrates without an α-aminogroup or an aminogroup in β-position. These compounds have a very low affinity for the renal transporter whereas in the intestine these structural aspects do not restrict substrate affinity [10,11,1,5].

In vitro translation of the cDNA of the intestinal clone revealed a glycosylated gene product with an apparent molecular weight of approx. 71 kDa [29]. This is lower than the 105 kDa protein identified in the present study. Although we can not exclude entirely an overestimation of the size of 105 kDa protein, we favor the hypothesis that there are different proteins in the two tissues mediating peptide/proton cotransport. A claim, that the renal transporter may not only display different functional characteristics, but may indeed represent a different gene product, is supported by the observation that northern blot analysis with a full length antisense cRNA from the intestinal clone showed a very low signal [28] in rabbit kidney. Whereas after 3 h of exposure a strong hybridization signal in the intestine was observed, demonstration of a signal of similar size and strength in the kidney sample needed 8 days of exposure, indicating a very low abundance of a comparable RNA. The question of whether similar or identical proteins mediate peptide/proton cotransport in intestine and kidney therefore remains to be answered.

In conclusion, our studies provide direct evidence for the presence of a 105 kDa protein in the kidney brush-border membrane that appears to be involved in peptide

and aminocephalosporin/H^+-symport across the brush-border membrane. The apparent functional molecular size (RIS) of the transporter was found to be 414 ± 16 kDa which could correspond to a homo- or heteroterameric arrangement.

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