Epidermal growth factor decreases PEPT2 transport capacity and expression in the rat kidney proximal tubule cell line SKPT0193 cl.2

Silvina A. Bravo,1 Carsten Uhd Nielsen,1 Jan Amstrup,2 Sven Frokjaer,1 and Birger Brodin1

1Department of Pharmaceutics, The Danish University of Pharmaceutical Sciences, DK-2100 Copenhagen; and 2Department of Zoophysiology, August Krogh Institute, DK-2100 Copenhagen, Denmark

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Bravo, Silvina A., Carsten Uhd Nielsen, Jan Amstrup, Sven Frokjaer, and Birger Brodin. Epidermal growth factor decreases PEPT2 transport capacity and expression in the rat kidney proximal tubule cell line SKPT0193 cl.2. Am J Physiol Renal Physiol 286: F385–F393, 2004. First published October 14, 2003; 10.1152/ajprenal.00226.2003.—The renal peptide transporter PEPT2 plays an important role in absorption of di- and tripeptides in the proximal tubule; however, knowledge of regulation of PEPT2 by growth factors and hormones is limited. In the present study, we examined the effects of epidermal growth factor (EGF) on PEPT2 transport capacity and expression in the rat proximal tubule cell line SKPT0193 cl.2 (SKPT), which expresses rat PEPT2 (rPEPT2) in the apical membrane. Treatment of SKPT cells with EGF during cell culture growth caused a dose-dependent decrease in rPEPT2 transport capacity and expression, as determined by studies of apical uptake of [14C]glycylsarcosine, rPepT2 mRNA levels, and immunostaining of SKPT cells with a rPEPT2-specific antibody. On the contrary, apical uptake of glucose and lysine was increased in EGF-treated cells, indicating that EGF was not acting generally to decrease apical nutrient uptake mechanisms in the proximal tubule cells. Our findings indicate that EGF decreases rPepT2 expression by lowering transcription of the rat Pept2 gene or by decreasing rat PepT2 mRNA stability. Previous investigators routinely used SKPT cell culture media with a high (10 ng/ml) EGF concentration. Our study suggests that this might be disadvantageous when studying PEPT2-mediated transport phenomena. These findings demonstrate for the first time EGF-mediated regulation of PEPT2 expression in a kidney cell line. The relevance for kidney regulation of peptide transport activity in physiological and/or pathophysiological situations, where EGF and EGF receptor levels change drastically, remains to be established.

Materials and Methods

Materials. If not otherwise stated, compounds were supplied by Sigma (St. Louis, MO).

Cell culture. SKPT cells at passage 44, donated by Dr. M. Brandsch (Biozentrum, Halle, Germany) with kind permission of Dr. U. Hopfer (Case Western Reserve University, Cleveland, OH), were seeded on culture flasks and passaged in 1:1 DMEM/Nutrient mixture F-12 (F12), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 5 μg/ml insulin, 4 μg/ml dexamethasone, 5 μg/ml apotransferrin and varying amounts of EGF, all from Life Technologies (Taasstrup, Denmark). When cells reached passages 45–63, they were seeded onto tissue culture-treated Transwells (1 cm², 0.4-μm pore size; Costar, Cambridge, MA) at a density of 5 × 10⁴ cells/cm². Monolayers were grown in an atmosphere of 5% CO₂-95% O₂ at 37°C. Growth media were replaced every other day.

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Address for reprint requests and other correspondence: B. Brodin, Dept. of Pharmaceutics, The Danish Univ. of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen, Denmark (E-mail: bbr@dfh.dk).

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Gly-Sar, d-(-)-glucose, and l-lysine uptake experiments. [14C]Gly-Sar with a specific activity of 49.94 mCi/mmol, [3H]mannitol with a specific activity of 51.50 mCi/mmol, d-2-[3H]glucose with a specific activity of 12.4 mCi/mmol, and l-[4,5-3H(N)]-lysine with a specific activity of 84.80 mCi/mmol were obtained from New England Nuclear (Boston, MA). Uptake of [14C]Gly-Sar was measured in HBSS supplemented with 0.05% BSA. Apical media were buffered with 10 mM HEPES and adjusted to pH 7.4. Cells were placed on a shaking plate, preheated to 37°C (SWIP, EB), and allowed to equilibrate for 15 min in HBSS and buffered apical buffer solutions. The experiment was started by adding fresh apical buffer containing the relevant Gly-Sar concentration and 0.5 μCi [14C]Gly-Sar per well. In certain experiments, 0.5 μCi [3H]mannitol per well was added to the apical solution, as a marker of extracellular space. Uptake experiments were terminated after 10 min, unless otherwise stated, by gentle suction of the uptake medium, followed by four washes of the monolayers with ice-cold HBSS. The filters were cut out and placed in scintillation vials. Radioactivity was determined in a Packard Tri Carb liquid scintillation counter, using Ultima Gold scintillation fluid (Packard, Canbera). Apical uptake experiments with d-(-)-glucose and l-lysine were performed in a similar manner. Fresh apical buffer containing 0.1 mM d-(-)-glucose or l-lysine was added to the apical chamber, and the rest of the protocol was identical to the Gly-Sar uptake experiments.

Kinetic analysis. Uptake of Gly-Sar as a function of apical Gly-Sar concentration was fitted to a Michaelis-Menten type equation as previously used in Nielsen et al. (27). The ED50 value for EGF-mediated inhibition of Gly-Sar uptake was determined using the De Leans’ equation (7).

Protein determination. Filters were placed in NP-40 lysis buffer (10 mM Tris•HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 2% NP-40) in Eppendorf tubes. Cells were removed from the filters by a gentle treatment with a pistil. The tubes were centrifuged at 10,000 g for 10 min. The protein content in lysates free of cellular debris was determined by the method of Bradford (1), using the Bio-Rad Protein Assay Kit according to the manufacturer’s instructions (Bio-Rad, Hemel Hempstead, UK).

RNA isolation, cDNA synthesis, and PCR. RNA was isolated from SKPT cells grown for 4 days in the absence and presence of 10 ng/ml EGF using total RNA isolation reagent (Advanced Biotechnologies, Epsom, UK) according to the manufacturer’s instructions. First-strand cDNA was synthesized using 1 μg of total RNA and an anchored oligo(dt) primer using Reverse-it First Strand Synthesis Kit (Advanced Biotechnologies). PCR was carried out using 5 μl of reverse-transcribed RNA added to a solution consisting of 0.5 μM of forward and reverse primers for rPepT2 and for the internal control glucose 6-phosphate dehydrogenase (G6PD; DNA Technology, Aarhus, Denmark), 1.5 mM MgCl2, 5 μl of PCR buffer, 200 μM of each dNTP, and 1.25 U of HotStar Taq DNA polymerase (all from Advanced Biotechnologies) in a total volume of 50 μl. Temperature cycling proceeded as follows: 1 cycle at 94°C for 15 min and 28 cycles at 94°C for 60 s, 68°C for 60 s, and 72°C for 90 s, followed by 72°C for 10 min. Linearity was observed until 28 cycles of amplification. rPepT2 expression was measured using the sense strand 5’-GCATCTCATCCGAGATGTG-3’ (corresponding to bases 1030 to 1048) and the antisense strand 5’-TGACTGGAATGTCCTCTG-3’ (corresponding to bases 1953 to 1971) used previously by Fujita et al. (9), resulting in a 471-bp PCR product. The PCR products were electrophoresed on 1.7% agarose gels. To confirm the identity of the rPepT2 PCR product, which were subjected to restriction enzyme digestion using PstI, which should give two fragments of 806 and 135 bp. DNA from gel bands was purified using Gel Band Purification Kit (Amersham Biosciences, Upsala, Sweden) according to the manufacturer’s instructions. The purified DNA was then subjected to the action of the restriction enzyme PstI for 2 h using the appropriate buffers. A second PCR using nested primers was performed. Five microfilters (in a total volume of 50 μl) of the first PCR product were used as template. Temperature cycling in this second PCR amplification proceeded as follows: 1 cycle at 94°C for 15 min and 15 cycles at 94°C for 60 s, 60°C for 60 s, and 72°C for 90 s, followed by 72°C for 10 min. rPepT2 expression was measured using the sense strand 5’-GGGTACTTAGATGACCCCACA-3’ (corresponding to bases 1318 to 1336) and the antisense strand 5’-AGTCTTGGCCACGCTGA-3’ (corresponding to bases 1771 to 1789), resulting in a 471-bp PCR product. The PCR products were electrophoresed on a 2% agarose gel stained with SYBR-Gold from Molecular Probes (Eugene, OR). Images were obtained and analyzed on a Kodak Image Station 1000 (Kodak, Rochester, NY).

Confocal laser scanning microscopy. Cells grown on filters were fixed for 10 min in HBSS with 3% paraformaldehyde, permeabilized for 5 min in 0.1% Triton X-100 in PBS, and blocked for 30 min in a solution of 2% BSA in PBS. Cell morphology was visualized by staining actin filaments with Alexa 488-conjugated phalloidin and cell nuclei with propidium iodide, both from Molecular Probes. The cells were incubated with Alexa 488-conjugated phalloidin (1 IU in 200 μl of PBS + 2% BSA) for 30 min, treated with 100 μg/ml RNase in 2% SSC buffer solution (300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 20 min, and counterstained with 0.5 μM propidium iodide in 2% SSC buffer solution for 5 min.

EGF receptor (EGF-R) localization was studied using an anti-EGF-R antibody raised against EGF-R of human origin but with reactivity toward EGF-R protein of rat, mouse, and human (determined by the manufacturer using Western blot analysis). Anti-EGF-R antibody and blocking peptide were from Santa Cruz Biotechnology (Santa Cruz, CA). After fixation and permeabilization, cells were incubated with rabbit anti-EGF-R antibody (1:200) for 2 h. The filters were rinsed twice in PBS and then incubated with the secondary antibody (Alexa 488-conjugated goat anti-rabbit IgG from Molecular Probes) for 90 min. Thereafter, the cells were treated with RNase and stained with propidium iodide as described above. As a control, the antibody was preincubated with EGF-R blocking peptide (1:200) before the staining procedure.

The localization and expression level of rPEPT2 in SKPT cells were studied by immunostaining. Rabbit-anti-rPEPT2 antibody raised against the peptide sequence MNPFQKSNESKETLSFC corresponding to the first 15 NH2-terminal amino acid residues of the rat PEPT2 protein plus a cysteine added for conjugation purposes was custom made by Sigma-Genosys (Cambridge, UK). After fixation and permeabilization, the SKPT cells were incubated with anti-rPEPT2 (1:200) antibody for 2 h. The filters were rinsed twice in PBS and incubated with secondary antibody (Alexa 488-conjugated goat anti-rabbit IgG from Molecular Probes) for 90 min. Then, cells were treated with RNAse and counterstained with propidium iodide as described above. As a control, the antibody was preincubated with EGF-R blocking peptide (1:200) before the staining procedure.

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Statistics analysis. Experiments were performed using several SKPT cell passages (n = 3–4) and in duplicates or triplicates (N = 2–3) within each passage. Values are given as means ± SE. The statistical significance of the results was determined using a two-tailed
Apical Gly-Sar uptake was decreased in SKPT cells cultured in the presence of EGF. The transport capacity of rPEPT2, as estimated by the apical uptake of [14C]Gly-Sar at a Gly-Sar concentration of 25 μM, was followed over a time period of 8 days after seeding the SKPT cells (Fig. 1A). Preliminary experiments showed that there was no significant transepithelial transport of Gly-Sar over the time period studied and only a very small uptake of Gly-Sar when iso- tope and substrate were added basolaterally, all [14C]Gly-Sar uptake was therefore assumed to be apical. The cells were grown in the absence or presence of 10 ng/ml EGF in the culture media. On day 2 and throughout the remaining part of the culture period, the uptake of Gly-Sar was significantly reduced (P < 0.05, n = 3) in the presence of 10 ng/ml EGF in the culture media. In SKPT cells, without added EGF, the maximum uptake values were found on days 3-5. When SKPT cells were grown in the presence of 10 ng/ml EGF, the Gly-Sar uptake remained approximately constant on days 3-8. Subsequent experiments were performed on day 3 or 4, unless otherwise stated.

Effect of EGF on peptide uptake was maximal after 48 h of stimulation. SKPT cells were seeded without EGF added to the culture media and the effect of introducing 10 ng/ml EGF to the culture media at different time points during a 3-day culture period was investigated (Fig. 1B). Addition of EGF 24 h before measuring Gly-Sar uptake decreased the uptake compared with nontreated cells (3.44 ± 0.05 vs. 6.37 ± 0.93 pmol·cm⁻²·min⁻¹, P < 0.05, n = 3). The maximal inhibition of Gly-Sar uptake was reached when SKPT cells were treated with EGF for 48 h (1.65 ± 0.34 pmol·cm⁻²·min⁻¹) or more.

EGF in the culture media did not affect protein content of the SKPT cells. The protein content of SKPT cells cultured in the absence and presence of 10 ng/ml EGF was followed over a time period of 8 days (Fig. 1C). No significant difference was observed throughout the period studied. The protein content was 0.25 ± 0.02 mg·cm⁻² (n = 2) and 0.29 ± 0.05 mg·cm⁻² (n = 2) in SKPT cells grown for 4 days in the presence and absence of EGF supplement, respectively.

Apical uptake of Gly-Sar was linear up to at least 60 min. The apical uptake of [14C]Gly-Sar at a Gly-Sar concentration of 520 μM was linear for the time period studied both for SKPT cells grown in the absence and presence of 10 ng/ml of EGF supplement, and subsequent uptake experiments were performed using either 10- or 40-min incubation (Fig. 1D, inset).

Apical uptake of Gly-Sar was inhibited by surplus of the competing substrate Gly-Pro in both nontreated and EGF-treated monolayers. Apical Gly-Sar uptake was determined in SKPT cells cultured during 4 days in media containing 10 ng/ml EGF or in EGF-free media (Fig. 1D). The uptake of [14C]Gly-Sar at a Gly-Sar concentration of 70 μM was measured over 40 min in the absence and presence of 20 mM glycylproline (Gly-Pro), a competing substrate for rPEPT2-mediated transport. Apical solutions contained 0.5 μCi of the extracellular space marker [3H]mannitol. When SKPT cells were cultured in media containing EGF, the Gly-Sar uptake values in the absence and presence of Gly-Pro were 3.46 ± 0.22 pmol·cm⁻²·min⁻¹ (P < 0.05, n = 3), respectively. Under these culture conditions, the component of apical Gly-Sar uptake that could not be inhibited by Gly-Pro represented 13% of the total uptake (Fig. 1D). However, when the Gly-Sar present in the apical extracellular space was...
subtracted from the values, Gly-Sar uptake in the presence of 20 mM Gly-Pro was not significantly different from zero \( (n = 3) \).

When EGF was removed from the culture media, only 3% of the total Gly-Sar uptake corresponded to a noninhibitable component. The \(^{14}C\)Gly-Sar uptake at 0.07 mM was 13.66 ± 0.88 pmol·cm\(^{-2}\)·min\(^{-1}\) in the absence of 20 mM Gly-Pro and 0.39 ± 0.20 pmol·cm\(^{-2}\)·min\(^{-1}\) when 20 mM Gly-Pro were present in the donor solution \( (P < 0.05, n = 3) \). When corrections for extracellular space were performed, Gly-Sar uptake in the presence of 20 mM Gly-Pro was not significantly different from zero \( (n = 3) \).

These data show that the uptake of Gly-Sar in both nontreated and EGF-treated cells can be completely abolished by adding surplus of a competing dipeptide substrate for the peptide transporter rPEPT2, indicating that the differences in uptake of radiolabel in nontreated and EGF-treated tissues are due to differences in peptide transporter capacity and not “unspecific” uptake. The remaining component of “uptake,” measured in the presence of 20 mM Gly-Pro, was due to apical extracellular \(^{14}C\)Gly-Sar, which had not been removed during the washing of the cell monolayers.

**EGF decreased peptide uptake when applied either apically or basolaterally.** To investigate whether the observed effects of EGF on apical Gly-Sar uptake were mediated by apical or basolateral stimulation, a series of experiments was performed where media containing 10 ng/ml EGF were added to the apical, basolateral, or both sides of the cells throughout the culture period and compared with controls without EGF added to the growth media. On day 3 after seeding of the SKPT cells, Gly-Sar uptake experiments were carried out. Apical, basolateral, or bilateral application of EGF decreased apical Gly-Sar uptake to the same extent, compared with nontreated controls, indicating that the effect of EGF is mediated by receptors located on both the apical and basolateral membranes of SKPT cells (Fig. 2A).

The expression of EGF-R was studied by anti-EGF-R immunostaining using confocal laser scanning microscopy (CLSM) (Fig. 3B). SKPT cells grown for 4 days in the absence or presence of 10 ng/ml EGF were treated with primary EGF-R antibody, followed by labeling with a secondary Alexa 488-conjugated goat anti-rabbit antibody. As a control, the primary antibody was coincubated with EGF-R blocking peptide, which abolished staining (data not shown). As additional controls, omission of either primary or secondary antibody gave negative results (data not shown). The EGF-R was localized in both apical and basolateral membranes, and in vesicles just below, in both EGF-treated and untreated cells.

**EGF decreased apical Gly-Sar uptake in a dose-dependent manner.** SKPT cells were grown in the presence of varying concentrations (0–50 ng/ml) of EGF in the culture medium for 4 days. The inhibition of apical Gly-Sar uptake by EGF showed a clear dose dependency (Fig. 2B). The ED\(_{50}\) for the EGF-mediated inhibition of apical Gly-Sar uptake in SKPT cells was estimated as 0.75 ± 0.20 ng/ml \( (n = 3) \) using De Leans’ equation (Fig. 2B, inset), and the uptake at maximal inhibition was 0.51 ± 0.36 pmol·cm\(^{-2}\)·min\(^{-1}\) \( (n = 3) \), corresponding to ~8% of the uptake value obtained in cells grown in the absence of EGF in the culture media.

**EGF-induced changes in SKPT cell morphology.** The morphology of 4-day-old SKPT cells was investigated by labeling the cell nuclei with propidium iodide and actin with Alexa 488-conjugated phalloidin. The network of actin filaments underlies the plasma membrane and thereby visualizes the outline of the cells. Differences were observed between untreated cells and SKPT cells treated with 10 ng/ml EGF during 4 days of culture (Fig. 3A). The vertical scans demonstrated that both groups of cells formed a monolayer when grown onto polycarbonate filters (Fig. 3A). A significantly higher number of cells per area were found when the culture media contained EGF supplement (256 ± 24 vs. 173 ± 20 cells/20,000 \( \mu \)m\(^2\), \( P < 0.05, n = 4 \)), EGF-treated cells appeared higher than untreated SKPT cells, with a height of 20.25 ± 1.31 and 12 ± 0.41 \( \mu \)m, respectively \( (P < 0.01, n = 4) \).

**EGF treatment changed \( V_{\text{max}} \) of apical Gly-Sar uptake.** Apical uptake of \(^{14}C\)Gly-Sar was measured over a concentration range of 30–520 \( \mu \)M Gly-Sar in the apical solution in control cells and cells treated throughout the culture period with 10 ng/ml EGF (Fig. 4). The obtained values for Gly-Sar uptake were corrected for Gly-Sar present in the extracellular fluid.
fluid using [3H]mannitol as the extracellular space marker. The experimental data were fitted to the Michaelis-Menten equation, and kinetic constants were calculated. The apparent $K_m$ values for Gly-Sar were 90 ± 3 and 114 ± 8 μM for nontreated and EGF-treated SKPT cells, respectively (not significantly different, $n = 3$). These values were comparable to the value of 86 μM obtained by Brandsch et al. (3). SKPT cells grown in the traditional EGF-containing culture medium displayed a $V_{max}$ of 9.02 ± 1.16 pmol·cm⁻²·min⁻¹. This value was lower than the value of ~15 pmol·cm⁻²·min⁻¹ described in the publication by Bransch et al. (3); however, this was probably caused by differences in the experimental set-ups and in the correction methods used. A significantly higher $V_{max}$ was obtained in cells grown in the absence of EGF ($V_{max} = 34.56 ± 4.14$ pmol·cm⁻²·min⁻¹) compared with EGF-treated cells ($P < 0.001$, $n = 3$).

SKPT cells cultured in the presence of EGF showed decreased expression of rPepT2 mRNA and rPEPT2 protein in the apical membrane. rPepT2 mRNA levels were investigated in 4-day-old SKPT cell monolayers cultured in the absence or presence of 10 ng/ml EGF (Fig. 5). Whereas rPepT2 mRNA was undetectable in EGF-treated cells, a product of the ex-

Fig. 3. A: morphology of SKPT cells cultured in the absence and presence of EGF. Cell nuclei were labeled with propidium iodide (red), and actin filaments were labeled with Alexa 488-conjugated phallolidin (green). Left: horizontal section through SKPT cells. Two filters, one treated (left) and the other untreated (right), were aligned on the same coverslip. Right: vertical sections of SKPT cells grown in presence (top) and absence (bottom) of 10 ng/ml EGF throughout the culture period. B: immunolocalization of EGF receptors (xyz sections). After fixation and permeabilization, SKPT cells grown in the absence (right) and presence (left) of 10 ng/ml EGF were treated with EGF receptor antibody and labeled with a secondary Alexa 488 goat anti-rabbit antibody (green). Cell nuclei were labeled with propidium iodide (red). C: immunolocalization of rPepT2 by anti-rPepT2 immunostaining. SKPT cells grown in the absence (right) and presence (left) of 10 ng/ml EGF supplement throughout the culture period. SKPT monolayers were treated with anti-rPepT2 antibody followed by labeling with a secondary Alexa 488 goat anti-rabbit antibody (green signal). Top: projected z series through the monolayers. Bottom: vertical section of SKPT cells grown in the absence and presence of 10 ng/ml EGF. All the images were performed 4 days after the SKPT cells were seeded. Top, right: monolayers treated with anti-rPepT2 antibody and blocking peptide. All images were representative of 3–5 individual preparations, each performed on different cell passages.
REGULATION OF PEPT2 BY EGF IN A KIDNEY CELL LINE

EGF-treated cells (Fig. 3C). The mean pixel intensity of confocal stacks (maximal projections) was used as a semi-quantitative measure of immunolabeling. The pixel intensity was calculated in four different images corresponding to different filters of the same passage. In SKPT cells grown in the absence of EGF, the mean pixel intensity was 101.02 ± 15.11, whereas in SKPT cells grown in media containing 10 ng/ml EGF the mean pixel intensity was 29.77 ± 8.54 (P < 0.001, N = 4).

**EGF treatment of SKPT cells increased the apical uptake of glucose and lysine transporters.** To assess whether the effect of EGF was general on apical membrane transporters in the SKPT cells, apical uptake of glucose and lysine was measured in cells cultured for 4 days in the absence and presence of 10 ng/ml EGF (Fig. 6). The apical uptake of glucose was significantly lower in untreated SKPT cells than in EGF-treated cells (3.11 ± 0.18 vs. 5.26 ± 0.28 pmol·cm⁻²·min⁻¹, P < 0.05, n = 3; Fig. 6A). The apical uptake of lysine was significantly lower in SKPT cells grown in the absence of EGF than in cells cultured in the presence of EGF supplement (23.40 ± 0.62 vs. 35.03 ± 1.81 pmol·cm⁻²·min⁻¹, P < 0.05, n = 3; Fig. 6B). This indicates that EGF treatment during the culture time increases the uptake of glucose and lysine via transport mechanisms and that the decrease in dipeptide uptake is not caused

**Fig. 4.** Gly-Sar concentration dependence of apical uptake of [¹⁴C]Gly-Sar in SKPT cells grown in the absence of EGF (○) and presence of 10 ng/ml EGF (●) was measured over 40 min. The DPM values of Gly-Sar were corrected for Gly-Sar present in the extracellular spaces (see MATERIALS AND METHODS). The corrected data points were fitted to the Michaelis-Menten equation. Each data point represents means ± SE of 3 individual passages.

**Fig. 5.** A: RT-PCR products of rPepT2 mRNA and glucose 6-phosphate dehydrogenase in SKPT cells cultured in the absence and presence of 10 ng/ml EGF. The PCR products were separated by agarose gel electrophoresis and visualized with SYBR-Gold. Numbers to the left indicate the molecular weight (bp). B: PCR amplification products of rPepT2 mRNA using nested primers as described in MATERIALS AND METHODS. PCR products were separated by agarose gel electrophoresis and visualized with SYBR-Gold.
by a general decrease in apical membrane transport protein expression.

DISCUSSION

The present study is to our knowledge the first demonstration of EGF-mediated regulation of PEPT2. EGF was shown to downregulate rPEPT2 in the rat kidney proximal tubule cell line SKPT. EGF in a concentration of 10 ng/ml in the culture media decreased the maximal uptake capacity to ~25% of the value obtained in untreated cells without changing $K_m$, suggesting that the EGF treatment reduced the number of active peptide transporters in the apical membrane of SKPT cells. This was not due to general changes in apical membrane transport protein expression, because the uptake of glucose and lysine actually decreased in cells grown in the absence of EGF. RT-PCR and immunostaining followed by confocal visualization confirmed that rPepT2 mRNA levels and rPEPT2 expression were increased when EGF was omitted from the culture media.

Regulation of PEPT2 by EGF. Little is known about regulation of PEPT2 transport activity. Wenzel et al. (38) demonstrated that the uptake of a dipeptide, d-Phe-L-Ala, in LLC-PK1 was regulated by the cellular concentration of Ca$^{2+}$, and PKC was found to transmit this effect. Takahashi et al. (32) found that the high-affinity type H$^+$-peptide cotransport activity in rat kidney was upregulated by 5/6 nephrectomy, accompanied by the increased expression of rPEPT2. However, regulation of PEPT2 by specific hormones and growth factors has not been studied previously. EGF is synthesized in a number of cell types and is inserted in the plasma membrane as proEGF, which can be cleaved, releasing soluble EGF. EGF acts via a tyrosine kinase receptor, EGF-R (23), which initiates a series of intracellular signaling events (13, 24). EGF has been reported to have numerous effects on a number of cellular events, ranging from short-term activation of sodium/proton exchangers to elongation of villi in intestinal cells. However, our data indicate that the changes in peptide uptake capacity and rPEPT2 protein expression observed in this study are caused by changes in rPepT2 mRNA levels. EGF-R activation has been reported to affect mRNA levels of various plasma membrane proteins involved in transport. Mischoulon et al. (26) demonstrated an EGF-mediated upregulation of GLUT-1 mRNA in cultured hepatocytes, and Ishida et al. (18) observed the same effect in cultured bovine corneal endothelial cells and presented evidence that PKC was involved. Kekuda et al. (20) showed that the serotonin transporter was upregulated by EGF in JAR human placental choriocarcinoma cells. In rat alveolar epithelial cells, EGF was shown to downregulate steady-state mRNA levels of rat epithelial Na$^+$ channel subunits (21). Xu et al. (39) demonstrated that EGF downregulates the intestinal sodium-phosphate co-transporter NaPi-IIb via regulation of the NaPi-IIb gene promoter and our group demonstrated previously that EGF down-regulates hPEPT1 in the intestinal cell line Caco-2 via changes in hPepT1 mRNA (27). The signaling pathway leading from EGF-R activation to changes in PepT2 mRNA levels still remains to be elucidated but is likely to include either regulation of transcription of the PepT2 gene, possibly via an EGF-responsive element, or regulation of PepT2 mRNA stability. It will have to be investigated whether the regulation of PEPT1 and PEPT2 by EGF shares common features.

Role of EGF in kidney proximal tubule cells. The relationship between EGF and peptide transport activity in the kidney has not been investigated. Most of the EGF produced in the kidney is in the form of the membrane-bound precursor pro-EGF. Pro-EGF is situated in the apical membrane of EGF-producing cells in the thick ascending limb and in the distal tubules, and large amounts of EGF are secreted into the urine. EGF-Rs are present in the basolateral membrane of epithelial cells in the proximal tubule, the cortical and the inner medullary collecting duct (4). In normal kidneys, they appear to be predominantly immunolocalized in the proximal tubule (4, 36). Urinary EGF will therefore only come into contact with basolateral receptors when the urothelium is damaged, indicating a role in renal tissue regeneration (much similar to the suggested role of EGF in the GI tract). A number of studies therefore investigated the potential role of EGF and EGF-R activation in renal tissue under pathophysiological conditions. A general pattern observed after drug- or ischemia-induced kidney damage is a fast decrease in kidney total EGF expression and an upregulation of EGF-R mRNA and expression (14, 28). Leonard et al. (22) showed that the decrease in EGF immunoreactivity was due to an overall decrease in tissue-bound pro-EGF, probably caused by a release of EGF (16) and a decrease in EGF mRNA.
As mentioned previously, Takahashi et al. (32) reported an increase in rPEPT2 activity in rats 2 wk after 5/6 nephrectomy, a situation where EGF-R immunostaining has been reported to change (31). Given the data presented in this study, there might be a connection between the large changes in EGF and EGF-R expression observed in proximal tubule cells after nephrectomy and the observed increases in rPEPT2 activity. However, the exact spatiotemporal pattern of changes in EGF-R expression as well as basolateral levels of EGF, TGF-α, and heparin-binding EGF in the vicinity of the EGF-Rs on proximal cells would have to be investigated and correlated with PEPT2 expression in vivo, thus investigating the in vivo relevance of the present findings.

Omission of EGF from standard SKPT cell culture media is favorable for rPEPT2 expression. The SKPT cell line has been used routinely to investigate PEPT2-mediated peptide and drug transport due to its ability to form confluent monolayers expressing rPEPT2 in the apical membrane (3). The cells have traditionally been cultured using standard supplements, as well as a cocktail of receptor agonists, i.e., EGF, insulin, dexamethasone, and apotransferrin (2, 3, 10, 19, 35). As demonstrated in the present paper, removal of EGF caused a drastic increase in peptide uptake activity and rPEPT2 protein and mRNA expression in SKPT cells grown for 4 days on permeable supports. The reasons for adding the supplements are not described in the original publication (3), but this was probably done to supply the cells with adequate amounts of growth factors and stimulants necessary for the cells to reach confluence quickly. However, we previously showed that when monolayers of the intestinal cell line Caco-2 are grown in the presence of EGF, the intestinal peptide transporter hPEPT1 is downregulated, accompanied by changes in tissue morphology, indicating that Caco-2 cells were kept in a proliferative state and did not fully differentiate when grown in the presence of >2 ng/ml EGF (27). The results from the present study indicate that this could be the case for the SKPT cell line as well. With removal of EGF from the culture medium, the $V_{\text{max}}$ values of Gly-Sar uptake increased by a factor of about four, nicely followed by the expression of PEPT2, which increased by a factor of about three as judged by confocal staining. The number of cells per area decreased (indicating less growth/ cell division). Peptide uptake capacity is fairly low in SKPT cells grown in the traditional EGF-containing culture medium. This necessitates corrections to be used when peptide uptake is measured in SKPT cells, i.e., corrections for isotope still present in the apical extracellular space after washing [corrections for a “nonsaturable component” (2)]. An increased uptake, as seen in the non-EGF-treated cells, yields more tissue DPM per extracellular DPM and thereby less error introduced by the correction. Direct comparisons with peptide transport parameters obtained in previous studies are not possible, because most investigators grow SKPT cells in the bottom of dishes, compared with the permeable supports used in our study, and this might cause differences in cell behavior and PEPT2 expression. As an example, protein content values in our setup are approximately twice as high as the values originally published by Brandsch et al. (3). This is probably due to the differences in culture conditions (Brandsch et al. cultured cells in 9.62-cm$^2$ petri dishes, whereas we cultured cells on 1-cm$^2$ permeable filters) and differences in the protein assay (Brandsch et al. used the Lowry method, whereas we used the Bradford method). $V_{\text{max}}$ values of Gly-Sar uptake of SKPT cells grown in the traditional EGF-containing culture medium also differed between our study and the original characterization study: $V_{\text{max}}$ was estimated to be ~9 pmol·cm$^{-2}$·min$^{-1}$ in the present study, compared with the value of ~15 pmol·cm$^{-2}$·min$^{-1}$ described in the publication by Bransch et al. (3). However, although we cannot predict how omission of EGF from the culture media will influence SKPT cells grown under different culture conditions, we suggest that investigators working with SKPT cells reevaluate the usefulness of the traditional EGF-containing SKPT culture media.

Conclusions. We demonstrated in the present study that long-term treatment with EGF in SKPT cells causes a decrease in Gly-Sar uptake. We showed that this effect was due to a decrease in rPEPT2 protein expression caused by a decrease in rPepT2 mRNA. Further studies are necessary to elucidate cellular events linking EGF-R activation and PEPT2 expression in SKPT cells. The physiological significance of these findings also has to be investigated in native tissue.

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