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Nrf2 regulates the expression of the peptide transporter PEPT1 in the human colon carcinoma cell line Caco-2

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ABSTRACT

Background: PEPT1 is a rheogenic transport protein in the apical membrane of intestinal epithelial cells capable of transporting essentially all possible di- and tripeptides that are generated from the luminal protein breakdown. In addition, several anticancer, antimicrobial and antiviral drugs are taken up from the intestinal lumen via PEPT1 and therefore PEPT1 is a target for efficient drug delivery via prodrug approaches. Thus, understanding PEPT1 gene regulation is not only of importance for dietary adaptation but also for drug treatment.
Methods: In silico analysis of the Pept1 promoter was performed using MatInspector. Pept1 promoter constructs were generated and cotransfected with an Nrf2 expression plasmid. Caco-2 cells were stimulated with Nrf2 inducers followed by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP). Biological relevance was investigated using western blot analysis and transport activity assays.
Results: Reporter gene assays showed transcriptional activation of the Pept1 promoter in response to Nrf2 overexpression. EMSA as well as ChIP analysis validated Nrf2 binding to the ARE located closest to the start codon (Pept1-ARE1). Induction of the Nrf2 pathway resulted in increased endogenous PEPT1 protein abundance as well as transport activity. Moreover, we demonstrate that also the induction of autophagy by MG132 resulted in elevated Nrf2 binding to Pept1-ARE1 and increased PEPT1 protein expression.
Conclusion: In summary, we identified a biologically active Nrf2 binding site within the Pept1 promoter which links Pept1 to the cellular defense program activated by Nrf2.
General significance: This study identifies Pept1 as an inducible target gene of the Nrf2 pathway.

1. Introduction

PEPT1 (SLC15A1) is mainly found in the apical membrane of the intestine, but is also detectable in smaller amounts in other tissues like the uterus and thymus [27]. The high capacity/low affinity transporter mediates the uptake of a wide variety of short chain peptides consisting of two or three amino acids, derivatives thereof and peptidomimetics like β-lactam antibiotics or antiviral drugs [11]. As a transporter that has a broad substrate specificity, PEPT1 is a preferred target to improve the bioavailability of drugs and therefore numerous studies have been performed to define its substrate specificity and to design prodrugs that become substrates of PEPT1 [18,24,40]. Although mice lacking Pept1 ($Pept1^{-/-}$) do not display a strong phenotype, intestinal absorption of model compounds such as the dipeptide GlySar [16] or valacyclovir [44] was strongly reduced in these animals. This indicates that any change in the expression level of PEPT1 will impair intestinal absorption not only of dietary compounds but also of pharmacological agents. Thus, it is of great clinical and pharmacologic interest to understand which conditions or factors alter Pept1 gene expression and function in humans.

The human intestinal cell line Caco-2 has been widely used to study Pept1 membrane abundance, activity and transcriptional regulation. Diurnal rhythm and fasting periods were found to regulate activity as well as protein expression of PEPT1 [31]. In addition, hormones like insulin, leptin and epidermal growth hormone were found to alter PEPT1 [29,30,41] and a reduced activity level of PEPT1 was reported after treatment of cells with hydrogen peroxide [1]. While Cdx2 was identified as the transcription factor that mediates the changes in Pept1 expression in response to butyrate and leptin treatments [10,29], transcriptional mechanisms which translate the effects of for example epidermal growth factor or those of hydrogen peroxide are not known.

In studies using the nematode *Caenorhabditis elegans* (*C. elegans*) a promoter analysis of the *C. elegans* homolog of Pept1 identified several putative transcription factor binding sites for SKN-1, the mammalian

Abbreviations: ARE, anti-oxidant response element; NRF2, nuclear factor erythroid 2-related factor 2; NQO1, NAD(P)H quinone oxidoreductase 1; γGCS, γ-glutamate-cysteine ligase; CNC, Cap 'n' collar; LAMP1, lysosomal associated membrane protein 1; GS, Glutathione synthetase; GPx4, Glutathione peroxidase 4; GI-GPx, gastrointestinal glutathione peroxidase; GSH, glutathione

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NRF2 (nuclear factor erythroid 2-related factor 2) homolog (data not shown). Nrf2, like SKN-1 orchestrates the expression of various cytoprotective genes encoding antioxidant or detoxification enzymes like NAD(P)H quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase (γGCS) and proteasomal subunits [39]. The basic leucin zipper transcription factor Nrf2 is part of the Cap 'n' collar (CNC) family. As a heterodimer in association with small Maf proteins, Nrf2 binds to antioxidant response elements (ARE) within the promoters of its target genes [19]. In the cytosol, Nrf2 is bound to Keap1, a component of an E3 ubiquitin ligase, which targets Nrf2 for proteasomal degradation [20,21]. Thiol modifications of Keap1 catalyzed by oxidative and electrophilic agents as well as UV radiation stabilize Nrf2 and, thus, activate the pathway with a translocation of Nrf2 into the nucleus. Various natural cancer-chemopreventive agents (e.g.: resveratrol, sulforaphane and curcumin) were found to initiate Nrf2-mediated transcription in a variety of target cells [22,33,39]. Proteins regulated by Nrf2 mediated gene expression include proteins involved in synthesis of glutathione as a major cellular antioxidant. PEPT1 is known to mediate the transport of glutathione precursors, cysteine, glutamate and glycine in peptide bound form such as cysteinylglycine [11] and could therefore indirectly contribute to increased glutathione synthesis upon Nrf2 stimulation. In addition, PEPT1 was found in lysosomal vesicles [5,38] suggesting that it may be involved in export of di- and tripeptides from lysosomal protein breakdown. Therefore, activation of Nrf2 by autophagy could also promote PEPT1 expression to ensure complete recycling of protein degradation products.

That PEPT1 may be embedded into pathways that regulate cellular redox status was suggested by studies in *C. elegans* showing that a lack of Pept1 resulted in alterations in glutathione homeostasis [35] whereas a knock down of a glutathione peroxidase resulted in elevated PEPT1 protein [4]. Here we now show that these pathways in Caco-2 cells involve Nrf2 by demonstrating Nrf2 binding to the promoter of the human Pept1 gene. Moreover, resveratrol and sulforaphane treatment of cells results in increased PEPT1 abundance and transport activity.

2. Material and methods

2.1. Chemicals

Cell culture media and supplements were purchased at PAA (Austria). Nucleotides for cloning, EMSA and ChIP analysis were synthesized by Thermo-Fisher Scientific (Germany). Sulforaphane, MG132, resveratrol, saponine, ATP, coenzyme A and Tween 20 were ordered at Sigma-Aldrich (Germany). Poly(dI-dC)·Poly(dI-dC) and Protein A Sepharose were purchased at Amersham Bioscience (Germany). BSA and RNaseA were obtained from Applichem (Germany) and glucose and salmon sperm were ordered at Merck (Germany). Antibodies against Actin (sc-1615) and Nrf2 (sc-13032) were purchased at Santa Cruz Biotechnologies (USA). Anti-Lamp1 was obtained from DSHB (USA). Secondary antibodies for western blot detection were ordered at Li-Cor Biosciences and for immunocytochemistry at Jackson Immuno Research (USA). All other chemicals were obtained from Roth (Germany), if not stated differently.

2.2. Plasmid construction

2 kb of the 5′ upstream region of the Pept1 gene was amplified from genomic DNA isolated from Caco-2 cells using the primers pept1_Prom_rev and pept1_Prom_4bds (Table S1). Truncated versions of the Pept1 promoter were constructed using pept1_Prom_rev in combination with the respective forward primer. PCR products were cloned into pGL3 basic vector (Promega, Germany) using HindIII and BglII restriction enzymes (New England Biolabs, USA) resulting in reporter constructs as listed in Fig. 1A.

2.3. Cell culture

Caco-2 cells were cultured in MEM with early salts medium from PAA with 1% non-essential amino acids, 10% FBS (fetal bovine serum), gentamycin (50 mg/ml), and endomycin (0.5 ml/500 ml). Cells were seeded into 6 well plates or petri dishes and medium was exchanged every third day. HepG2 cells (human liver carcinoma cells) were kept in RPMI 1640 with 2 mM L-alanyl-L-glutamine supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin.

2.4. Transfection and reporter gene assays

HepG2 cells were seeded in 24 well plates one day prior to transfection using Lipofectamine 2000 (Invitrogen, Germany) according to the manufacturer's instructions. Cells were transfected with 0.15 μg of either empty pGL3 basic vector (Promega, Germany) or PEPT1 promoter reporter constructs. In all cases 0.4 μg of pSV (β-gal) plasmid (Promega, Germany) was co-transfected for normalization of transfection efficiency. Overexpression of Nrf2 was accomplished by co-transfection with

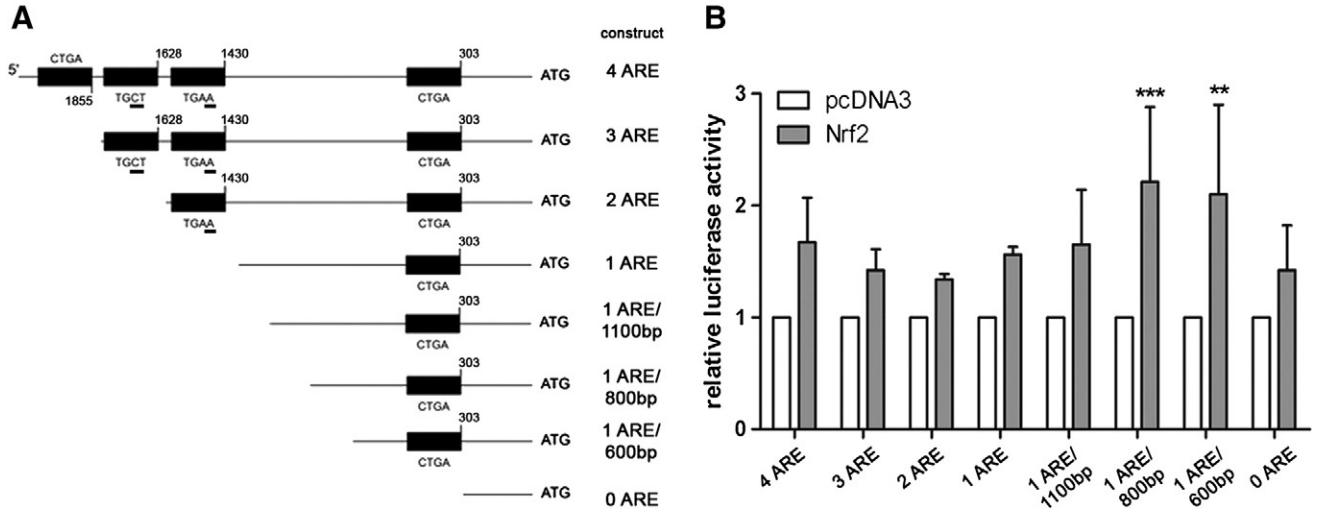

Fig. 1. Nrf2-dependent activation of the Pept1 promoter. (A) Pept1-promoter constructs of varying length were fused to the luciferase reporter gene in the pGL3basic vector. Predicted ARE sites are depicted schematically (black boxes), ARE core sequence is displayed at the according site and numbers indicate position 5′ upstream of the start codon. Mismatches to the ARE core sequence are underlined. (B) Pept1-promoter constructs were co-transfected into HepG2 cells with pcDNA3-mNrf2 or with empty pcDNA3. Relative luciferase activity of co-transfections with empty pcDNA3 was set as 1. Bars represent three independent biological experiments + standard deviation measured in triplicate. For statistical analysis student's *t*-test was applied $^{**}p < 0.01$; $^{***}p < 0.001$.

pcDNA3-mNrf2 (provided by Dr. M Yamamoto, University Tsukuba, Japan). Cells were harvested 48 h after transfection and lysed in 150 μl/well RLB-buffer under shaking for 15 min. 20 μl of lysate was placed into a white 96-well Plate. 6.2 mg of Coenzym A, 4.3 mg of Luciferin (P.J.K., Kleinbittersdorf), and 8.84 mg of ATP were dissolved in 5 ml of 100 mM Tris (pH 7.8), and 500 μl aliquots was subsequently lyophilized. One aliquot was resuspended in 3 ml of reaction buffer using as luciferin-mix. 100 μl of the luciferin-mix was added and measurement of bioluminescence was conducted immediately using a Luminoscan Ascent. To normalize for transfection efficiency β-galactosidase activity as determined by photometric measurement of conversion of O-nitrophenyl-β-D-galactopyranosid (ONPG, 4 mg/ml ONPG in 60 mM Na_2HPO_4, pH 7.5) to O-nitrophenol was measured. 50 μl of lysate and 70 μl of β-gal-buffer were placed into a 96 well Plate. 30 μl of ONPG was added to start the reaction. Absorbance was measured at a wavelength of 405 nm after 30, 60, 90, 120 and 180 min at 37 °C.

2.5. Protein extraction

Caco-2 cells were washed twice using ice-cold PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4) containing 1 mM PMSF. After addition of an appropriate volume of PBS and 1 mM PMSF, cells were scraped of the plates, transferred into 15 ml tubes and centrifuged (500 ×*g*, 5 min, 4 °C). After cells were washed, cell lysis was done in 300 μl PBS with 1 mM PMSF and by applying mechanical force using a 24′G syringe. The protein solution was cleared from cell debris (500 ×*g*, 5 min, 4 °C) and the supernatant was centrifuged for 60 min at 20,000 ×*g* at 4 °C. The cytosolic soluble fraction was collected, and the pellet containing the crude membrane protein fraction was suspended in an appropriate volume of PBS containing 1 mM PMSF using a 24′G syringe. Intestinal mucosa samples of $Nrf2^{-/-}$ and wild-type mice were kindly provided by Dr. Henryk Dreger (Charité Berlin). Tissue samples were lysed using an ultraturrax mixer in PBS containing 1 mM PMSF and the crude extract was treated as described for Caco-2 cells.

2.6. Western Blot

Protein extracts were separated on a 10% SDS-PAGE. Transfer of proteins to a nitrocellulose membrane was accomplished by wet blot technique as described by Geillinger et al. with minor modifications [14]. Incubation with the primary antibody (anti-PEPT1, customized 1:5000; anti-actin, 1:1000) was conducted at 4 °C overnight during constant shaking. After three rounds of 10 min washing with PBST (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4, 0.01% Tween 20) the secondary antibody was applied (1:10 000) for 2 h at room temperature. Detection of antibody binding was done by fluorometric analysis using an Odessey scanner. Quantitative densitometric analysis was accomplished by the use of the Odyssey software. The intensity of the target protein was normalized by adjustment to actin bands and for further analysis intensities were set into relation to the proper experimental control sample.

2.7. ^{14}C-GlySar uptake

Activity of PEPT1 was determined as described previously [4]. Briefly, after cells were washed with 1 ml of MES-Tris buffer (140 mM NaCl, 5.36 mM KCl, 1.76 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 27.5 mM Mes Puffer) at a pH of 6.0 according to [6,26] uptake was initiated by adding 20 μM ^{14}C-GlySar (^{14}C-glycyl-sarcosine) in Mes–Tris buffer. Plates were incubated for 10 min at 37 °C. The saturable transport component was determined using 10 mM GlyGly as competitor. Removal of the substrate terminated uptake. Cells were washed twice using 1 ml of cold MES-Tris buffer and disrupted in 1 ml of Igepal lysis buffer (50 mM Tris, 140 mM NaCl, 1.5 mM $MgSO_4$, 0.5% Igepal, pH 8.0). Cell lysates were mixed with 3 ml of scintillation cocktail. Radioactivity was measured by liquid scintillation spectrometry in a TriCarbCounter (Perkin Elmer) and uptake was normalized to the protein content of the sample (Bradford reagent, Biorad, Germany).

2.8. Nuclear extracts and EMSA

Caco-2 cells were grown for 7 days in 75 cm^2 tissue bottles. Resveratrol [8] (10 μM; 3 h) or MG132 [32] (25 μM; 6 h) was added into serum-free medium. Cells were washed with PBS and harvested by scraping. Each cell pellet was lysed in 1 ml of hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol [DTT], pH 7.9), containing protease and phosphatase inhibitor cocktail (Roche, Germany), by using a 25′G syringe and incubated for 10 min on ice. Cell lysates including nuclei were pelleted by centrifugation (11,000 ×*g*, 20 min, 4 °C). The supernatant was removed and a hypertonic extraction buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 1 mM DTT, 25% glycerol, pH 7.9), containing protease and phosphatase inhibitor cocktail, was added to the pellet. Nuclei were lysed for 15 min at 4 °C under rotation and nuclear extracts were centrifuged for 1 min at 11,000 ×*g*. Protein concentrations were determined by the Biorad protein assay.

A synthetic 5′-Cy5-labeled oligonucleotide probe of Nrf2 (5′-CCGA CCTCCTGAGTCAGCTGGCC GGG-3′ Pept1-ARE1) was annealed for 5 min at 85 °C. Binding reactions were performed on ice in a total volume of 10 μl containing 5 μg of nuclear extract, 1 ng of labeled DNA probe, 5× binding buffer (20% glycerol [v/v], 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris [pH 7.5]), and 450 ng of poly(dIdC) for 30 min. For supershift analysis 2 μg of anti-Nrf2 or control IgG was added 30 min prior to the addition of the labeled probe. Afterwards reaction mixtures were mixed with 10× loading buffer (250 mM TrisHCl pH 7.5, 0.2% Orange G, 40% [v/v] glycerol) and incubated for 10 min. Electrophoretic mobility shift assay (EMSA) was carried out on a native 5.3% polyacrylamide gel in 0.5× Tris-borate-EDTA for 3 h at 190 V and 4 °C. Cy5-fluorescence was analyzed by Typhoon Trio scanner (GE Health care, Bioscience) at 650 nm. For competition experiments 50-fold and 100-fold molar excess of unlabelled Pept1-ARE1 oligonucleotide were added to the reaction mixture. Additionally, a 10-fold molar excess of unlabelled mutant Pept1-ARE1 probe, containing a mutation in its core sequence, was added to the reaction mixture.

2.9. CHIP analysis

For the chromatin immunoprecipitation, Caco-2 cells were grown in 150 cm^2 tissue bottles for 4 days before 10 μM of the Nrf2 inducer sulforaphane was added for 4 h in serum-free medium. The protocol was used as described [2] with minor modifications. Very briefly, cells were fixed in 1% formaldehyde and cross-linking stopped by adding 125 mM glycine. Cells were lysed in 1.5 ml of lysis buffer (10 mM HEPES, 1 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.35 M sucrose, 0.5 mM DTT, pH 7.9) containing protease inhibitor cocktail (PIC) and PhosphoSTOP (Roche, Germany). After centrifugation (15 min, 4000 ×*g*, 4 °C) cell pellet was incubated in extraction buffer (20 mM HEPES, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, pH 7.9) containing PIC and PhosphoSTOP. DNA fragments (200 to 1000 bp) were harvested using ultrasonification (2 × 30 s at amplitude 100% and cycle 1.0). After centrifugation (18,000 ×*g*, 10 min, 4 °C) 50 μl input in RIPA buffer (50 mM TrisHCl, 150 mM NaCl, 0.25 mM EDTA, 1.0% Triton X-100, 0.1% sodium deoxycholate, pH 8.1) was removed. Protein A sepharose slurry, pre-blocked with sonicated salmon sperm DNA and BSA, was added to the solution and samples (1:10 in RIPA buffer) were pre-cleared. Half of each sample was incubated with or without 3 μg of specific Nrf2 (H-300) antibody (sc-13032; Santa Cruz Biotechnology, USA) overnight at 4 °C. Immunoprecipitation was done using 25 μl of the A-Sepharose slurry (2 h, 4 °C). After washing, protein/DNA complexes were eluted in 150 μl of 0.1 M $NaHCO_3$ and 1%

sodium dodecyl sulfate (SDS). Cross-linking was reversed using 0.3 M NaCl and 20 μg RNase A for 4 h at 65 °C. Proteins were digested using 100 μl proteinase K (Roche, Germany) (40 mM TrisHCl, 10 mM EDTA, 1 h, 55 °C). After phenol/chloroform extraction and DNA precipitation using ethanol DNA pellet was re-dissolved in 30 μl of water. For PCR, a 200 bp region of the GI-GPx promoter, a known Nrf2 target gene served as the positive control [2]. Primers spanning a 200 bp region of the PEPT1 promoter containing the ARE 1 were used. PCR was conducted in 25 μl reaction mixtures containing 50 pmol of each primer, 200 μM of each dNTP, 2.5 units/reaction Taq DNA polymerase (Qiagen, Germany), 2.5 μl of 10× reaction buffer and 1 μl DNA sample. Initial denaturation step (5 min, 95 °C) was followed by 40 cycles (GI-GP) or 35 cycles (PEPT1) of 45 s denaturation at 94 °C, 45 s at 65.8 °C (GI-GPx) or 66 °C (PEPT1), and 2 min at 72 °C, completed by 10 min at 72 °C. PCR products were separated on a 1.5% agarose gel.

2.10. Immunocytochemistry

Caco-2 cells were seeded into 12-well plates. At 6 days postconfluency, the medium was removed and the wells were washed with PBS. Fixation was conducted using 4% PFA for 15 min following permeabilization with 0.5% saponin for another 15 min. Accordingly, cells were blocked with 2% BSA for 1 h. Primary antibodies (anti-PEPT1 customized, anti-LAMP1purchased from DSHB) were diluted 1:500 in PBS. Cells were incubated with the primary antibody overnight at 4 °C. After 3 × 5 min of washing with PBS the cells were incubated for 1 h at room temperature with the secondary antibody, which was diluted 1:250 in PBS. Microscopy was performed using a Leica epifluorescence microscope equipped with a Leica EL6000 light source, computer, and laser scanning microscope image analysis software.

2.11. Statistical analysis

Statistical analysis was conducted using GraphPad Prism 4.0 (USA). If not stated otherwise student's *t*-test was applied and a p-value < 0.05 regarded as significant.

3. Results

3.1. The human Pept1 promoter contains four potential Nrf2 binding sites

Since the *pept-1* promoter region of *C. elegans* displayed three potential SKN-1 binding sites, the human PEPT1 promoter was analyzed using Matinspector (Genomatix) for the presence of putative antioxidant response elements (ARE). Indeed, 4 AREs were found, all located in the reverse direction starting at −303 bp (Pept1-ARE1), −1430 bp (Pept1-ARE2), −1628 bp (Pept1-ARE3) or −1855 bp (Pept1-ARE4), respectively (Fig. 1A). Pept1-ARE1 and ARE4 displayed a perfect ARE core sequence (TGAC), while ARE2 had one and ARE3 two mismatches in the ARE core sequence (Fig. 1A). To elucidate their potential biological relevance, 2 kb of the human PEPT1 promoter were cloned in front of a luciferase reporter gene. In addition, shorter promoter constructs were generated to analyze the functional role of the four putative ARE sites in response to Nrf2 co-transfection. First, the constructs 4 ARE, 3 ARE, 2 ARE, 1 ARE, and 0 ARE were co-transfected with empty pcDNA3 or Nrf2 expression vector. This resulted in a small but not significant increase in luciferase activity upon Nrf2 co-transfection that was also detectable in the 0 ARE construct without a putative Nrf2 binding site. Shimakura et al. [34] described a strong repressor element located between −1430 bp and −308 bp within the Pept1 promoter. Therefore, additional constructs containing Pept1-ARE1 were generated that had a length of 1100 bp, 800 bp or 600 bp (Fig. 1A). Only the constructs of 800 and 600 bp lengths showed a significant response to Nrf2 overexpression (Fig. 1B). In accordance with Shimakura et al., this indicates that a repressor element is located between −1100 and −800 bp of the Pept1 promoter that interferes with the Nrf2-dependent activation. In addition, these results provide evidence for a functional ARE within the Pept1 promoter that is essential for its activation in response to Nrf2. To further prove this, Nrf2 binding to the Pept1-ARE1 was analyzed using electrophoretic mobility shift assay (EMSA), as well as chromatin immunoprecipitation (ChIP).

3.2. EMSA and ChIP confirm binding of Nrf2 to the Pept1-ARE1 site

To investigate the direct interaction of Nrf2 with the ARE1 located in the Pept1 promoter, oligonucleotides were synthesized containing the Pept1-ARE1 fused to the fluorophore Cy3. Oligonucleotides were tested using nuclear extracts of Caco-2 cells collected 6 days after confluency. To evaluate specific shifts, unlabeled Pept1-ARE1 oligonucleotides were used in increasing concentrations (Fig. 2A). In addition, mutated unlabeled Pept1-ARE1 oligonucleotides were used in competition assays. While increasing the concentration of unlabeled Pept1-ARE1 resulted in the decrease of the Nrf2 complex, mutated oligonucleotides did not affect the Nrf2 complex. Supershift analysis using specific anti-Nrf2 antibody showed in contrast to control IgG a decrease of the Nrf2 complex (Fig. 2B). Binding of the antibody to the transcription factor can interfere with its nucleotide binding capacity [7], which might explain the reduction in the signal of the Nrf2 complex and the absence of a retarded Nrf2 complex. We next exposed Caco-2 cells to resveratrol, considered to be a prototypical Nrf2 activator. Treatment of Caco-2 cells with 10 μM of resveratrol prior to nuclear extraction, showed an increase in DNA binding to the Pept1-ARE1 (Fig. 2C).

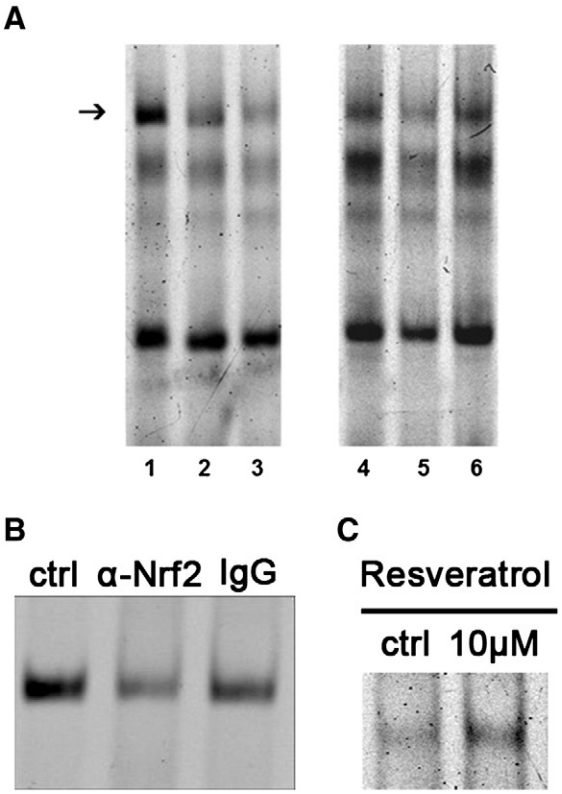

Fig. 2. Binding of Nrf2 to Pept1-ARE1 is increased in response to Nrf2 inducers. (A) Nuclear extracts of Caco-2 cells were incubated with Pept1-ARE1-Cy3 probe. Competition experiments were carried out with 50-fold (lane 2) and 100-fold (lane 3) molar excess of unlabeled Pept1-ARE1 probe compared to control with only Pept1-ARE1-Cy3 probe (lane 1). Effect of the unspecific mutant probe was assessed by using 10-fold molar excess (lane 6) compared to control (lane 4) and 10-fold molar excess of unlabeled Pept1-ARE1 probe (lane 5). (B) Nuclear extracts were incubated with or without 2 μg of anti-Nrf2 antibody or control IgG prior to addition of labeled probe. (C) Nuclear extracts of Caco-2 cells were prepared after incubation of cells with 10 μM of resveratrol for 3 h or corresponding solvent control. Results are representative of three independent experiments.

To assess endogenous binding of Nrf2 to the Pept1-ARE1 sequence ChIP analysis was performed. Caco-2 cells were treated with 10 μM of sulforaphane for 4 h and chromatin was precipitated using the Nrf2 specific antibody. Subsequent PCR was conducted using primers spanning either Pept1-ARE1 or GI-GPx ARE, an already known Nrf2 target [2]. ChIP analysis using Caco-2 cells exposed to 10 μM of sulforaphane confirmed the results obtained by EMSA (Fig. 3). Pept1-ARE1 showed increased Nrf2 binding in response to sulforaphane treatment comparable with the positive control GI-GPx [2], while input signals remained constant. In summary, Nrf2 binds to the Pept1-ARE1 in the basal state and to a greater extent after activation using sulforaphane as shown by ChIP or resveratrol as shown by EMSA.

3.3. PEPT1 protein expression as well as its activity in response to Nrf2 activation

Results of EMSA and ChIP analysis showed that Nrf2 binds to the Pept1-ARE1 sequence in the Pept1 promoter. To test the relevance of Nrf2 for endogenous Pept1 regulation, PEPT1 protein expression and transport activity were determined after treatment of Caco-2 cells with resveratrol or sulforaphane. Incubation of Caco2 cells with sulforaphane resulted in significantly increased nuclear Nrf2 localization (Fig. 4A), while resveratrol only tended to enhance nuclear Nrf2. Nevertheless, sulforaphane and resveratrol were able to increase PEPT1 protein levels (Fig. 4B). In accordance with the results obtained by EMSA, treatment of Caco-2 cells with resveratrol induced a significant increase in PEPT1 protein whereas induction of Nrf2 using sulforaphane increased PEPT1 protein levels only marginally. Transport activity determined with ^{14}C-GlySar as a substrate was increased by both compounds but effects of resveratrol were slightly stronger than those of sulforaphane (Fig. 4C). When analyzing tissues obtained from animals lacking Nrf2, however, we demonstrated that PEPT1 protein abundance in the brush border membrane of the small intestine remains unchanged (Fig. 4D). This suggests that Nrf2 does not participate in expression control of Pept1 under basal conditions but may play a critical role in response to a stressor. Alteheld et al. [1] showed that treating Caco-2 cells with hydrogen peroxide – another Nrf2 modulator – reduced PEPT1 protein status and activity. When we treated our Caco-2 cell with 1 mM hydrogen peroxide we similarly recorded decreased PEPT1 protein levels (data not shown). Based on these findings we reasoned that no alterations in the redox state per se may affect PEPT1 expression levels and therefore tested next activators of Nrf2 which alter protein degradation processes rather than the redox potential of the cell.

3.4. Induction of autophagy enhances Nrf2 binding to Pept1-ARE1 and PEPT1 protein

Nrf2 is not only activated by changes in the redox state but also by ER stress and autophagy. Resveratrol as well as sulforaphane have been shown to also stimulate autophagic processes. Sulforaphane induces autophagy by increasing expression and association of LC3 with autophagosomes [15] whereas resveratrol enhances autophagy in a SIRT1 dependent manner by acting on Atg proteins as well as on LC3 which are essential for the maturation of the autophagosome [25]. We tested the proteasomal inhibitor MG132, which is known to be a strong autophagy stimulant [9] and exposed Caco-2 cells for 6 h to 25 μM of MG132. This resulted in an increased nuclear Nrf2 abundance (Fig. 5A) and elevated density of the Nrf2 complex associated with the Pept1-ARE1 (Fig. 5B). In line with an increased binding of Nrf2 to the Pept1-ARE1, protein abundance increased also in cells treated with 1 μM of MG132 for 24 h (Fig. 5C). However, MG132 failed to change PEPT1 transport activity (Fig. 5D). Since PEPT1 was previously described to be found in lysosomal membranes of the pancreas as well as liver [5,38] we speculated that induction of autophagy could increase lysosomal PEPT1 protein abundance and not that in the apical membrane. PEPT1 in the lysosomal membrane could similarly transport di- and tripeptides produced by the intralysosomal protein breakdown in a proton-dependent manner into the cytosol. To elucidate a possible localization of PEPT1 in lysosomal vesicles of Caco-2 cells, a co-staining of PEPT1 and the lysosomal associated membrane protein LAMP1 was performed in untreated cells or cells exposed to MG132. After fixation,

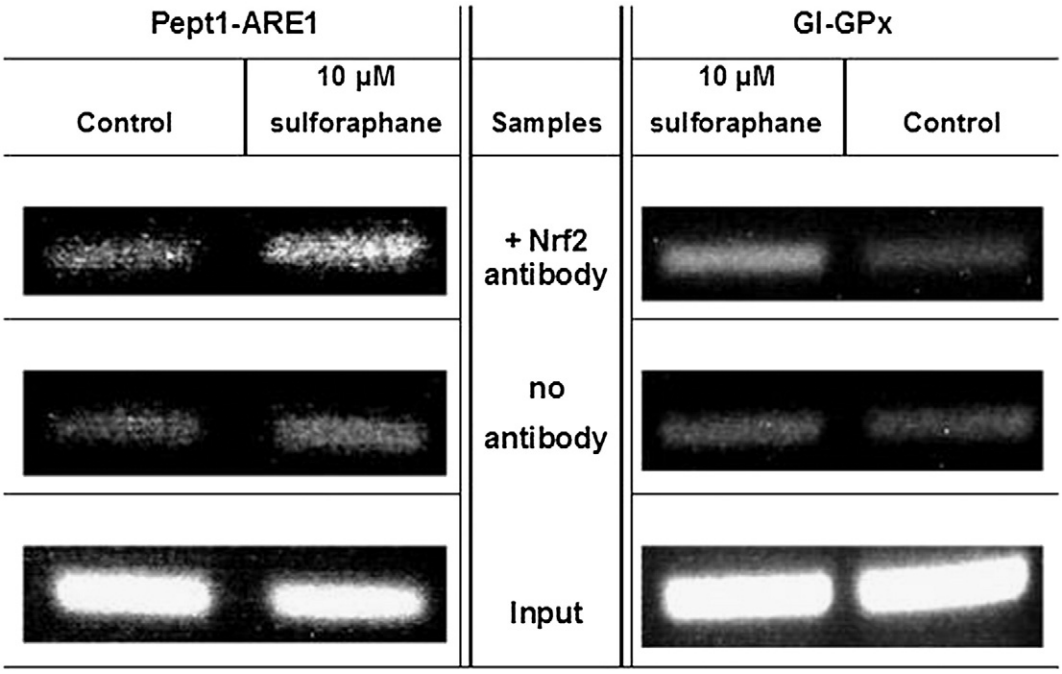

Fig. 3. Nrf2 binding to the PEPT1 promoter in response to sulforaphane analyzed by ChIP. Caco-2 cells were grown for four days postconfluent before stimulation with 10 μM of sulforaphane or solvent control for 4 h. DNA–protein complexes from fixed cells were immunoprecipitated by Nrf2 antibody and cleared DNA was amplified by using control GI-GPx and Pept1-ARE1 primers. Input samples were used as a control for the cell amount used of un-/stimulated cells. Results are representative of three biological replicates.

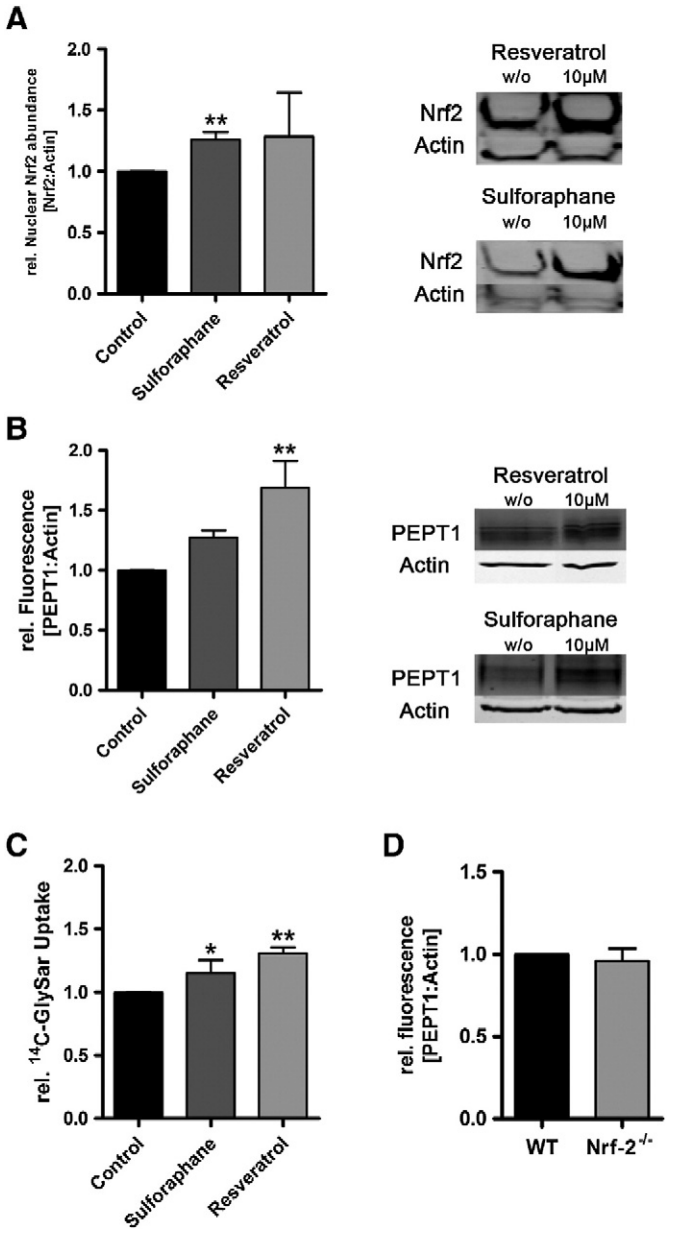

Fig. 4. Activation of Nrf2 results in increased endogenous PEPT1 protein abundance and function. (A) Caco-2 cells, 7 days postconfluent, were treated with 10 μM of resveratrol, sulforaphane or respective solvent control. Nuclear extracts were analyzed for Nrf2 abundance using Western Blot. (B) Caco-2 cells, 7 days postconfluent, were incubated for 24 h with 10 μM of resveratrol, sulforaphane or respective solvent control. Crude membrane fraction was analyzed using Western Blot. Protein abundance was normalized to actin. Control values were set as 1. (C) Caco-2 cells were treated with 10 μM of resveratrol, sulforaphane or respective solvent control for 24 h. PEPT1 activity was determined using ^{14}C-GlySar. Uptake was normalized to protein content and set in relation to respective solvent controls. Values are means of at least two biological replicates ± standard deviation, for statistical analysis student's *t*-test was conducted, *$p < 0.05$; **$p < 0.01$. (D) Membrane proteins of small intestinal mucosa of wild-type and Nrf2$^{-/-}$ mice were isolated and PEPT1 expression was analyzed by Western blot. Values represent means of five mice ± standard deviation.

cells were stained using DAPI (nuclear staining), anti-LAMP1 as well as anti-PEPT1 primary antibodies. Treatment with MG132 increased punctual structures of LAMP1 (red staining) in Caco-2 cells, indicating enhanced autophagy. Co-localization of PEPT1 (green staining) and LAMP1 indicated by yellow staining was visible only in some cells treated with MG132 but was completely undetectable in control cells (Fig. 5E). These preliminary findings may be taken as an indication that PEPT1 in Caco-2 cells might also reside in lysosomal vesicles but only after induction of autophagy.

4. Discussion

We here report the identification of a functional ARE in the Pept1 promoter using reporter gene assays, EMSA and ChIP. However, basal promoter activity was not reduced upon loss of the functional ARE (data not shown) and moreover, PEPT1 levels were not changed in intestinal samples of mice that lacked Nrf2. We therefore reasoned that Nrf2 does not contribute to basal expression of Pept1 but is essential for its up-regulation in response to Nrf2-activating stimuli. And indeed, Nrf2 activators like resveratrol and sulforaphane were able to increase binding of Nrf2 to the Pept1-ARE1 and this was associated with an increased PEPT1 protein level and increased ^{14}C-GlySar transport.

An increased apical density of PEPT1 after stimulation of Nrf2 using sulforaphane or resveratrol provides an enhanced capacity for the uptake of amino acids in peptide bound form, amongst them cysteine, glutamate and glycine. These amino acids serve as precursors for glutathione (GSH) biosynthesis and especially cysteine availability is thought to regulate de novo GSH synthesis [3]. Catalysis of γ-glutamylcysteine by γ-GCS is the rate limiting step which is followed by the addition of glycine to the γ-glutamylcysteine catalyzed by GSH synthetase (GS). Glutathione itself acts as antioxidant and serves as a substrate for various antioxidant enzymes, like glutathione peroxidases. Hence, up-regulation of Pept1 might indirectly contribute to the antioxidant defense system by providing precursors for glutathione synthesis. Interestingly, we previously reported glutathione homeostasis to be altered upon a loss of Pept1 in *C. elegans* [35] linking Pept1 to the control of the cellular redox status. In addition, siRNA-mediated down-regulation of glutathione peroxidase 4 (GPx4) resulted in up-regulated Pept1 expression in Caco-2 cells [4]. Together with Pept1, expression of Nqo1, a well-known target gene of Nrf2, was increased upon GPx4 silencing (data not shown). Thus, it is likely that the Nrf2/Keap1 pathway is involved in GPx4-mediated Pept1 induction, linking the cellular antioxidant defense system to the transport of di- and tripeptides in intestinal epithelial cells. Thus, loss of Pept1 might modulate the susceptibility towards oxidative damage.

However, increased oxidative stress after treatment of cells with H_2O_2, which is known to activate Nrf2, was reported to inhibit rather than increase PEPT1 protein and function [1]. Time-dependent resolution of the Nrf2/Keap1 activation after hydrogen peroxide treatment showed that oxidation of Keap1 was maximally after 5 min and already returned to levels comparable to controls after 40 min [12]. Due to the short time of Keap1 oxidation, Nrf2 was only activated marginally for up to 20 min. The authors related the limited effects of hydrogen peroxide to the very short-lived nature of the stressor. Thus, these results of Alteheld et al. are not per se contradictory to the effects we observed after prolonged activation of Nrf2, but raise the question of whether membrane or DNA damage in cells treated with hydrogen peroxide which occur even at lower concentrations [42] are responsible for the decline in PEPT1 protein.

Recently, an alternative pathway for Nrf2 activation was identified. The selective autophagy substrate carrier p62 was found to compete with Nrf2 for binding of Keap1, thus, activating Nrf2-driven gene expression by accelerating the degradation of Keap1 [23,36]. The Nrf2 pathway is activated via this alternative route without any redox modification of Keap1. This could be an additional explanation why Pept1 expression was not enhanced by H_2O_2 treatment while it was enhanced by resveratrol and sulforaphane and both compounds were previously shown to stimulate autophagic processes besides acting as Nrf2 activator [15,25]. We demonstrated that MG132 which is known to induce autophagy [13] increased binding of Nrf2 to Pept1-ARE1 and elevated PEPT1 protein abundance although transport activity remained unaffected. Autophagy is a process in which large-scale lysosomal protein degradation during starvation is used to recover free amino acids. Starvation was also shown to induce PEPT1 in animal studies [17,28,37] and Thamotharan et al. as well as Bockman et al. identified PEPT1 in the membranes of lysosomes of pancreas and liver tissues [5,38]. We observed increased protein levels of PEPT1 but not increased apical uptake activity when autophagy was

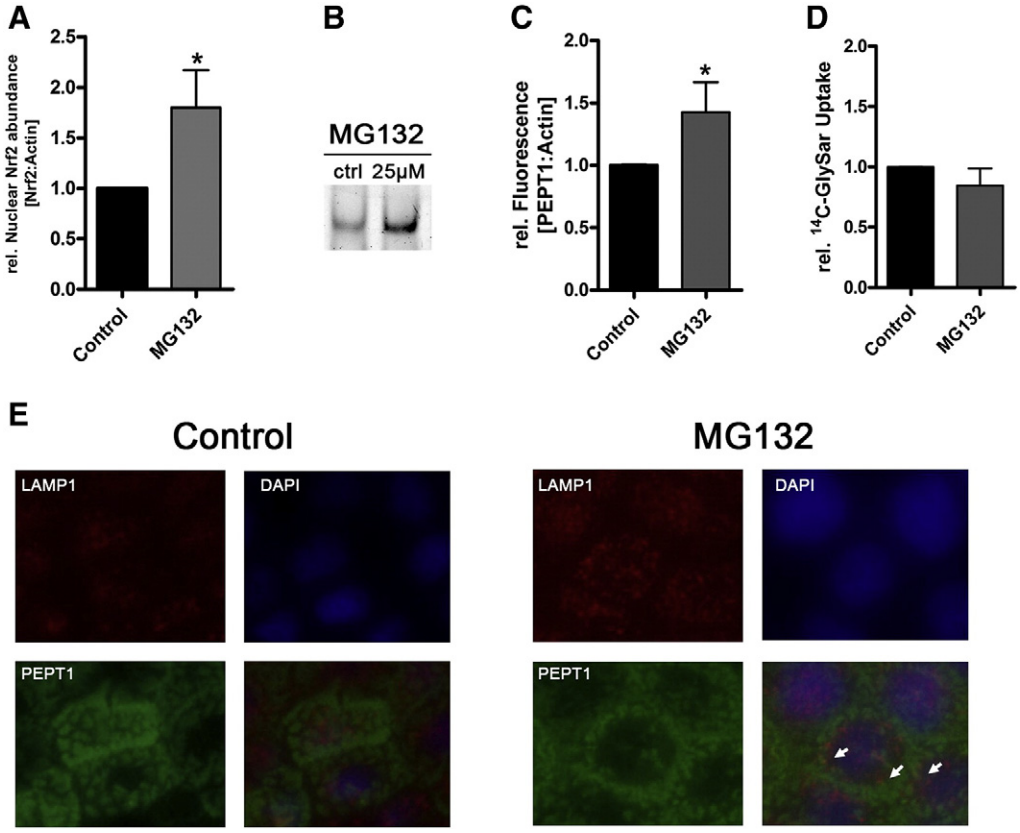

Fig. 5. Induction of autophagy increases PEPT1 protein. (A) Caco-2 cells, 7 days postconfluent, were treated with 25 μM of MG132 or respective solvent control for 6 h. Nuclear extracts were analyzed for Nrf2 abundance using Western Blot. (B) Representative results of EMSA analysis using nuclear extracts of Caco-2 cells prepared after incubation of cells with 25 μM of MG132 for 6 h or corresponding solvent controls. Results are representative of three individual experiments. (C + D) Caco-2 cells were treated with 1 μM of MG132 for 24 h. PEPT1 protein (C) was determined using Western Blot analysis. PEPT1 function (D) was determined using ^{14}C-GlySar. Values represent three independent replicates ± standard deviation. For statistical analysis student's *t*-test was applied, $^{*}p < 0.05$. (E) Immunocytochemistry of Caco-2 cells treated with 1 μM of MG132 for 24 h. PEPT1 (green), LAMP1 (red) as well as the nucleus (DAPI), were visualized using fluorescence microscopy. Arrows indicate co-localization of PEPT1 and LAMP1. Results are representative of three individual experiments.

induced, suggesting that more protein is located in lysosomes with a change in intracellular localization of PEPT1. We therefore tested whether PEPT1 can be found in lysosomal membranes in Caco-2 cells treated with MG132 and found that PEPT1 co-localized with LAMP-1 positive membranes in individual cells but we never found this in untreated Caco-2 cells. Lysosomal PEPT1 exports di- and tripeptides originating from the degradation of proteins via autophagy. Increased PEPT1 levels in response to elevated protein degradation by autophagy might ensure a complete and efficient transport of di-and tripeptides from the lysosomes to the cytosol. The low pH in lysosomes established by a proton-ATPase allows proton-coupled peptide export in analogy to proton-coupled influx across the plasma membrane. Since unspecific dipeptidases are missing in lysosomes [43], export of short chain peptides into cytosol followed by hydrolysis in cytosol which contains various dipeptidases with high activity generates the free amino acids needed.

Taken together, we provide evidence that PEPT1 is a subject to regulation by Nrf2 in intestinal Caco-2 cells that involves an ARE domain in the Pept1 promoter. This however seems to play a role only when cells are submitted to stress and in particular in response to signals that promote autophagy. Protein degradation in lysosomes during autophagy requires an increased export of short chain peptides into the cytosol for final degradation and this export pathway seems to involve PEPT1.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.12.026.

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