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The selenoproteins GPx2, TrxR2 and TrxR3 are regulated by Wnt signalling in the intestinal epithelium

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ABSTRACT

Background: The glutathione peroxidase 2 (GPx2) is expressed at crypt bases of the intestinal epithelium and in tumour tissue. The GPx2 promoter is activated by the Wnt pathway, which might be the reason for the specific expression pattern of GPx2. Together with additional selenoproteins, thioredoxin reductases TrxR2 and TrxR3, which are putative Wnt targets based on microarray analysis, Wnt-dependent GPx2 expression was analysed.
Methods: Two cell culture models for either an activated (3T3 cells with Wnt3a overexpression) or an inhibited Wnt pathway (HT-29 APC cells) were analysed. To provide physiological relevance, crypt base epithelial cells of the jejunum and colon of mice were compared to cells of the villus or crypt table, respectively. In addition, β-catenin was deleted in crypt base cells ex vivo.
Results: In cancer cell lines, the endogenous expression of all three selenoproteins was consistently dependent on Wnt pathway activity. Expression was higher in the proliferative crypt compartment, where also the Wnt pathway is active. An inducible knockout of β-catenin in isolated colonic crypt base cells reduced basal GPx2 expression. We, thus, demonstrated the regulation of GPx2 expression by the Wnt pathway in vitro and in vivo. Furthermore, the selenoproteins TrxR2 and TrxR3 have been identified as novel Wnt targets. This may imply a role of GPx2, TrxR2 and TrxR3 in proliferation, apoptosis and, therefore, also during cancer development.
General significance: Selenium which is essential for the biosynthesis of Wnt-dependent selenoproteins might be important for the renewal of the intestinal epithelium and during carcinogenesis.

1. Introduction

The continuously renewing intestinal epithelium is an appropriate model system to study characteristics of epithelial stem cells, and to compare proliferating and differentiated epithelial cells under physiological conditions. The stem cell compartment is strictly localised at crypt bases, while in the mid crypt region progenitor cells start to differentiate into distinct epithelial cell lineages. The Wnt pathway is the main regulator of the continuous renewal of the intestinal epithelium and is active in the stem cell compartment at crypt bases [1]. The key player in this pathway is β-catenin, which upon activation translocates into the nucleus and induces the expression of its target genes by interacting with proteins of the TCF/LEF family (Fig. S1). In the cytosol, β-catenin levels are regulated via a glycogen synthase kinase 3β (Gsk3β)-dependent degradation. Bound to a multiprotein complex composed of APC (adenomatous polyposis coli), axin, and Gsk3β, β-catenin becomes phosphorylated, ubiquitinated and degraded via the proteasome. Accordingly, this complex is called destruction complex. Dishevelled (Dvl) is required for Wnt-dependent inhibition of this process resulting in the stabilisation and nuclear accumulation of β-catenin. Dvl is activated upon binding of a Wnt-ligand to a frizzled family receptor and its co-receptor low density lipoprotein receptor-related protein 5 and 6 (Lrp5/6) by a yet unknown mechanism (reviewed in [2,3]). The destabilisation of the destruction complex appears to involve Lrp–Dvl complex formation and degradation of axin [4]. In more than 90% of human colon cancer cases the Wnt pathway is constitutively activated [5,6] by mutations e.g. in the APC or β-catenin gene which are early events in the crypt stem cell compartment [7] leading to unrestricted proliferation and reduced differentiation of stem cells.

GPx2, the gastrointestinal glutathione peroxidase, is a member of the glutathione peroxidase family [8]. All glutathione peroxidases from which five are selenoproteins in humans are able to reduce hydroperoxides but differ in their substrate specificity and localisation [9]. In healthy organisms, GPx2 is mainly expressed in the epithelium lining the gastrointestinal tract [10]. A GPx2 knockout highly increases the number of apoptotic cells at crypt bases [11], where GPx2 is preferentially expressed indicating that GPx2 might be

Abbreviations: GPx, glutathione peroxidase; TrxR, thioredoxin reductase; Gsk3β, glycogen synthase kinase 3β; APC, adenomatous polyposis coli; Dvl, dishevelled; Lgr5, leucine-rich repeat containing G protein-coupled receptor 5; Lrp, low density lipoprotein receptor-related protein; dnTCF4, dominant-negative TCF4; TGR, thioredoxin glutathione reductase; IFN, interferon; PCNA, proliferating cell nuclear antigen; Fabp2, intestinal fatty acid binding protein 2; RAR, retinoic acid receptor

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needed to maintain the functional state of proliferating cells. A possible link between GPx2 and the Wnt pathway was first observed by van de Wetering et al. [12], who inhibited the Wnt pathway in different cancer cell lines by overexpression of dominant-negative TCF4 (dnTCF4). dnTCF4 turns proliferating cells into differentiating cells and, by doing so, also down-regulates GPx2 expression as analysed by a microarray approach [12]. Based on this finding, a functional TCF binding element was identified in the GPx2 promoter, which responded to Wnt signals [13]. Thus, GPx2 might be a novel target of the Wnt pathway.

Apart from glutathione peroxidases the family of thioredoxin reductases belongs to the best studied selenoproteins. The three mammalian isoforms are all characterised as flavoprotein oxidoreductases required for normal thioredoxin function, but also for maintenance of reduced forms of several other thiol substrates (reviewed in [14]). While TrxR1 is ubiquitously expressed and mainly localised in the cytosol, TrxR2 is predominantly a mitochondrial protein. TrxR3 is highly expressed in maturing spermatids [15]. It does not only contain the thioredoxin motif but also a glutaredoxin domain and is, therefore, also called thioredoxin glutathione reductase (TGR). TrxR1 and TrxR2 are essential for development, since both knockout mice are embryonically lethal [16,17]. A TrxR3 knockout mouse model so far does not exist. All three thioredoxin reductases are expressed in the intestine at least at the mRNA level, which is relatively unaffected by marginal selenium deficiency and in the case of TrxR2 and 3 rather increased when selenium becomes limited [18]. A possible link between thioredoxin reductases, namely TrxR2 and TrxR3, and the Wnt pathway was first shown by microarray analysis studying gene expression upon a conditional knockout of APC, c-Myc or both in the intestine [19], where TrxR2 and TrxR3 mRNAs were up-regulated. Until now, transcriptional regulation of these two enzymes has not been investigated.

The aim of the present study, therefore, was to analyse the Wnt-dependent regulation of selenoproteins that are putative Wnt target genes. To this end promoters of TrxR2 and TrxR3 were analysed for putative binding sites. Furthermore, Wnt-dependent endogenous expression of TrxR2, TrxR3 and GPx2 was confirmed in vitro as well as in vivo.

2. Materials and methods

2.1. Cell culture

Cells were grown in a 5% CO_2 atmosphere in medium containing 10% heat-inactivated fetal calf serum (FCS; Biochrom, Berlin, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco, Karlsruhe, Germany). HepG2 cells (human liver carcinoma cells; ATCC HB8065) were cultured in RPMI 1640 with 2 mM L-alanyl-L-glutamine, NIH-3T3 cells (murine embryonic fibroblasts; ACC 59) in DMEM (high glucose) with 2 mM L-alanyl-L-glutamine and HT-29 cells (human colon adenocarcinoma cells, ACC 299) in DMEM (high glucose) with 1% nonessential amino acids. NIH-3T3 Wnt3a overexpressing cells were a kind gift of Rolf Kemler and HT-29 APC cells were provided by Bert Vogelstein. For selecting stably transfected cells, NIH-3T3 Wnt3a cells were supplied with 400 μg/ml Geneticin (Calbiochem, Bad Soden, Germany) and HT-29 APC cells with 600 μg/ml Hygromycin B (Roth, Karlsruhe, Germany). Cells were cultured with a selenium-poor FCS (Biochrom) [20]. For selenium repletion they were supplied with 50 nM sodium selenite (Sigma, Taufkirchen, Germany).

2.2. Animal experiments

Animal experiments and husbandry were carried out in accordance with the guidelines from Federation of European Laboratory Animal Science Association (FELASA). All animals were housed under specific pathogen free (SPF) conditions with a 12 h dark light cycle and free access to food and water. For studies on mice adjusted to a specific selenium status, male C57BL/6J mice (reproduced by in-house breeding) were randomly assigned to a selenium-poor or a selenium adequate diet group directly after weaning. The selenium-poor diet based on torula yeast (no. C1045, with 50% carbohydrates, 17% protein, 5% fat, 4% fibre, and a mixture of micronutrients; Altromin, Lage, Germany) contained 0.086 mg Se/kg diet and was filled up with selenomethionine (Acros, Geel, Belgium) to a selenium content of 0.15 mg Se/kg that meets the recommendations for mice [21]. The experimental diets were fed as pellets for 16 weeks.

Mx-1 Cre β-catenin$^{Wbm/flox}$ mice were generated by in-house breeding of Mx-1 Cre mice [22] with β-catenin$^{Wbm/flox}$ mice [23]. Both strains had a C57BL/6J background. These mice were fed a standard chow (V1534-3, with 58% carbohydrates, 33% protein, and 9% fat, and a mixture of micronutrients; Ssniff, Soest, Germany) with a selenium content of 0.36 mg/kg diet for at least 8 weeks. Afterwards the animals were anesthetised with isofluran, blood was taken by cardiac puncture and the animals were killed by cervical dislocation. To induce loss of β-catenin, intestinal epithelial cells of the crypt base (see isolation procedure below) were cultured ex vivo in DMEM with 5 U/ml γ-interferon (IFN) for 24 or 48 h for RNA or protein analysis, respectively.

2.3. Isolation of intestinal epithelial cells along the crypt–villus axis

The distal colon and distal jejunum were cut into sections of approximately 2 cm length and perfused first with ice cold PBS (140 mM NaCl, 10 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.4) and then with ice cold PBS containing 1 mM DTT. The sections were inverted, wrapped up on a bent piece of wire and placed in 12-well cell culture plates containing ice cold PBS. After an incubation in citrate buffer (96 mM NaCl, 1.5 mM KCl, 27 mM sodium citrate, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, pH 7.3) for 15 min at 37 °C, sections were shifted into new 12 well plates with PBS containing 1.5 mM EDTA, 1 mg/ml BSA and either 0.5 mM DTT (jejunum) or 1, 5 or 10 mM DTT depending on the fractionation step (colon). Incubation in this buffer was performed at 37 °C while shaking for defined periods of time. The detached cells were collected and washed twice in ice cold PBS. Fractions representative for villus or crypt table of the jejunum or colon, respectively, or the crypt base of both intestinal sections were proceeded for further analyses. The above described procedure was modified after [24,25].

2.4. Transfection and reporter gene assays

The firefly luciferase reporter plasmid containing the human GPx2 promoter (GI-prom 1, 2111 bp) in pGL3-basic was generated as described [26]. Reporter gene plasmids containing 2062 or 1683 bp of the human TrxR2 or TrxR3 promoter, respectively, were generated via PCR from genomic DNA of HepG2 cells using the primers listed in Table 1. For cloning into pGL3-basic, primers contained an XhoI restriction site at the 5′ end and a HindIII site at the 3′ end in the case of TrxR2 and MluI and BglII restriction sites in the case of TrxR3 (Table 1). All clones to be used were sequenced (SeqLab, Heidelberg, Germany). The expression plasmids for human dnTCF4 and S33Y β-catenin were kindly provided by Oliver Müller, the TCF expression plasmid by Bert Vogelstein, and the expression plasmid for c-Myc by Martin Eilers. For control, the empty plasmid pcDNA3 (Invitrogen, Karlsruhe, Germany) was used. pSV-β-galactosidase (Promega, Mannheim, Germany) or Renilla luciferase (phRL-TK-Renilla; Promega) were cotransfected and utilised for normalisation of firefly luciferase activity. Cells (2.0×10^5) were seeded into 24-well plates 24 h prior to transfection. Using Tfx-20 (Promega), 0.3 μg pSV-β-galactosidase vector, 0.15 μg firefly luciferase reporter plasmid and indicated amounts of the S33Y β-catenin and TCF, the dnTCF4 or the c-Myc expression plasmids were cotransfected according to the manufacturer's instructions into HepG2