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Advances in Brief

Genetic Disruption of *Ptgs-1*, as well as of *Ptgs-2*, Reduces Intestinal Tumorigenesis in *Min* Mice

Patricia C. Chulada,[1] Morrow B. Thompson, Joel F. Mahler, Christine M. Doyle, Beth W. Gaul, Christopher Lee, Howard F. Tiano, Scott G. Morham, Oliver Smithies, and Robert Langenbach[1]

Laboratory of Experimental Carcinogenesis and Mutagenesis [P. C. C., M. B. T., C. L., H. F. T., C. D. L., R. L.], Laboratory of Experimental Pathology [J. F. M.], NIH, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709; Experimental Pathology Laboratories, Research Triangle Park, North Carolina 27709 [C. M. D., B. W. G.]; and Department of Pathology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599 [S. G. M., O. S.]

Abstract

Two isoforms of cyclooxygenase (COX) are known, and to date most studies have implicated COX-2, rather than COX-1, as the isoform involved in colon carcinogenesis. In the present study, we show that homologous disruption of either *Ptgs-1* or *Ptgs-2* (genes coding for COX-1 or COX-2, respectively) reduced polyp formation in *Min*/+ mice by ~80%. Only COX-1 protein was immunohistochemically detected in normal intestinal tissue, whereas both COX-1 and variable levels of COX-2 protein were detected in polyps. Prostaglandin E_2 was increased in polyps compared with normal tissue, and both COX-1 and COX-2 contributed to the PGE_2 produced. The results indicate that COX-1, as well as COX-2, plays a key role in intestinal tumorigenesis and that COX-1 may also be a chemotherapeutic target for nonsteroidal anti-inflammatory drugs.

Introduction

Colorectal cancer is the most common cancer in North America and the second leading cause of cancer deaths in the United States. Several independent lines of research support a chemopreventive association between NSAIDs[2] and a reduced risk for colorectal cancer (1–6). Although the mechanism(s) by which NSAIDs reduce intestinal tumors are not precisely known, their inhibition of COX activity (7) is thought to be important. Two isoforms of COX, COX-1 and COX-2, have been characterized (8), and studies with *Ptgs-1* and *Ptgs-2* (genes coding for COX-1 and COX-2, respectively) knockout mice indicate that the isoforms have different physiological roles (9–11). Several types of studies have suggested that COX-2, rather than COX-1, is the isoform contributing to colorectal cancer development (12–15).

To determine whether COX-1 may also contribute to the development of intestinal cancer, we bred mice disrupted for the *Ptgs-1* (9) or *Ptgs-2* (10) genes to the *Min*/+ mouse (16). The *Min*/+ mouse contains a truncating mutation in the *Apc* gene and spontaneously develops intestinal adenomas. In the present study, we used the *Min*/+ mouse to demonstrate that the deficiency of COX-1, as well as of COX-2, reduces intestinal polyp formation.

Materials and Methods

Development of Mice. In an attempt to have all mice on a homogeneous background, the *Ptgs-1* (9) and *Ptgs-2* (10) mutations were transferred from the 129 Ola/C57Bl/6 background onto the C57Bl/6 background prior to crossing with the *Min*/+ mouse (Jackson Laboratories) already on the C57Bl/6 background. The *Ptgs-1* knockout was transferred without difficulty. However, backcrossing the *Ptgs-2* knockout into the C57Bl/6 strain was detrimental to the generation of *Ptgs-2*(−/−) mice. Therefore, *Ptgs-1*(+/−) or *Ptgs-2*(+/−) mice at three generations into the C57Bl/6 background were used to cross with the *Min*/+ line. All mice used in the study were four to five generations into the C57Bl/6 background. Because COX(+/+), COX(+/−), and COX(−/−) *Min* mice were obtained by similar breeding strategies, differences in modifying genes, other than *Ptgs-1* and *Ptgs-2*, were not considered to be factors when the tumorigenic responses of wild-type and COX-deficient mice were compared.

Determination of Tumor Numbers and Histological Analysis. To determine the number of intestinal tumors, the entire intestinal tract was removed, opened longitudinally, and washed with cold saline, and the proximal, mid, and distal sections of the small intestine, along with the colon, were spread flat, mucosal surface up, on filter paper for counting of nodules. Macroscopic nodules were scored with a limit of detectability of 1 mm. Selected sections of the tract were fixed in 10% neutral buffered formalin (NBF), paraffin embedded and histologically sectioned for immunohistochemistry. Swiss rolls (17) of intestinal sections from mice of each genotype were likewise fixed in 10% neutral buffered formalin and sectioned for microscopic examination to assess correlations between genotype and the incidence of preneoplastic lesions, as well as the histomorphology of adenomas. Microscopic examination of Swiss rolls also confirmed that nodules corresponding to gut-associated lymphoid tissue were few in number relative to adenoma nodules and did not effect tumor numbers obtained by macroscopic counting.

Immunohistochemisty Protocol. Paraffin-embedded sections of intestinal tissue were stained according to the protocol from the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA). The polyclonal antibodies used were goat-antimouse COX-1 (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit-antimouse COX-2 (1:4000; Cayman Chemical, Ann Arbor, MI). Immunoreactivity was detected with 3,3-diaminobenzidine (Sigma Chemical Co., St. Louis, MO), and slides were counterstained with Mayer's hematoxylin (Sigma). Intestinal tissues from COX-1(−/−) or COX-2(−/−) were run as negative controls to check the specificities of the respective antibodies.

Prostaglandin E_2 Analyses. For PGE_2 analysis, normal or tumor tissue was excised and snap frozen in liquid N_2. The frozen tissues were thawed, weighed, and homogenized in 0.5–1.5 ml of 50 mM Tris-HCl (pH 7.4) containing 5 μg/ml indomethacin. Often, it was necessary to pool two to three polyps from a particular mouse to obtain adequate tissue prior to homogenization. Homogenates were centrifuged at 1700 × *g*, 4°C, and the supernatant was analyzed for PGE_2 levels using the Amersham-Pharmacia Biotech (Piscataway, NJ) ^{125}I-labeled PGE_2 RIA.

Statistical Analyses. For tumor counts and PGE_2 levels, ANOVA procedures were used to assess sex and genotype differences. No significant differences were observed between males and females. Therefore, the data were pooled from the two sexes. The Freeman-Tukey Transformation for Poisson data (18) was used as a variance stabilizing logarithmic transformation. Although some degree of extra Poisson variability was present in the tumor counts, the Freeman-Tukey transformation was successful in eliminating the heterogeneity of variances across groups. If overall differences among genotypes were detected, pairwise comparisons were made by Fisher's LSD test (19).

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[1] To whom requests for reprints should be addressed, at National Institute of Environmental Health Sciences, 111 Alexander Drive, Research Triangle Park, NC 27709. E-mail: chulada@niehs.nih.gov or langenbach@niehs.nih.gov.

[2] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; *Min*, multiple intestinal neoplasia; *Apc*, adenomatous polyposis coli gene; PGE_2, prostaglandin E_2; LSD, least significant difference.

Results

Genetic Disruption of *Ptgs-1* or *Ptgs-2* Reduces the Number of Polyps in *Min*/+ Mice. As shown in Fig. 1*A*, a statistically significant gene dosage-dependent reduction (43 and 77%, respectively) in the number of intestinal tumors was observed when *Ptgs-1*(+/−) and *Ptgs-1*(−/−) *Min*/+ mice were compared with *Ptgs-1*(+/+) *Min*/+ mice at 6 months of age. Similar results were obtained from *Ptgs-1*(+/+) *Min*/+, *Ptgs-1*(+/−) *Min*/+, and *Ptgs-1*(−/−) mice at 8 months of age (data not shown). Survival studies showed that *Ptgs-1*(+/−) *Min*/+ mice lived ~10 months, and *Ptgs-1*(−/−) mice lived 12 months or longer compared with the 7–8-month life span of *Ptgs-1*(+/+) *Min*/+ mice. In 1-year-old *Ptgs-1*(−/−) *Min*/+ mice, the numbers of tumors (2.5 ± 0.8 in proximal; 5.5 ± 2.0 in mid; 8.8 ± 3.0 in distal small intestines; and 0.5 ± 0.5 in colon) were only slightly increased over those in 6-month-old mice with equivalent genotypes.

Oshima *et al.* (15) had shown previously that *Ptgs-2* deficiency decreased intestinal tumor formation in an *Apc* knockout mouse. Therefore, to determine whether *Ptgs-2* deficiency could also reduce tumorigenesis in the *Min*/+ mouse, the tumor numbers in *Ptgs-2*(+/+) *Min*/+ mice were compared with *Ptgs-2*(+/−) and *Ptgs-2*(−/−) *Min* mice. The data in Fig. 2*B* show that a significant reduction (84%) in polyps was also observed in *Ptgs-2*(−/−) *Min*/+ mice. For *Ptgs-2*(+/−) *Min*/+, the decrease in polyps was ~10% compared with *Ptgs-2*(+/+) *Min*/+ mice (Fig. 1*B*). Like the *Ptgs-1*(−/−) *Min*/+ mice, the survival of *Ptgs-2*(−/−) *Min*/+ mice ($n = 2$) was also increased to ~1 year. Therefore, our observation that COX-2 deficiency causes effects in the *Min*/+ mouse similar to those seen by Oshima *et al.* (15) in the *Apc*^{716} knockout mouse suggests that the *Ptgs-2*, as well as the *Ptgs-1*, effects observed in the present study are not limited to the *Min*/+ mouse.

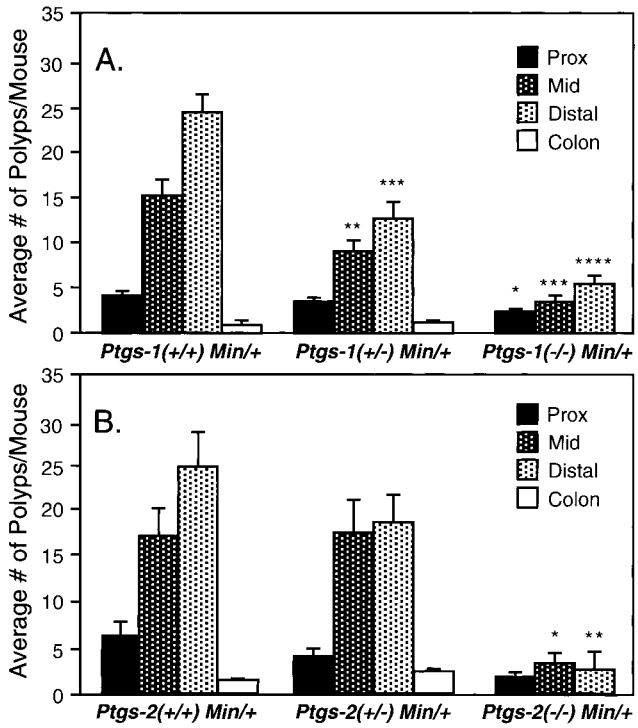

Fig. 1. Targeted gene disruption of *Ptgs-1* (*A*) or *Ptgs-2* (*B*) reduces the number of polyps in the *Min*/+ mouse. *Columns*, the number of tumors (means) in the proximal, mid, distal sections of the small intestines and colons of 6-month-old mice; *bars*, SE. For the *COX-1 Min*/+ mice, 13–17 mice were used for each genotype to determine the number of tumors. For the *COX-2 Min*/+ mice, 10–14 mice were used, except for the *COX-2*(−/−) *Min*/+ group where only two mice were available. Fishers LSD test was used to determine statistical significance from *Min*/+ mice: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

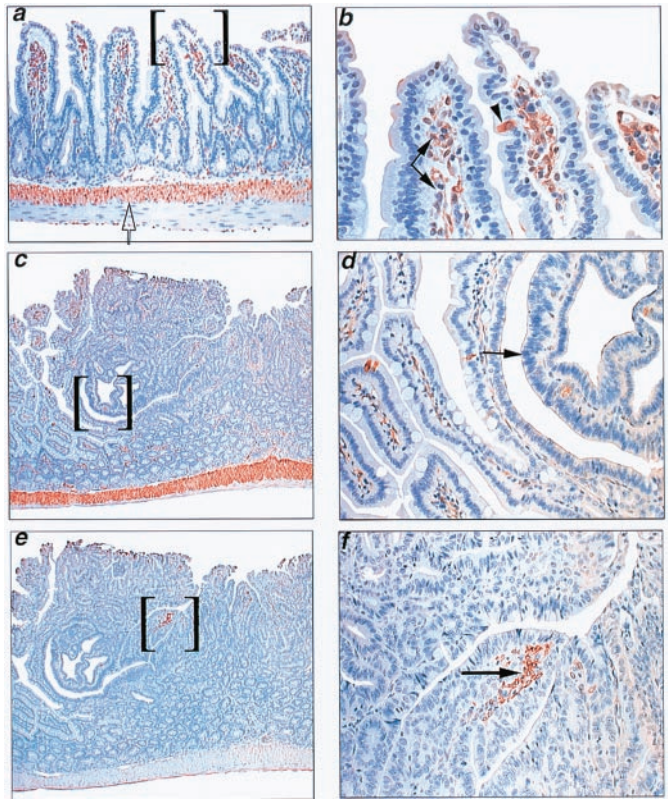

Fig. 2. Immunohistochemical localization of COX-1 and COX-2 in normal and neoplastic tissue. *a* and *b*, low (×50) and high (×150) power images of a section of normal small intestine from a 6-month-old *Min*/+ mouse stained with antibody to COX-1. Immunolocalization was seen in the cells of the lamina propria (*closed arrows*), inner muscular layer (*open arrow*), and occasional villous epithelial cell (*arrowhead*). *c* and *d*, low (×25) and high (×150) power images of an intestinal adenoma from a 6-month-old *Min*/+ mouse stained with antibody to COX-1. At low magnification, the tumor lacks COX-1 immunoreactivity relative to the adjacent lamina propria and underlying muscularis. Higher magnification (*d*) of the *bracketed area* in (*c*) shows unstained neoplastic epithelial cells (*arrow*) adjacent to positive normal mucosa. *e* and *f*, low (×25) and high (×150) power images of the same tumor in *c* and *d* stained with an antibody to COX-2. Tumor and adjacent normal tissue are negative for COX-2 protein except for a small focus (in *brackets*, *e*), which on higher magnification (*f*) is composed of a cluster of positive-staining lamina propria cells.

The distribution of tumors was similar in all *Ptgs* genotypes of *Min*/+ mice, with a proximal to distal increase in tumor burden in the small intestine and with few tumors in the colon. In *Min*/+ mice with wild-type *Ptgs* alleles, tumors ranged from 1 mm (the limit of macroscopic detectability) to 7 mm in size; the larger tumors were typically broad based with depressed centers. Those in the colon were fewer in number and more polypoid in shape. Intestinal tumors in *Ptgs-1*(−/−) and *Ptgs-2*(−/−) *Min*/+ mice ranged from 1 to 4 mm. Analysis of Swiss rolls (17) prepared from the intestinal tracts from mice of all genotypes indicated that most tumors were adenomas, based on irregular glands that were raised above the mucosal surface and were lined with atypical epithelial cells. The numbers of adenomas determined by analysis of the Swiss roll sections (data not shown) correlated with those determined by gross counts shown in Fig. 1.

COX-1 Is Immunologically Detected in Normal Tissue Whereas Both COX-1 and COX-2 Are Detected in Polyps. Normal and neoplastic tissues from all mouse genotypes were immunostained with antibodies specific for COX-1 or COX-2. Only COX-1 was detected in normal tissue of all mouse genotypes except from mice lacking a functional *Ptgs-1* gene. COX-1 was localized to the inner muscular layer, cells in the lamina propria, and a few rare villous epithelial cells

in the mucosa (Fig. 2, *a* and *b*). This pattern of COX-1 immunoreactivity was similar in both normal and neoplastic tissue (Fig. 2, *c* and *d*). Whereas COX-2 protein was generally not detectable in normal intestinal tissue, localized areas of COX-2 immunostaining were detected in cells of the lamina propria in many adenomas [except those from *Ptgs-2*(−/−) mice; Fig. 2, *e* and *f*]. However, the size of the positive regions and intensity of COX-2 immunostaining varied from polyp to polyp, with smaller polyps generally showing less detectable COX-2 protein.

PGE_2 Production in Normal Intestinal Tissue and in Polyps. To determine the relative contribution of the COX isoforms to intestinal prostaglandin production, PGE_2 was used as an indicator of prostaglandin synthesis because it is a prostaglandin that is increased in polyps compared with normal intestinal tissue (20). The data in Fig. 3 compare PGE_2 levels in normal distal intestinal tissue (tissue surrounding the adenomas) and adenomas from *Ptgs-1* and *Ptgs-2 Min*/+ mice. The data show that COX-1 is the major isoform responsible for basal PGE_2 production in normal tissue, because PGE_2 levels are reduced by 99% in *Ptgs-1*(−/−) mice. PGE_2 levels were increased in polyps compared with normal tissue in the distal intestine (Fig. 3) in wild-type mice and the data from the COX-1 and COX–2 deficient mice indicate that both COX-1 and COX-2 contribute to PGE_2 production in the polyp. Similar results were obtained when colonic normal tissue and polyps were compared (data not shown). In summary, the data show that COX-1 is the major source of PGE_2 in normal tissue and that both COX-1 and COX-2 contribute to PGE_2 production in polyps.

Discussion

The data demonstrate that the deficiency of either COX-1 or COX-2 reduces intestinal tumorigenesis in the *Min*/+ mouse. However, the deficiency of either isoform still allowed some tumor formation (Fig. 1) and thus raises the question as to whether the isoforms can partially substitute for one another, or if COX independent mechanisms are involved in the development of these tumors. Notwithstanding, the data show that the functional presence of both COX-1 and COX-2 is required to produce the expected level of polyp formation in the *Min*/+ mouse.

The deficiency of either COX-1 or COX-2 caused similar decreases in intestinal tumorigenesis in the *Min*/+ mouse (*i.e.*, 77 and 84%, respectively; Fig. 1). Furthermore, it was observed that both COX-1 and COX-2 contributed to PGE_2 production in polyps (Fig. 3). Simplistically, one possible interpretation of the data may be that it is the total prostaglandin level in the incipient polyp that is important for adenoma development, and that decreased prostaglandin production attributable to the loss of either isoform significantly reduces tumor formation. In support of this possibility, it has recently been reported

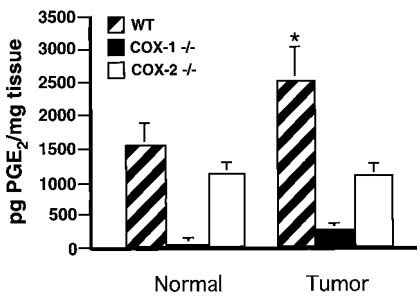

Fig. 3. Comparison of PGE_2 levels in polyps and normal surrounding distal intestinal tissue from *Min*/+, *Ptgs-1*(−/−) *Min*/+, and *Ptgs-2*(−/−) *Min*/+ mice. *Columns*, the PGE_2 levels (means) from polyps and normal tissue from 6-month-old mice; *bars*, SE. Fishers LSD test was used to determine statistical significance of normal tissue from adenomas. *, statistically different from normal intestinal tissue at $P < 0.1$.

that mice deficient in the PGE_2 receptor, EP_1, show about a 40% decrease in aberrant crypt foci after azoxymethane treatment (21). Furthermore, an EP_1 antagonist decreased the number and size of polyps formed in the *Min*/+ mouse (21). Both COX-1 and COX-2 could contribute to the production of PGE_2, which interacts with the EP_1 receptor. Alternatively, the COX isoforms could lead to the production of different prostaglandins and thereby influence tumorigenesis through different receptor-mediated pathways. As discussed below, it is also possible that the individual COX isoforms contribute to polyp formation at different stages of the tumorigenesis process.

Recent studies have provided some insight into possible roles of COX-2 in intestinal tumorigenesis (22). Oshima *et al.* (15) demonstrated previously that COX-2 deficiency decreased intestinal tumorigenesis in an *Apc* knockout mouse. Additionally, these authors observed that in *Ptgs-2*(+/+) mice COX-2 protein was detectable in the intestinal polyps after they reached a size of ~2 mm. Prescott and White (22), in their discussion of the work of Oshima *et al.* (15), postulated that COX-2 was up-regulated after the loss of the wild-type *Apc* allele and that COX-2-derived prostaglandins contributed to tumor promotion. The data we obtained with the COX-2(−/−) *Min*/+ mouse are essentially the same as those reported by Oshima *et al.* (15), and therefore COX-2-derived prostaglandins could also contribute to tumor promotion in the *Min*/+ mouse. Similar to Oshima *et al.* (15), we observed that COX-2 expression was in the interstitial cells rather than the epithelial cells of the polyp. It has been reported recently that macrophages in the lamina propria of the polyps of the *Min*/+ mouse were responsible for the increased COX-2 expression (23). Therefore, our data, and those of Oshima *et al.* (15), Hull *et al.* (23), and Shattuck-Brandt *et al.* (24) raise the question as to whether COX-2 expression is required in the epithelial tumor cells or whether COX-2-derived prostaglandins from the interstitial cells at this stage of tumor development can act by a paracrine mechanism on the neoplastic epithelial cells.

The possible role(s) of COX-1 in intestinal tumorigenesis have received less attention than those for COX-2. Our data show that COX-1 is constitutively expressed in normal intestinal tissue (Fig. 2) and that although it is the primary source of prostaglandins as measured by PGE_2 production in intestinal tissue (Fig. 3), no pathology of intestinal tissue was detected in COX-1(−/−) mice (9). However, both COX-1 and COX-2 contribute to PGE_2 levels in the adenoma (Fig. 3). Therefore, COX-1 could exert its effects in the tumorigenesis process both at an early stage and at later stages in tumor development. In support of an early role for COX-1, studies have indicated that COX-1 can metabolically activate procarcinogens to mutagenic intermediates (3), and that aspirin can inhibit this metabolic activation. Craven and DeRubertis (4) demonstrated that aspirin, a more effective inhibitor of COX-1 than COX-2 (7), administered at the time of 1,2-dimethylhydrazine treatment reduced intestinal tumorigenesis by 60%, whereas starting aspirin administration after the 1,2-dimethylhydrazine had little effect on tumorigenesis. In addition to the possible activation of dietary procarcinogens, normal COX-1 metabolism of endogenous arachidonic acid can lead to the generation of a known mutagen, malondialdehyde (3). Because the *Min*/+ mouse is already genetically mutated at one *Apc* allele, a second mutagenic event according to the two-hit mechanism of Knudson (25) is required. It has been shown that 100% of the spontaneous polyps in the C57 Bl/6-*Min*/+ mouse lose the wild-type *Apc* allele (16). It is possible that in COX-1(−/−) mice, malondialdehyde production decreases, and that a second mutagenic event, possibly leading to the loss of the wild-type allele, is less frequent, and therefore, fewer tumors result. Alternatively, independent of contributing to mutagen production, COX-1 has been shown to protect colonic stem cells after gamma irradiation. Cohn *et al.* (26) demonstrated that after *in vivo*

gamma irradiation, COX-1 produced prostaglandins that significantly enhanced stem cell survival and growth *ex vivo*. In the absence of COX-1 in the *Min*/+ mouse, genetically damaged cells and/or cells undergoing the loss of the wild-type *Apc* allele may have impaired survival and thus lead to less adenoma formation. However, this possible early role for COX-1 in intestinal tumorigenesis does not preclude COX-1-derived prostaglandins from also contributing to tumor promotion.

In the present study, we have demonstrated that both COX-1 and COX-2 deficiencies reduce the tumorigenic responses in the *Min*/+ mouse. Although our data do not allow us to define separate roles for COX-1 and COX-2, based on our observations and data from previous studies, we have speculated that the two isoforms function via different mechanisms and/or during different stages of the tumorigenesis process. The possibility that the COX isoforms act at different stages during tumor development suggests that COX dual inhibitors may be effective at both early and late stages, whereas selective inhibitors might be more effective when administered either early (COX-1 specific) or late (COX-2 specific). In summary, our data show that genetic ablation of either COX isoform can significantly impact the course of intestinal tumorigenesis in the *Min*/+ mouse.

Acknowledgments

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