Cyclooxygenase-2, a Colorectal Cancer Nonsteroidal Anti-inflammatory Drug Target, Is Regulated by c-MYB

Robert G. Ramsay, Annabel Friend, Yotta Vizantios, Ruth Freeman, Catherine Sicurella, Fleur Hammett, Jane Armes, and Deon Venter

Peter MacCallum Cancer Institute, East Melbourne, 8006, Victoria, Australia

Abstract

Cyclooxygenase-2 (COX-2) is an important pharmacological target with great promise in the prevention and treatment of colorectal cancer (CRC). The mechanism underlying COX-2 overexpression in CRC is unresolved. On the basis of the coincident high levels of the transcription factor c-MYB and COX-2 in CRC, we hypothesized that c-MYB is a candidate activator of COX-2 transcription. We identified 13 c-Myb binding sites in the human COX-2 promoter. Eight of these sites were moderate to high-affinity DNA binding targets. Promoter studies indicated that c-Myb can activate COX-2 transcription, whereas dominant-negative Myb mediated repression. These data provide the first rational basis for overexpression of COX-2 in CRC and offer an additional potential target for managing this disease.

Introduction

COX-2 has been implicated in CRC progression and metastases. Most studies show that the long-term treatment of individuals with aspirin or NSAIDS, which target COX-2 activity, reduces colon cancer risk. Similarly, CRC family cohort members treated with NSAIDS have reduced CRC incidence (1). In addition, experimental mouse models using COX-2 knock-out strains crossed with gastrointestinal cancer prone Min (adenomatous polyposis coli mutant) mice show a marked reduction in polyp formation and tumor burden (2). Finally, exogenous overexpression of COX-2 accelerates colon cancer in mice (3). c-MYB overexpression is also a consistent feature of colon tumors and tumor-derived cell lines (4) and is required for cell growth (5, 6). We have identified an unprecedented number of c-MYB binding sites in the COX-2 promoter and shown that c-MYB can efficiently transactivate COX-2 transcription. In addition, CRC cell lines express both c-MYB and COX-2 mRNA and coincident high expression of COX-2 and c-MYB mRNA by in situ hybridization on colon cancer tissue arrays. These data suggest that c-MYB is responsible for the elevated levels of COX-2 in colon epithelial neoplasia and may in itself constitute a possible therapeutic target for this malignancy.

Materials and Methods

In Situ Hybridization Probes. c-MYB riboprobe was generated by insertion of a cDNA corresponding to the HindIII to BglII (nucleotides 664–1516) sites encompassing c-MYB unique sequences that do not cross-react with other MYB family members. The COX-2 riboprobe (pcRII-huCOX2) was a gift from Dr. Raymond De Bois incorporating a 278-bp fragment corresponding to nucleotides 952–1220.

Hybridization Protocol. Paraffin-embedded CRC specimens were identified, and core samples were punched from the block and pooled into arrays for re-embedding. These were then sectioned to deliver multiple CRC specimens on the same slide. Slides were incubated in prehybridization solution [60% denatured formamide, 25 mM Tris (pH 7.4), 1 mM EDTA (pH 8.4), 375 mM NaCl, 12% (w/v) dextran sulfate, and 1.2× Denhart’s solution] for 2 h at 42°C. Digoxigenin-UTP-labeled RNA at 200 ng/ml in hybridization buffer was incubated in hybridization buffer with 0.1 mM DTT, 0.1% sodium thiosulfate, and 0.1% SDS under slide covers overnight at 42°C. Slides were then washed twice in 2× SSC 15 min at room temperature and then at 42°C, followed by 15-min washes in 1× SSC and 0.1× SSC. Section were blocked for 30 min at room temperature in 1% Blocking reagent, followed by anti-digoxigenin antibody conjugated with alkaline phosphatase 1:1000 in 1× blocking solution 60 min at room temperature, and washed with two buffer changes (0.1 M maleic acid and 150 mM NaCl) for 15 min each. Color was developed as described by the manufacturer (Roche). Finally, slides were rinsed in water and counterstained with 0.1% methyl green, rinsed in water again, and mounted with Kaiser’s glycerol gelatin.

EMSA. Specific DNA binding by CTL c-MYB protein has been described in detail elsewhere (7). Briefly, double-stranded end-filled radiolabeled oligonucleotides corresponding to putative c-MYB binding sites were incubated in the presence of Bal31-corrupted-expressed recombinant c-MYB for 15 min prior to running on 6% acrylamide, 0.5× Tris borate ET gels. At least three replicate experiments were performed with 2, 1, and 0.5 ng of oligonucleotide. Relative binding was quantified using a Molecular Dynamics PhosphorImager. The oligonucleotide sequences for the COX-2 promoter used in this study are listed in Table 1. All oligonucleotide pairs had 5′-ggtc- overhangs to allow labeling.

Cloning of the COX-2 Promoter Region. The COX-2 promoter was amplified as two subclones designated as COX-2 5′ and 3′ fragments. The 5′ fragment oligonucleotide primers corresponding to −2355 to −1255 from the transcription start site (forward, 5′-aagctatgacatcagttgcag-3′) and (reverse, 5′-tggagattggtggtctggagcag-3′); noting the presence of an internal NcoI restriction site used for subsequent cloning purposes. The 3′ fragment oligonucleotide primers −1255 to +35 (forward, 5′-atggtcattgagatcagttgcag-3′) and (reverse, 5′-gagttggtcattgagagcag-3′). Touchdown PCRs were carried out at 55°C for 40 cycles on a Perkin-Elmer Thermocycler using Taq polymerase and Taq start antibody (Bresatec). Fragments corresponding to the 5′ plus 3′ promoter regions were either joined via an internal NcoI site or the 3′ fragment alone was subcloned into pCATBasic (Promega).

CAT Assays. Fugene 6 (Boehringer Mannheim) transfection of Colo201 and 293HEK cells were performed according to the manufacturer’s instructions. Cells were harvested and lysed, adjusted for total protein, and tested for β-galactosidase activity (8). Ten µl of protein extract (adjusted for β-galactosidase activity), 50 µl of 0.25 M Tris (pH 8), 10 µl of acetyl-CoA, and 2 µl of [14C]choloramphenicol (Amersham) were incubated for 2–4 h at 37°C. Reactions were extracted in 0.5 ml of ethyl acetate and dried. The dehydrated pellet was resuspended in 15 µl of ethyl acetate and “spotted” on a TLC plate and chromatographed in 5% methanol and 95% chloroform. The plate was air dried, wrapped in plastic, and placed in a PhosphorImager (Molecular Dynamics).
Expression Plasmids. Full-length murine c-MYB cDNA was expressed from a pACT vector described elsewhere (9) and the DN expression construct containing a c-MYB DNA binding domain fused to the *Drosophila* engrailed repressor protein and estrogen receptor (pMERT; Ref. 10). Modulation of the DN-Myb construct was achieved using 10−8 M 4-OHT (Sigma) added immediately after the transfection mix was replaced with fresh medium.

### Results and Discussion

c-MYB and COX-2 overexpression is a consistent feature of colon tumors and tumor-derived cell lines; however, they have not been examined contemporaneously. Therefore, we examined the relationship between c-MYB and COX-2 expression in CRC COX-2, c-MYB, and β2-microglobulin. Five of five CRC cell lines (Colo201, LIM1215, LIM1863, LIM2405, and LIM2412) had detectable c-MYB and COX-2 expression, but the epithelial cell line HEK293 did not (data not shown). It was notable that Colo201 cells that have an amplified c-MYB locus with high c-MYB transcription and protein level also had the highest COX-2 expression (see below).

Similarly, a retrospective survey of the literature is consistent with the view that c-MYB and COX-2 are overexpressed in the majority of CRCs; however, an examination of the expression of both of these genes in the same primary tumor samples has not been performed. Hence, we investigated 20 CRC specimens from the Peter MacCallum Cancer Institute archives by *in situ* hybridization using antisense RNA encoded by c-MYB and COX-2 cDNAs. These specimens were chosen on the basis of clear histological features of CRC. Fig. 1A shows high expression of both COX-2 and c-MYB mRNA in the epithelial components of two representative CRC biopsy sections examined as indicated by the purple staining (D2 and E3). These images are taken at low power magnification (×25) to show the distribution of mRNA within a field of tumor tissue and were consistent for 20 matched core samples. Sense and antisense probe hybridization for either c-MYB or COX-2 conducted on consecutive sections of the two independent core samples examined at higher power magnification (×100) shows the colocalization of COX-2 and c-MYB to the epithelial cells in these tumors (Fig. 1B). In general, this analysis confirmed the restricted expression of COX-2 and c-MYB to the epithelial cells and low expression in stroma and muscle. As expected, specific c-MYB expression was evident in the normal epithelial mucosa confined to the columnar cells of the colonic crypt (Ref. 4; Fig. 1C).

Having established the colocalization and expression of c-MYB and COX-2, we next considered the possibility that c-MYB may act upon the COX-2 promoter. Examination of the human *cox-2* gene in the 5′ region upstream of the first exon (11) revealed 13 putative c-MYB binding sites that conform to the core binding consensus c/tAACt/g (Ref. 12; Fig. 2). Analysis of the human *COX-1* promoter region (13) did not detect any such sequences. To examine whether these putative sites can be recognized by c-MYB protein, double-stranded oligonucleotide duplexes corresponding to these sites sequentially identified as sites “A” through “M” were synthesized. These were radiolabeled by end-filling reaction in the presence of [α-32P]dATP, incubated in the presence of recombinant c-MYB, and subjected to EMSA (7).

Each oligonucleotide pair was tested in comparison to at least three different pairs corresponding to other putative sites to assemble a hierarchy of relative binding affinities. An additional site called “R” was examined because it was the only perfectly aligned (but not sequence-conserved) site present in a comparison of the rat (14) and human *COX-2* 5′ sequences (see below). Three oligonucleotide concentrations were used to allow an estimate of binding, to achieve linear binding, and finally to detect minimal binding of c-MYB to the lowest affinity sites. All EMSA reactions were controlled for nonspecific DNA binding and used the same batch of CTL recombinant c-MYB. Table 1 lists the sites in order of binding affinity, whereby sites L, C, D, F, and I belong to the high-affinity group; sites K, E, and H have intermediate relative affinity, and sites B, G, and A fall into the low-affinity group. Sites J, M, and R serve as poor c-MYB binding sites. From studies of the specific DNA binding of c-MYB (12), it is clear that flanking nucleotides adjacent to the core consensus sequence (pyAACt/g) influence high-affinity binding. Of utmost importance to high-affinity binding is a nucleotide residue position 6 that is ideally a guanine. The high-affinity sites L, C, D, F, and I conform to this sequence requirement as does site K, which is the next best bound site that has been assigned to the moderate-affinity group.

Putative c-MYB target genes have been reported elsewhere (15); however, the presence of 13 theoretical and 8 high-affinity c-MYB binding sites within the promoter of a gene is unprecedented. The absence of putative c-MYB sites within a similar nucleotide stretch in the promoter region of the *COX-1* gene that encodes a highly related isoform of COX-2 reinforces the nonrandomness of this observation.

To determine whether the COX-2 promoter could be transactivated by c-MYB, we cloned the 5′ region of the human *COX-2* gene in two parts, subcloned them collectively or as the most proximal (3′) part of the promoter region, into the pCATbasic vector and conducted transactivation studies. A histogram depicted in Fig. 3A indicates that the most proximal region of the *COX-2* promoter (3′) is very active in the colon cell line Colo201 (~8-fold activation) compared with the chicken β-actin promoter (2.5-fold) driving the CAT reporter gene.

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**Table 1** Hierarchy of c-Myb binding sites in the COX-2 promoter determined by EMSA

<table>
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<tr>
<th>Site</th>
<th>Relative binding</th>
<th>5′ flanking</th>
<th>Position 1a</th>
<th>Position 2a</th>
<th>Position 3a</th>
<th>Position 4a</th>
<th>Position 5a</th>
<th>Position 6a</th>
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<td>T</td>
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* Core binding sequence c/tAACt/g.
* Bottom strand.

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*COX-2 REGULATION BY c-MYB*
Notably, Colo201 expresses the highest level of endogenous COX-2 mRNA and c-MYB.

To further characterize the cox-2 promoter, we transfected the 3' and the 5' plus 3' COX-2 promoter regions into the human epithelial cell line HEK293, which has undetectable c-MYB and COX-2 mRNA. Fig. 3B shows that full-length c-MYB can transactivate the 3' cox-2 promoter 4-fold compared with the basal level of transcription observed in HEK293 cells, whereas the combined 5' + 3' promoter fragment allows ~ 5-fold transactivation. Exogenous c-MYB expression in the HEK293 cells was confirmed by Western blotting (data not shown).

Additional evidence that the c-MYB binding sites in COX-2 are important is demonstrated when transactivation of either the 5' + 3' or 3' promoter constructs are examined in the presence of a DN (DN-Myb) form of c-MYB. The activity of this construct is dependent upon the addition of 4-OHT. DN-MYB plus 4-OHT substantially reduced the transactivation of both COX-2 reporter constructs. 4-OHT alone did not affect full-length c-MYB-dependent transactivation (data not shown). These data show that c-MYB can activate the COX-2 promoter. They further show that the DN-MYB construct inhibits the basal level of transcription most likely by the direct binding to, and repression through, the numerous c-MYB binding sites characterized in the EMSA studies documented above.

An analysis of potential c-MYB binding sites within the rat (14) and mouse (16) COX-2 promoters revealed 10 and 13 probable sites, respectively, with 4 in each of these satisfying the criteria for high-affinity binding sites. The distribution of probable high-affinity c-MYB binding sites within the mammalian COX-2 promoters is similar, and sequence comparisons show 85% similarity between the rat and mouse and 69% similarity between the human and mouse. However, the sites do not align perfectly with each other, suggesting that substantial evolutionary divergence has occurred between the three mammalian species. Nevertheless, these observations are consistent with COX-2 regulation by c-MYB during colon tumorigenesis in rodents. Further support of these views is the observation of overexpression of c-MYB in rat colon tumors (17).

The data presented here suggest that the high level of COX-2 mRNA associated with colon tumor epithelial cells may be attributable to the transcriptional activation of the COX-2 promoter by c-MYB. Perhaps because of the historical association of c-MYB and hematopoiesis, the role of c-MYB in other cell types has been largely
ignored. Nevertheless, we have shown that c-MYB appears to be an
important regulator of BCL-2 and thus apoptosis in developing mu-
rine colon and in human colon tumor cell lines (4, 18). The observa-
tions presented here highlight an additional connection between c-
MYB and the inhibition of apoptosis because COX-2 overexpression
also appears to be protective against apoptosis (19). Thus, c-MYB
overexpression in CRC may inhibit apoptosis by direct regulation of
both BCL-2 and COX-2.

COX-2 appears to inhibit apoptosis, and it is likely that therapeutic
targeting of COX-2 activity in CRC prevention and treatment may
restore normal apoptosis. We have also suggested that c-MYB regu-
lates apoptosis in normal and malignant colon tissues through the
transcriptional control of BCL-2. The ablation of c-MYB expression in
tumors may lead to the reduction of downstream transcriptional tar-
gets. To this end, successful inhibition of c-MYB expression has been
achieved in vivo using antisense oligonucleotide treatment (20), and
these methodologies have been used to inhibit malignant cell growth
and reduced tumor burden. These end points have been achieved with

Fig. 2. Sequence analysis of the human COX-2 promoter region highlighting potential c-MYB binding sites (c/tAACt/g) as boxes with their alphabetical designation noted in the left-hand margin from A to M plus site R identified in the rat COX-2 promoter region. The TATA box and transcription start site (arrow) are also indicated. Sites L and M are in the reverse orientation. Boxes with underlined sequences are predicted to be high-affinity sites because of the presence of a guanine residue at position 6. The location of the NcoI restriction site is noted as the joining point for the 5’ and 3’ segments of the COX-2 promoter sequence.

Fig. 3. Transactivation of the COX-2 promoter in c-Myb-expressing colon cell line Colo201 and in c-Myb-negative cell line HEK293 by exogenous c-MYB. A, the 3’ COX-2 promoter region results in ~8-fold activation of the CAT reporter gene in Colo201 cells compared with the β-actin-driven CAT construct (pACT-CAT). This cell line expresses relatively high MYB levels. In c-MYB-negative HEK293 cells, the addition of exogenous MYB transactivates the 3’ region of the COX-2 reporter construct 4-fold, whereas the addition of a DN-MYB construct has no effect unless activated by the addition of 4-OHT. C, the combined 5’ and 3’ regions of the COX-2 promoter allows slightly higher transactivation compared with the 3’ region alone. In addition, the DN construct also inhibits its activity. Bars, SE.
minimal apparent effects on tissues that normally express c-MYB. These kinds of studies are the basis of Phase II clinical trials (National Cancer Institute-sponsored Protocols UPCC-3492 and NCI-H94–0532). These data offer encouraging prospects for targeting CRC with elevated COX-2 and BCL-2 expression through the inhibition of c-MYB expression.

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