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

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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 Du 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 resectioned to deliver multiple CRC specimens on the same slide. Slides were incubated in prehybridization solution [60% deionized formamide, 25 mM Tris (pH 7.4), 1 mM EDTA (pH 8.5), 375 mM NaCl, 12% (w/v) dextran sulfate, and 1.2 × Denhardt’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.

EMSAs. 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 Baccalurius-expressed recombinant c-MYB for 15 min prior to running on 6% acrylamide, 0.5× Tris borate ETDA 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′-ggtcc 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′-aagacctgtgtacacatagccag-3′) and (reverse, 5′-aagctgccaggtcagcaactc-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′-aagctgccaggtcagcaactc-3′) (and reverse, 5′-aagctgccaggtcagcaactc-3′) and 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]Chloramphenicol (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 was “spotted” on to 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).
diately after the transfection mix was replaced with fresh medium.

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COX-2

MYB

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(data not shown). It was notable that Colo201 cells that have an

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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 to 4-OHT (Sigma) added imme-
diately 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 relation-
ship between c-MYB and COX-2 expression in CRC COX-2, c-MYB,
and B2-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 com-
ponents 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/tAACU/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 oligonu-
cleotide 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 as-
semble 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 oligonu-
cleotide 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 (pyAACU/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 and do not 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 trans-
activation 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.

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

<table>
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<tr>
<th>Site number</th>
<th>Relative binding</th>
<th>5′ flanking</th>
<th>Position 1a</th>
<th>Position 2a</th>
<th>Position 3b</th>
<th>Position 4a</th>
<th>Position 5a</th>
<th>Position 6</th>
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<td>A</td>
<td>C</td>
<td>C</td>
<td>T</td>
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</table>

*M Core binding sequence c/tAACU/g.
* Bottom strand.

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 to 4-OHT (Sigma) added imme-
diately after the transfection mix was replaced with fresh medium.
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 murine colon and in human colon tumor cell lines (4, 18). The observations 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 regulates 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 targets. 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...
Acknowledgments

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