Brief Definitive Report

Regulation of Cyclooxygenase-2 Expression by the Translational Silencer TIA-1

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Abstract

The cyclooxygenase-2 (COX-2) enzyme catalyzes the rate-limiting step of prostaglandin formation in inflammatory states, and COX-2 overexpression plays a key role in carcinogenesis. To understand the mechanisms regulating COX-2 expression, we examined its posttranscriptional regulation mediated through the AU-rich element (ARE) within the COX-2 mRNA 3′-untranslated region (3′UTR). RNA binding studies, performed to identify ARE-binding regulatory factors, demonstrated binding of the translational repressor protein TIA-1 to COX-2 mRNA. The significance of TIA-1-mediated regulation of COX-2 expression was observed in TIA-1 null fibroblasts that produced significantly more COX-2 protein than wild-type fibroblasts. However, TIA-1 deficiency did not alter *COX-2* transcription or mRNA turnover. Colon cancer cells demonstrated to overexpress COX-2 through increased polysome association with COX-2 mRNA also showed defective TIA-1 binding both in vitro and in vivo. These findings implicate that TIA-1 functions as a translational silencer of COX-2 expression and support the hypothesis that dysregulated RNA-binding of TIA-1 promotes COX-2 expression in neoplasia.

Key words: COX-2 • cyclooxygenase-2 • prostaglandins • TIA-1 • AU-rich element

Introduction

Metabolites of arachadonic acid participate in normal and aberrant growth responses, including chronic inflammation and carcinogenesis (1). Cyclooxygenases are key enzymes in the conversion of free arachidonic acid to prostaglandins. The inducible isoform of cyclooxygenase, COX-2, is an immediate-early response gene not expressed constitutively in most cells. However, tight molecular regulation allows for rapid COX-2 expression and increased prostaglandin synthesis when necessary. Several lines of evidence demonstrate that unregulated COX-2 expression occurs at multiple stages in colon carcinogenesis and is important in the promotion of tumorigenesis (1, 2). These findings, coupled with recent evidence demonstrating the benefits of COX-2 inhibitors in many cancer models, underscore the significance of aberrant COX-2 expression and suggest that pharmacologic inhibition of COX-2 and/or regulation of its expression may limit cancer progression.

COX-2 expression is regulated through both transcriptional and posttranscriptional mechanisms (3). We previously identified an AU-rich element (ARE) within the 3′ untranslated region (3′UTR) of COX-2 mRNA that confers posttranscriptional regulation by controlling both mRNA decay and protein translation (4, 5). The ability of this cis-acting mRNA element to regulate COX-2 protein levels and associated prostaglandin synthesis was observed in cells maintaining low to undetectable COX-2 levels (4). In contrast, stabilization of COX-2 mRNA occurs in cancer cells, implicating that loss of ARE-function promotes heightened COX-2 expression in neoplasia (3, 6).

Posttranscriptional regulation mediated by the COX-2 ARE is facilitated through trans-acting ARE-binding factors. These regulatory proteins form stable complexes with the COX-2 3′UTR and regulate both COX-2 mRNA stability and translation (4, 7). A number of ARE-binding proteins have been identified with several proposed func-

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tions including promotion of rapid mRNA decay, increase in mRNA stability, and regulation of translational efficiency (8). For example, HuR protein binds to the COX-2 ARE and when overexpressed promotes the stabilization of ARE-containing transcripts; this effect is observed in tumors where HuR protein is overexpressed (6). In contrast, AUF1/hnRNP D protein binding of the COX-2 ARE is proposed to regulate rapid mRNA decay similar to other ARE-containing transcripts (7). Thus, the relative abundance of these functionally distinct ARE-binding proteins can determine the fate of COX-2 transcript levels and impact COX-2 protein levels.

We have identified the apoptosis-associated protein TIA-1 as a regulator of COX-2 expression at the posttranscriptional level. Through its ability to bind the COX-2 ARE, this RNA-binding protein acts as a translational silencer of COX-2 expression but does not affect mRNA stability. More importantly, deficiencies in TIA-1 mRNA binding are observed in colon cancer cells overexpressing COX-2 protein through increased polysome association with COX-2 mRNA. These findings suggest that misregulated association of the TIA-1 RNA-binding protein with COX-2 mRNA contributes to enhanced expression and perhaps the overall neoplastic potential of cancer cells.

Materials and Methods

Cell Culture and DNA Transfections. HeLa, HT29, and LoVo cells were maintained in DMEM containing 10% FBS. Wild-type (TIA-$1^{+/+}$) and TIA-1 deficient (TIA-$1^{-/-}$) primary mouse embryonic fibroblasts (MEFs) were prepared as described previously (9). MEFs were grown in DMEM containing 10% FBS and used before exceeding 8 passages. TIA-$1^{-/-}$ MEFs stably transfected with a human TIA-1 cDNA were prepared as described previously (10). MEFs were transfected with a 1.8-kb human COX-2 promoter luciferase reporter construct (6) along with control pSV-βgal using Lipofectamine Plus (Invitrogen) according to the vendor's protocol. Reporter gene activities were normalized to cotransfected controls and all results represent three averaged experiments, each performed in triplicate.

Western Blot and Prostaglandin Analysis. Confluent cell monolayers were lysed in RIPA buffer and denatured lysates (50 μg) were separated by 10% SDS-PAGE as described previously (6). After electrophoresis, proteins were transferred to PVDF membranes and probed with antibodies against COX-2 (C-20), TIA-1 (C-20), TIAR (C-18), HuR (3A2), and β-actin (Santa Cruz). PGE_2 levels were assayed in culture media by ELISA (R&D Systems) as described previously (6). Relative PGE_2 levels were normalized to total protein and are the average of triplicate experiments.

mRNA Analysis. RNase protection assays were used to examine COX-2, c-myc, and 28S RNA levels in 10 μg of total RNA as described previously (4). RNA decay experiments were initiated by adding ActD (5 μg/ml) to the growth medium. RT-PCR analysis was conducted using primers designed to amplify the entire coding region of human TIA-1 (1139 bp) and a region of β-actin (514 bp) from oligo(dT)-primed cDNA as described (4).

Analysis of TIA-1 Protein/RNA Interactions. Radiolabeled TIA-1 protein was prepared by in vitro transcription/translation of the human TIA-1 cDNA to incorporate ^{35}S-methionine. In vitro transcription reactions incorporating biotin-16-UTP into sense RNAs for COX-2 and GM-CSF AREs (4) were performed using the MAXIscript kit (Ambion) according to the conditions specified by the manufacturer. pTRI-CAT (Ambion) was used to create a control CAT RNA. Biotin-labeled RNA (10 μg/well) was immobilized to NeutrAvidin-coated polystyrene 96-well strip plates blocked with BSA (Pierce Chemical Co.) in 50 μl of 1× RNA binding buffer (4) for 2 h at 25°C and then washed three times with binding buffer. ^{35}S-labeled TIA-1 protein (2 μl/well) was added in binding buffer in a total volume of 50 μl. Each well was incubated for 30 min and next 5 mg/ml heparin was added and incubation continued for 15 min. Wells were washed with binding buffer and RNA-bound TIA-1 was extracted and electrophoresed in 10% SDS-PAGE. The ^{35}S-labeled TIA-1 protein was visualized by autoradiography and quantitated by phosphorimage analysis.

In vitro UV-light cross-linking/label transfer experiments were conducted as described (4) to examine cytoplasmic protein binding to in vitro transcribed ^{32}P-labeled COX-2 ARE RNA. Immunoprecipitation of TIA-1 bound to the COX-2 ARE was performed as described previously (6) using 1 μg of polyclonal anti-TIA-1 antibody (Santa Cruz Biotechnology, Inc.) or isotype-matched IgG.

In vivo cross-linking analysis was performed as described (11). Confluent monolayers of HT29 and LoVo (5×10^6) cells were UV-irradiated in a Stratalinker 2400 (Stratagene) for 3 min and cytoplasmic poly(A) mRNA was isolated using Oligotex beads according to the vendor's protocol. After RNase digestion, samples were separated on 10% SDS-PAGE, transferred to PVDF membranes, and TIA-1 detection was determined by Western blot.

Polysome Profile Analysis. Analysis of polysomes by sucrose density gradient centrifugation was done as described (12). Cytoplasmic lysates were layered on a 15–40% linear sucrose gradient and centrifuged at 38,000 rpm for 127 min at 4°C in a Beckman SW40Ti rotor. The RNA from each gradient fraction (500 μl) was extracted by phenol/chloroform and aliquots were analyzed on a 1% agarose gel to resolve the polysome profile. COX-2 and GAPDH mRNA levels in each fraction were determined as described previously (4).

Results and Discussion

The Translational Silencer TIA-1 Binds the COX-2 AU-Rich Element. Best characterized for their ability to mediate rapid mRNA decay, AREs also function to regulate gene expression by acting as translation inhibitory elements (13, 14). Recent evidence has demonstrated that a central point in COX-2 regulation is mediated through the conserved ARE present in the 3′UTR (4, 5, 7). In normal cells, the COX-2 ARE can serve as a translation inhibitory element to specifically attenuate protein levels independent of rapid mRNA decay (4). We demonstrated that a complex of proteins with molecular weights ranging from 35 to 90-kD regulates posttranscriptional control by binding the ARE-containing 3′UTR of COX-2 (4). However, the trans-acting factors have not been definitively characterized and their role in regulating COX-2 expression is currently unknown. The TIA-1 protein functions as a translational silencer of nonheat shock mRNAs in cells subjected to environmental stress (15, 16). More pertinent to this study, this ubiquitously expressed RNA-binding protein has been demonstrated to confer translational repression of TNF-α

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through binding of the ARE present within the TNF-α 3′UTR (9).

We sought to determine if the COX-2 ARE is a target of TIA-1. RNA-binding assays were conducted using sense RNA for COX-2 and GM-CSF AREs (4) that were transcribed to incorporate biotin-16-UTP. ^{35}S-labeled TIA-1 was incubated with immobilized RNA and allowed to bind. TIA-1 bound to RNA was detected by SDS-PAGE (Fig. 1 A). Approximately 25% of the input TIA-1 was observed to bind the COX-2 ARE; similar levels of binding (29%) were detected using the well-characterized ARE from GM-CSF. A low level of background TIA-1 binding was detected in reactions using a control biotin-labeled RNA of similar length (CAT) or in the absence of RNA ligand (-RNA).

To determine if TIA-1 protein is a component of the COX-2 ARE-binding complex (4), the RNA-binding proteins present in crude (S10) cytoplasmic extracts were covalently cross-linked to the ^{32}P-labeled COX-2 ARE through UV-light irradiation and then subjected to TIA-1 immunoprecipitation (Fig. 1 B). A protein complex ranging from 35 to 90-kD present in HeLa cell lysates associates with the COX-2 ARE (4). Specific immunoprecipitation of UV-cross-linked lysates using anti-TIA-1 antibody identified a ^{32}P-labeled polypeptide migrating at 45-kD. No cross-linked proteins were immunoprecipitated using isotype-matched IgG. Similar results were obtained with immunoprecipitation when using ^{32}P-labeled GM-CSF ARE (unpublished data). These findings indicate that TIA-1 is a component of the COX-2 ARE-binding protein complex and has an ability to interact with other AREs (9, 14).

TIA-1 Protein Regulates COX-2 Expression. The ability of TIA-1 to bind the COX-2 ARE implicates it in COX-2 posttranscriptional regulation and suggests that defects in TIA-1 binding may alter normal COX-2 expression. To examine potential TIA-1 effects on COX-2 expression, endogenous COX-2 mRNA and protein expression were compared in wild-type and TIA-1–deficient (TIA-1$^{-/-}$) mouse embryonic fibroblasts (MEFs). Early passage MEFs derived from wild-type and TIA-1$^{-/-}$ mice were cultured under identical conditions in the presence of serum and assayed for the expression of COX-2 protein. As shown in Fig. 2 A, an ∼two- to threefold increase in COX-2 protein expression was consistently observed in the TIA-1$^{-/-}$ MEFs. We and others previously demonstrated the ability of the ARE-binding proteins TIAR and HuR to regulate the expression of COX-2 and other ARE-containing mRNAs (6, 14). The levels of the ARE-binding proteins TIAR and HuR were examined in both wild-type and TIA-1$^{-/-}$ MEFs. No differences in the levels of TIAR or HuR were detected in either cell type, whereas TIA-1 was detected only in the wild-type cells (Fig. 2 A). In addition, cyclooxygenase activity present in TIA-1$^{-/-}$ MEFs pro-

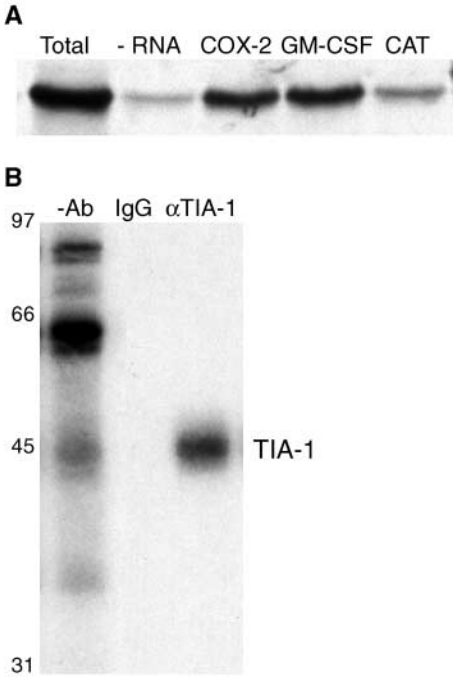

Figure 1. TIA-1 binds the COX-2 ARE. (A) Biotin-labeled RNA containing the COX-2 ARE, GM-CSF ARE, or control CAT RNA were incubated with in vitro translated ^{35}S-labeled TIA-1 protein. TIA-1 bound to immobilized RNA was detected by electrophoresis on SDS-PAGE. Total TIA-1 protein used per reaction is shown in the first lane and nonspecific binding is shown in the second lane. (B) Cytoplasmic HeLa lysate was incubated with ^{32}P-labeled COX-2 ARE and bound proteins were cross-linked to RNA by UV-light irradiation. Reactions were immunoprecipitated using anti-TIA-1 antibody (α-TIA-1) or IgG. The -Ab lane shows the bound proteins in 1/10 of the cross-linking reaction before immunoprecipitation. Molecular weight standards (kD) are listed on the left.

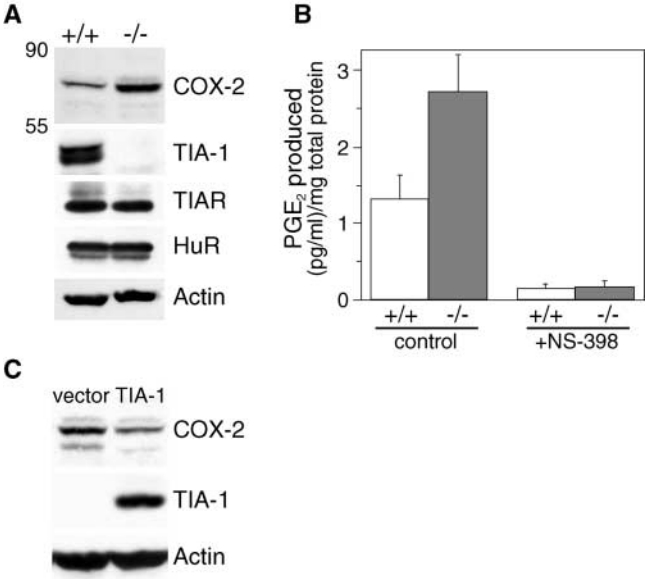

Figure 2. TIA-1 inhibits COX-2 expression. (A) MEFs derived from wild-type or TIA-1$^{-/-}$ mice were examined for COX-2 protein expression by Western blot. Molecular weight standards (kD) are listed on the left. (B) COX activity measured by PGE_2 production in wild-type (open bars) and TIA-1$^{-/-}$ (filled bars) MEFs treated with carrier or NS-398. (C) Western blot of COX-2 protein in TIA-1$^{-/-}$ MEFs stably transfected with a TIA-1 expression construct or vector.

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duced approximately twofold more prostaglandin PGE_2 than wild-type MEFs (Fig. 2 B). Blockade of COX-2 activity with the COX-2 inhibitor NS-398 left trace levels of PGE_2 in both cell lines. To determine if down-regulation of COX-2 could be restored, TIA-$1^{-/-}$ MEFs were stably transfected with a TIA-1 expression construct. In TIA-$1^{-/-}$ MEFs cells stably expressing TIA-1, the expression of COX-2 protein was only one-half of that observed in vector-transfected cells (Fig. 2 C). We conclude that the difference in COX-2 expression detected between TIA-$1^{-/-}$ and wild-type cells resulted from the absence of TIA-1.

To determine whether the enhanced expression of COX-2 protein observed in TIA-$1^{-/-}$ cells is a result of increased *COX-2* transcription, the steady-state level of COX-2 mRNA in TIA-$1^{-/-}$ and wild-type MEFs was examined. Growth in serum-containing media resulted in similar steady-state COX-2 mRNA observed in both cells (Fig. 3 A). In addition, a reporter construct containing a 1.8-kb fragment of the COX-2 promoter demonstrated similar levels of *COX-2* transcription in both cell types (Fig. 3 B). MEFs grown under serum-free conditions resulted in low-to-undetectable COX-2 mRNA and protein expression, suggesting that the induction of COX-2 transcription in both cell types is a result of growth factor stimulation (unpublished data). As both the wild-type and TIA-$1^{-/-}$ MEFs displayed similar levels of COX-2 mRNA, it is unlikely that loss of TIA-1 altered the rapid decay of COX-2 mRNA. To directly assess this, we measured the half-life of endogenous COX-2 mRNA in both cell types. The results shown in Fig. 3 C demonstrate that rapid COX-2 mRNA decay is seen in both wild-type ($t_{1/2}$ = 14 min) and TIA-$1^{-/-}$ MEFs ($t_{1/2}$ = 15 min). Rapid turnover of constitutively expressed c-myc mRNA was seen in both wild-type and TIA-$1^{-/-}$ cells ($t_{1/2}$ = 15 and 16 min, respectively). These results demonstrate that absence of TIA-1 does not alter either the transcription of the *COX-2* gene or the stability of its mRNA. This is consistent with the ability of AREs present within various cytokine mRNAs to act as translation inhibitory elements similar to the COX-2 ARE. In these studies, the mRNA levels of the respective genes were virtually unaffected, demonstrating the ability of AREs to act as translation inhibitory elements by imposing translational blockade (13, 14).

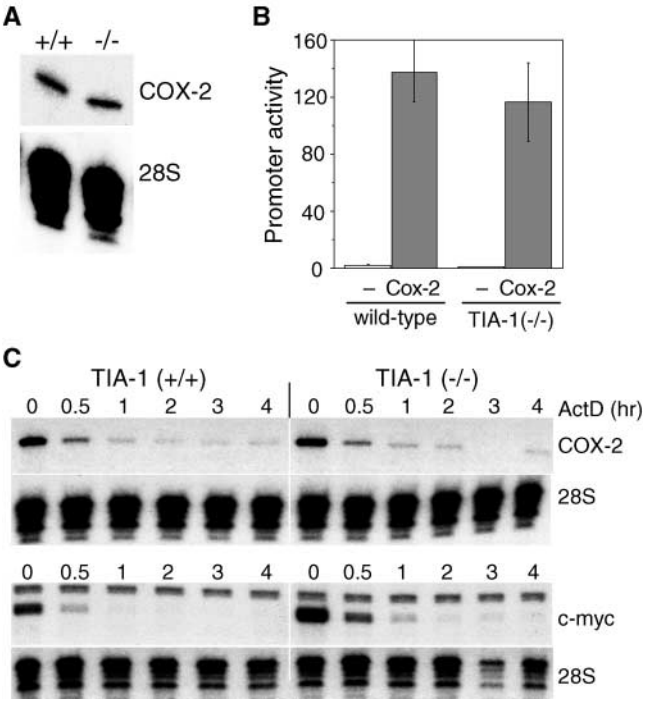

Figure 3. TIA-1 does not alter rapid COX-2 mRNA decay. (A) Steady-state COX-2 mRNA in wild-type and TIA-$1^{-/-}$ MEFs levels were detected by RNase protection assay. (B) COX-2 transcription was determined using a luciferase reporter construct containing either the COX-2 promoter (filled bars) or vector (open bars) cotransfected with pSV-βgal. (C) COX-2 and c-myc mRNA half-life experiments were initiated by adding 5 μg/ml ActD to MEFs for the indicated times and decay was analyzed by RNase protection assay. 28S RNA is shown as a control for RNA loading.

Loss of Translational Control Promotes COX-2 Expression in Colon Cancer Cells. It has been shown that constitutive transcription of *COX-2* initiates unregulated expression of the protein in colon cancer (3), yet growing evidence supports the notion that posttranscriptional regulation of COX-2 expression also plays a central role (4, 5, 7). Cellular defects in this facet of gene regulation result in elevated COX-2 protein expression, which in turn promotes cell growth through enhanced prostaglandin synthesis (5, 6). Previously, we demonstrated that altered recognition of the COX-2 ARE in colon cancer cells correlates with enhanced expression of COX-2 (6). We postulated that the binding of cellular trans-acting regulatory proteins directly influences stability and translation of COX-2 mRNA. The human colon cancer cell lines HT29 and LoVo display constitutive transcription of *COX-2* (6). Yet, HT29 cells have six- to sevenfold more COX-2 protein and associated prostaglandin synthesis than LoVo cells (6). This increase in COX-2 protein despite constitutive transcription of COX-2 mRNA in both cell types suggests that the translational control of COX-2 is altered in the HT29 cells.

This was directly examined through polysome profile analysis of the translational status of COX-2 mRNA in colon cancer cells. HT29 and LoVo cytoplasmic extracts were fractionated over sucrose gradients and evaluated for differences in distribution of COX-2 mRNA. As shown in Fig. 4, COX-2 transcripts were primarily associated with the nonpolysomal fractions in LoVo cells. This is consistent with polysome profiles demonstrating the ability of functional AREs to exclude transcripts from polysome association (17) and suggests that the COX-2 ARE acts similarly to block mRNA translation at the initiation step involving the formation of 80S particles (18). In contrast to this, there is a clear shift of COX-2 mRNA toward the polysomal fractions in the HT29 cells suggesting the loss of ARE-mediated translational repression. Control polysome profile of endogenous GAPDH mRNA showed similar profiles for each cell type (unpublished data). These results reflect the increased levels of COX-2 protein expression observed in HT29 cells when compared with LoVo cells (6) and agree

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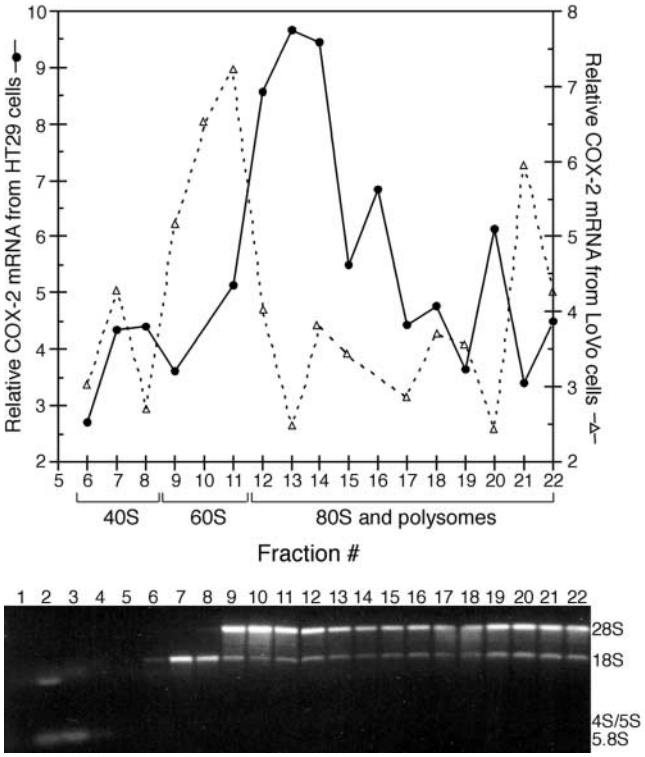

Figure 4. COX-2 polysome profile in colon cancer cells. HT29 and LoVo cytoplasmic lysates were fractionated on a continuous 15–40% sucrose gradient. Polysome profiles were obtained by running RNA samples from each fraction on an agarose gel to identify the 40S and 60S ribosomal subunits along with 80S and polysomes (bottom panel). The distribution of COX-2 mRNA from HT29 cells (filled circles) and LoVo cells (open triangles) was detected by RNase protection assay of gradient fractions.

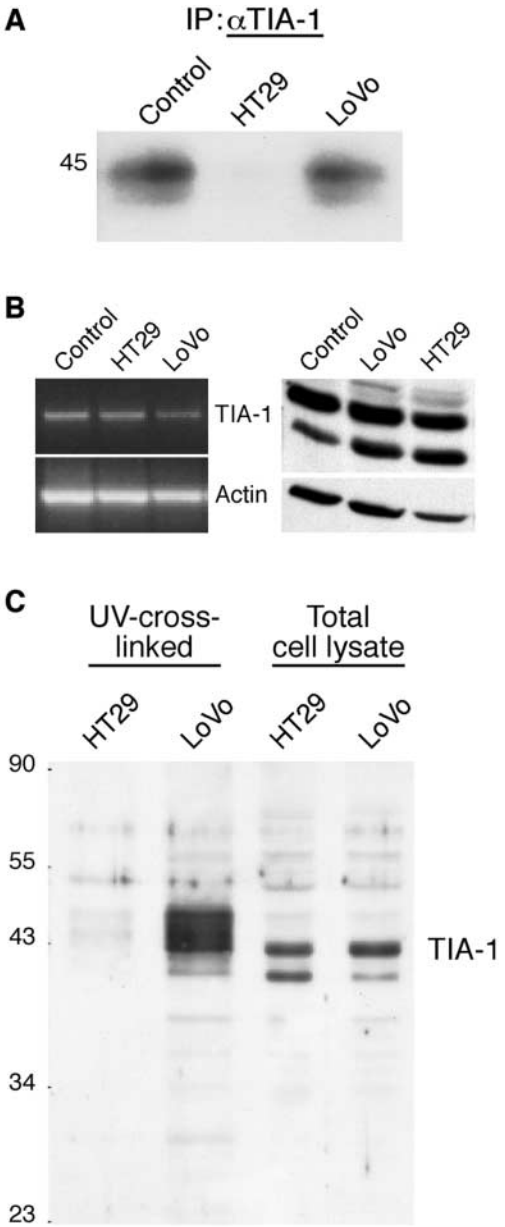

Figure 5. Deficient binding of TIA-1 occurs in HT29 cells. (A) Cytoplasmic protein complexes from control HeLa, HT29, and LoVo cells bound to the ^{32}P-labeled COX-2 ARE were UV-cross-linked and immunoprecipitated using TIA-1 antibody. (B) RT-PCR (left panel) and Western blot (right panel) analysis of TIA-1 and β-actin from control HeLa, HT29, and LoVo cells. (C) In vivo cross-linking of TIA-1 protein to poly(A) mRNA was examined in HT29 and LoVo cells.

with previous findings demonstrating the ability of the COX-2 3′UTR to block mRNA translation in cells displaying ARE-mediated posttranscriptional regulation of COX-2 (4).

Defective TIA-1 Binding Promotes COX-2 Expression in Colon Cancer Cells. Based on these results that implicated TIA-1 as a translational silencer of COX-2 expression, we sought to determine whether defects in its ability to bind the COX-2 ARE contribute to enhance COX-2 translation and promote overexpression in HT29 colon cancer cells. The ability of TIA-1 to bind the COX-2 ARE was examined by immunoprecipitation of cross-linked lysates from HT29 and LoVo cells. Immunoprecipitation of labeled proteins of 45-kD were detected in both LoVo and control HeLa cells; however, no detectable binding was observed in lysates from HT29 cells (Fig. 5 A). This apparent defect is not due to a lack of expression in HT29 cells since similar levels of TIA-1 mRNA and protein were detected in all cells (Fig. 5 B).

The lack of ARE-binding by TIA-1 in HT29 cells was examined further by UV-cross-linking studies of TIA-1 with poly(A) RNA in intact cells. After exposure to UV-light, poly(A) RNA was isolated from equal numbers of HT29 and LoVo cells. The cross-linked RNA-binding proteins were separated by SDS-PAGE and TIA-1 was detected by immunoblotting. Consistent with the immunoprecipitation results, TIA-1 protein was cross-linked to mRNA in LoVo cells and there was no association of TIA-1 with mRNA in HT29 cells (Fig. 5 C). TIA-1 is known to have alternative splicing (19) which accounts for the protein doublet. The slightly higher mobility of cross-linked TIA-1 is presumably due to covalent attachment of the RNA moiety; no protein binding to mRNA was detected when UV-irradiation was omitted (unpublished data).

As LoVo cells display intact TIA-1 binding and translational repression of COX-2, we attribute loss of ARE-mediated translational regulation in HT29 cells, in part, to defects in the ability of TIA-1 to bind the COX-2 ARE. Accordingly, inhibition of TIA-1 activity using a dominant-negative mutant of TIA-1 promotes expression of COX-2 in LoVo cells (unpublished data). Based on this evidence, it is likely that TIA-1 represses COX-2 mRNA translation through the assembly of nonpolysomal complexes, similar to that seen with TNF-α transcripts (16). Interestingly, lack of TIA-1 expression does not explain the differences in COX-2 expression between HT29 and LoVo cells and ectopic overexpression of TIA-1 in HT29 cells does not promote COX-2 translational repression (unpublished data). These findings suggest that binding of TIA-1 to the COX-2 ARE is influenced by other factors. Our previous observations demonstrated increased expression of the ARE-binding protein HuR in HT29 cells, resulting in a lengthened COX-2 mRNA half-life (6). This suggests that limited TIA-1 binding observed in HT29 cells may be a direct result of increased competition for ARE-binding sites due to elevated HuR levels. With regard to this, we also observe decreased ARE-binding of the TIA-1–related protein TIAR in HT29 cells (unpublished data). More importantly, this effect may extend to colon tumors where increased HuR levels are also observed (6). Consistent with this notion is the ability of the ARE-binding protein AUF1 to promote tumorigenesis when overexpressed in mice (20) and the observed competition for ARE-binding between HuR and TTP proteins after T lymphocyte activation (21).

The results presented here expand on our original observations linking tumorigenesis to the loss of posttranscriptional regulation of COX-2 expression. It is well accepted that transcriptional activation of *COX-2* is an early event in the initiation of colon tumorigenesis (3, 6). However, enhanced expression of COX-2 protein may also require aberrant posttranscriptional regulation in many instances. Our results demonstrate a novel link between control of COX-2 expression and the RNA-binding protein TIA-1. While other components of ARE-mediated regulation remain to be elucidated, these studies implicate a role for TIA-1 as a tumor suppressor in its ability to function as a translational silencer of COX-2. Defects in TIA-1 activity may result in unregulated expression of COX-2 during the later stages of tumor progression.

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