Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 5544–5549, May 1996
Pharmacology

Interaction of cyclooxygenases with an apoptosis- and autoimmunity-associated protein

(prostaglandins/systemic lupus erythematosis/programmed cell death/yeast two-hybrid system)

BRYAN A. BALLIF, NATALIE V. MINCEK, JUSTIN T. BARRATT, MICHAEL L. WILSON, AND DANIEL L. SIMMONS*

Department of Chemistry and Biochemistry, Brigham Young University, E280 Benson Science Building, Provo, UT 84602

Communicated by John Vane, The William Harvey Research Institute, London, United Kingdom, January 2, 1996 (received for review December 7, 1995)

ABSTRACT **Cyclooxygenases (COXs) 1 and 2 are 72-kDa, intralumenal residents of the endoplasmic reticulum (ER) and nuclear envelope, where they catalyze the rate-limiting steps in the conversion of arachidonate to the physiologically dynamic prostanoids. Recent studies, including the generation of knockout mice, show COX-1 and COX-2 to have biologically distinct roles within cells and organisms. Also apparent is that arachidonate substrate is selectably metabolized by COX-2 after mitogen stimulation in many cells that contain both isoforms. Because COX-1 and COX-2 are highly conserved in all residues needed for catalysis and in their purified forms have almost identical kinetic properties, we have searched for COX-interacting ER proteins that might mediate these unique isoenzymic properties. Using COXs as bait in the yeast two-hybrid system, we identified autoimmunity- and apoptosis-associated nucleobindin (Nuc) as a protein that specifically interacts with both isoenzymes. COX–Nuc binding was substantiated by immunoprecipitation experiments, which showed that COX-1 and, to a lesser extent, COX-2 form complexes with Nuc *in vitro*. When overexpressed in COS-1 cells, Nuc was found to be extracellularly released. However, when Nuc was co-overexpressed with COX-1 or COX-2, its release was reduced by >80%. This finding suggests that COX isoenzymes participate in the retention of Nuc within the lumen of the ER, where COX may regulate the release of Nuc from the cell. It also identifies Nuc as a potential regulator of COXs through this interaction.**

Two cyclooxygenase (COX) isoforms (COX-1 and COX-2) that differ significantly in their pattern of expression in higher organisms have been identified. COX-1 is typically expressed as a constitutive enzyme, whereas COX-2 is expressed in most tissues and cells at very low levels unless induced by mitogenic or hormonal stimuli (1–4). After this stimulation, cells expressing COX-2 synthesize and release increased levels of prostanoids, often prostaglandin E_2, into extracellular fluids.

COX-1 and COX-2 are very similar in structure and share all critical amino acids required for prostaglandin H_2 synthesis from arachidonate. In their purified forms, the COX isoforms show nearly identical catalytic properties toward arachidonate metabolism (5). Yet, the recent generation of mouse strains deficient in COX-1 and COX-2 shows that these isoenzymes have separate and distinct roles, as the mouse mutants differ in phenotype (6, 7). This concept is further confirmed by cell studies *in vitro*. For example, in cells expressing high levels of both isoforms, the marked increase in prostaglandin synthesis that follows mitogen-induced arachidonate release is prevented by COX-2-specific inhibitors, such as dexamethasone or COX-2 antisense oligonucleotides (8). Furthermore, antisense oligonucleotides against 85-kDa cytosolic phospholipase also inhibit this mitogen-stimulated prostaglandin synthesis (9). Taken together, these observations imply the existence of a COX-2-specific substrate delivery system, possibly through cytosolic phospholipase.

COX isoenzymes in different cell types often differ radically in their sensitivity to nonsteroidal anti-inflammatory drugs (NSAIDs). For example, NSAIDs frequently inhibit COX activity in macrophages at doses that are orders of magnitude lower than those required in fibroblasts (refs. 10 and 11; unpublished observations). Mechanisms that alter intracellular drug concentration (e.g., catabolism and drug pumps) may in some cases be responsible for this difference in NSAID reactivity between cell types. However, cell-specific NSAID sensitivity may also indicate that COX isoenzymes can have distinct microenvironments that alter their reactivities to NSAIDs and influence their catalytic properties.

The above data raise the question of whether COX-1 and COX-2 form complexes with other proteins, resulting in isoenzymic differences in substrate delivery, NSAID inhibition, signal transduction, and catalytic regulation. To examine this possibility, we used the yeast two-hybrid system to search for COX-interacting proteins. This approach identified nucleobindin (Nuc) as a protein that binds specifically to COX enzymes, a finding that was confirmed by biochemical and cellular studies.

Unlike COX enzymes that are firmly bound to the lumenal surface of the endoplasmic reticulum (ER) and nuclear envelope, the 55-kDa, hydrophilic Nuc contains features of a secretory protein, including (as shown here) a functional amino-terminal signal peptide and the lack of a consensus carboxyl terminal ER-retention sequence. In spite of these features promoting its secretion, Nuc is not typically found in blood plasma or in the media of cultured cells, even though the mRNA of Nuc is widely expressed in cells and tissues (12, 13). However, Nuc has been isolated from the sera of mice prone to the autoimmune disorder systemic lupus erythematosis (SLE), as well as from the growth media of a lymphocyte cell line established from these mice (12, 14, 15). When isolated from these sources, Nuc is found bound to nucleosomal-laddered DNA. Normal mice injected with Nuc develop thymic apoptosis and many of the symptoms of SLE, indicating that Nuc may play a role in the pathogenesis of this disease (16–18). The results presented here suggest that COXs interact with Nuc within the lumen of the ER and there participate in Nuc's normal intracellular retention and function. This interaction also suggests that Nuc and COX may be functionally important in regulating each other during inflammation, apoptosis, or autoimmune disease.

MATERIALS AND METHODS

Plasmids. Constructs for expression of murine COX-1, COX-2, and Nuc in mammalian cells were made by ligating

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: COX, cyclooxygenase; Nuc, Nucleobindin; SLE, systemic lupus erythematosis; NSAID, nonsteroidal anti-inflammatory drug; ER, endoplasmic reticulum.

*To whom reprint requests should be sent.

their respective cDNA coding regions into pSG5 (Stratagene). For yeast two-hybrid screening (19), COX cDNA fragments were cloned into pBTM116 (P. Bartel and S. Fields, State University of New York at Stony Brook) so as to fuse COX-1 or COX-2 segments to the LexA DNA-binding transcription factor (20). The cDNA library screened was in pVP16 (S. Hollenberg, Fred Hutchinson Cancer Research Center), where library inserts were fused with the VP16 transcription activating domain (20). cDNAs used to construct this library were generated by random priming of mRNA template from 9.5- to 10.5-day mouse embryos and were size-fractionated to ≈350–750 bp (20). All constructs used for expression of COX isoforms or Nuc were verified by restriction endonuclease analysis and dideoxy DNA sequencing. In addition to carrying ampicillin resistance genes, plasmids pBTM116 and pVP16 carry the selectable markers *TRP1* and *LEU2*, respectively.

Yeast Two-Hybrid Screening and Testing of Positive Clones. The yeast two-hybrid system for identifying protein–protein interactions has been described in detail by others (19, 20). Briefly, yeast strain L40 (*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ GAL4 gal8*; S. Hollenberg) harboring plasmids for LexA–COX expression (Fig. 1) was transfected with the pVP16 mouse embryo cDNA library. Yeast transfection was a modification of the lithium acetate carrier technique (20, 22). Double-transfectants encoding COX-interacting proteins were screened for on Yc (-)thull media deficient in tryptophan, histidine, uracil, leucine, and lysine and containing 1 mM 1,2,4-3-aminotriazole (Sigma). Surviving yeast colonies were subsequently assayed for β-galactosidase activity as described (20). Plasmid DNA from β-galactosidase-positive colonies was extracted from yeast (23) and was shuttled via electroporation into *Escherichia coli* strain MH_4 (auxotrophic for leucine). Colonies containing pVP16 library plasmids were selected for on minimal media plates containing 0.1 mg/ml of ampicillin (Sigma). Plasmids were isolated from bacteria surviving this selection and were then transfected again into L40 yeast expressing LexA–COX fusions. Double transfectants were subjected to a second round of testing to verify the ability of the library plasmids to induce transactivation of the *HIS3* and *lacZ* reporter genes. All pVP16 library plasmids that passed the above tests were transfected alone into L40 yeast. If their encoded proteins possessed intrinsic transactivating activity toward the reporter genes, they were discarded. The positive plasmids remaining were paired with plasmids encoding lamin C (24) and the murine homolog of chicken embryo fibroblast (CEF) 10, a secreted cytokine (25), fused to LexA. This pairing tested for promiscuous binding of the pVP16-encoded proteins to polypeptides other than LexA–COX. Clones encoding proteins that interacted with either of these proteins were discarded. Plasmids that passed the above tests were sequenced by the dideoxy method.

***In Vitro* Translation and COX–Nuc Binding Assays.** Full-length COX-1, COX-2, and Nuc cDNAs in Bluescript (Stratagene) plasmids were transcribed using T3 or T7 RNA polymerases (United States Biochemical) as described (2). For this procedure, a full-length Nuc cDNA was isolated by plaque hybridization from a λZAP (Stratagene) murine fibroblast cDNA phage library. Transcripts were placed individually into nuclease-treated rabbit reticulocyte lysate (Promega) containing [^{35}S]methionine in the presence or absence of canine pancreatic microsomes (Promega), and translation products were analyzed by PAGE as described (2).

To assay for COX–Nuc complexes, COX and Nuc translation products in rabbit reticulocyte lysate were mixed with each other to allow heterodimerization of COX and Nuc. COX–Nuc mixtures were incubated at 37°C for 2 hr, and complexes were then subjected to immunoprecipitation using polyclonal anti-sheep COX-1 antisera (L. Marnett, Vanderbilt University). This antiserum reacts well with both COX-1 and COX-2 from mice (data not shown). Various ratios of COX/Nuc, were tested by varying the volume of COX- or Nuc-containing lysate that was added to the binding reaction. The final volume of each reaction mixture was then equalized with unprogrammed translation reaction mixture. Because Nuc is known to bind calcium, which potentially could affect its binding (26), the binding reaction was done multiple times in the presence and absence of 7 mM calcium chloride.

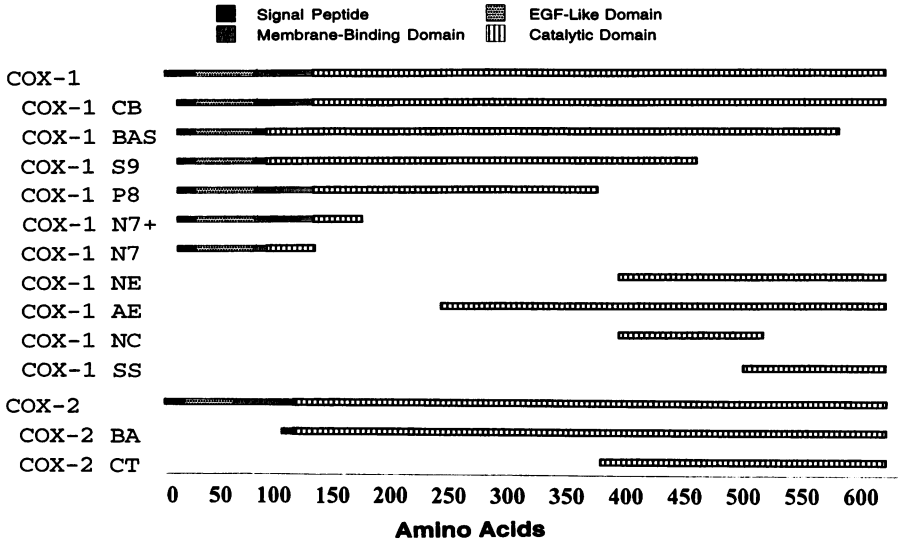

FIG. 1. LexA–COX fusions used in the yeast two-hybrid system. The 14 COX constructs depicted were fused to LexA in pBTM116. All but COX-1 NC and COX-2 CT were used for library screening. NC and CT were used in determining the Nuc-binding domain in COX. Constructs COX-1 BAS, S9, and N7 have amino acids Trp^{77}–Val^{113} replaced with the sequence Gly-Thr-Asp-Gln-Asp-Pro to render nonfunctional the predicted membrane-binding domain of Picot and coworkers (21), and thus, this region appears shortened in the figure. The COX-1 amino acids present in constructs are as follows: BAS, Pro^{11}–Leu^{602}; S9, Pro^{11}–Gln^{481}; N7, Pro^{11}–Thr^{163}; NE, Met^{381}–Leu^{602}; CB, Pro^{11}–Leu^{602}; P8, Pro^{11}–Gln^{358}; N7+, Pro^{11}–Thr^{163}; SS, Thr^{484}–Thr^{600}; NC, Met^{381}–Ile^{500}, and AE, Asp^{231}–Leu^{602}. COX-2 constructs contained the following amino acids: BA, Lys^{100}–Leu^{604}, and CT, Phe^{364}–Leu^{604}. Constructs COX-1 and COX-2 contain the entire coding sequence of each isoenzyme, respectively. A crystal structure of COX-2 has not been published; predicted functional domains shown in this figure for COX-2 are based solely on amino acid alignment with COX-1. Nuc was first detected in the two-hybrid screen by construct COX-1 AE.

Immunoprecipitation Experiments. Analysis by immunoprecipitation of Nuc and COX was done in an aqueous solution containing 1% NP-40, 0.5% sodium deoxycholate, 100 mM NaCl, and 10 mM Tris (pH 7.4). We have described the antibody binding and washing procedure previously (27). The support matrix was protein A Sepharose (Sigma). Immunoprecipitates were analyzed by SDS/PAGE followed by autoradiography and densitometric analysis.

Production of Polyclonal Anti-Nuc Antibodies. Full-length Nuc cDNA was cleaved with *Sst*I to isolate a fragment containing the entire Nuc coding region minus a sequence encoding 82 amino acids of the amino terminus, which was cloned in-frame into the pGEX-3X (Pharmacia) prokaryotic expression vector and transformed into XL-1 Blue (Stratagene) *E. coli*. An SDS polyacrylamide gel slice containing the glutathione *S*-transferase–Nuc fusion protein expressed in these cells was added to Freund's complete adjuvant, and an emulsion that was injected into New Zealand White rabbits was made. Rabbits were subsequently boosted at weekly intervals with the same antigen in incomplete adjuvant. Immune sera, tested by immunoprecipitation of recombinant Nuc, contained high titers of anti-Nuc antibody, whereas pre-immune sera completely lacked anti-Nuc activity (data not shown).

Expression Plasmid Transfection into Mammalian Cells. COS-1 cells were obtained from the American Type Tissue Culture Collection and were cultured in 60-mm dishes in DMEM containing 10% fetal calf serum. Expression plasmids were transfected into cells by liposome transfer (ref. 28; Lipofectin Reagent, GIBCO/BRL). The procedure for radiolabeling transfected cells with [^{35}S]methionine has been described (27). After 3 hr of labeling, the growth media were collected and centrifuged at 105,000 × *g* for 1 hr at 4°C, and proteins in the supernatant were subjected to trichloroacetic acid precipitation and measurement by scintillation spectrometry as described (2) to ensure that equal amounts of radioactively labeled protein was used in Nuc immunoprecipitation experiments.

RESULTS

Cloning of Nuc by the Two-Hybrid Method. LexA–COX-1 and LexA–COX-2 constructs, differing in the regions of COX isoenzymes expressed, were inserted into pBTM16 and were used as bait in the yeast two-hybrid system. Approximately 4 × 10^7 library transfectants were screened for heterodimerization of encoded VP16-fusion proteins with LexA–COX in L40 yeast. Of these, 1.8 × 10^7 were paired with LexA–COX-1 constructs, and 2.2 × 10^7 were paired with LexA–COX-2 constructs. From these combined screens, a total of ≈1200 colonies survived selection on Yc (-)thull media, but only 543 of these contained detectable β-galactosidase activity and were studied further. When transfected alone, 272 of the 543 clones were eliminated because they showed intrinsic transactivation of reporter genes. Another 184 were eliminated for nonspecific binding activity to heterologous proteins lamin C and murine CEF-10. Eighty-six clones were not subjected to all tests because β-galactosidase assays showed that they exhibited a very strong transactivation of the *lacZ* reporter gene characteristic of the 272 clones we characterized whose proteins possessed intrinsic transactivating activity. Only two clones survived all tests and one of these contained Nuc cDNA sequence −21 to 369. This clone encoded amino acids 1–123 of Nuc plus seven additional amino acids at the amino terminus. The other clone showed a weaker interaction with COXs and was unknown to genetic data bases (data not shown). COX constructs used to screen libraries as well as those constructed to determine the Nuc-binding domain of COX are shown in Fig. 1.

During two-hybrid library screening, we found that the largest LexA–COX fusion proteins (e.g., COX-1 BAS, COX-1, and COX-2; Fig. 1) were either unstable or poorly expressed, a phenomenon described by Bartel and Fields (29). These large baits failed to produce colonies in our library screens, and immunoprecipitation experiments using anti-COX-1 sera failed to detect any fusion protein in the yeast cytosol in cells transfected with either the COX-1 or the COX-1 BAS constructs (data not shown).

The presence of the predicted membrane-binding domain of Picot and coworkers (21) in constructs such as COX-1 CB, P8, and N7+ (Fig. 1) also prevented reporter gene transactivation, as would be predicted for a monotopic method of membrane binding that could adhere the bait protein to membrane and prevent its translocation to the nucleus. For this reason, this region was modified by partial deletion and the insertion of an adaptor in constructs COX-1 BAS, S9, and N7 (Fig. 1).

As with large or membrane-binding domain-containing constructs, short carboxyl-terminal COX-1 baits NE and SS (Fig. 1) were also found to be unsuitable for library screening because they had intrinsic transactivation activity toward the reporter genes. This property was lost in the larger carboxyl-terminal construct, COX-1 AE (Fig. 1), which first identified Nuc as a COX-associated protein in the library screen. Baits that interacted with Nuc (COX-1 AE, COX-1 NC, and COX-2 CT) shared a region (Met^{381}–Gly^{498}; using the translation start site in murine COX-1 as reference) that was contained in fragment COX-1 NC (Fig. 1).

Fig. 2*A* delineates the known functional domains of Nuc. These include the COX-binding domain, a functional calcium-binding domain, a DNA-binding domain, a predicted nuclear localization signal, and a leucine zipper. Fig. 2 *B* and *C* use the x-ray crystallographic structure of COX-1 (21) to identify the three-dimensional position of Met^{381}–Gly^{498} in the tertiary structure of COX-1. Fig. 2*B* shows the position of the Nuc-binding site with regard to the membrane-binding and dimerization domains as proposed in the original crystallographic study (21). Fig. 2*C* depicts COX-1 from an alternate angle that better demonstrates the presence of two potential Nuc-binding domains on the hydrophilic exterior of the COX-1 catalytic domain; one of these two domains (NC domain 1) is adjacent to the peroxidase active site, and the other (NC domain 2) is more proximal to the membrane-binding and dimerization domains.

***In Vitro* Testing of COX–Nuc Interaction.** To test *in vitro* the binding of Nuc to COXs, full-length COX-1 and COX-2 cRNA transcripts were translated in rabbit reticulocyte lysate and then mixed with *in vitro*-translated, full-length Nuc. Immunoprecipitation using polyclonal anti-COX antibodies was performed on the resulting complex in an attempt to co-immunoprecipitate COXs and Nuc. Surprisingly, rather than retrieving the expected Nuc–COX complex, the anti-COX antibodies were blocked from COX epitopes in proportion to the amount of Nuc in the binding reaction (Fig. 3). At saturation (1:5, COX-1/Nuc), 72% (but as high as 90% in some instances) of COX-1 was blocked from immunoprecipitation (lane 4). In contrast, immunoprecipitation of COX-2 was in all cases less dramatically reduced (40%) at saturation levels (1:5, COX-2/Nuc) of Nuc (lane 8), which was the same reduction achieved for COX-1 at a ratio of 1:0.5 (lane 2). Hence, Nuc bound COX-1 significantly more efficiently than COX-2.

In separate experiments, we determined that unbound Nuc exhibits weak, nonspecific interaction with the protein A Sepharose used in the immunoprecipitation reaction (lane 9). We exploited this nonspecific binding to qualitatively evaluate the amount of unbound Nuc present after COX–Nuc mixing. The concentration of free Nuc after mixing inversely correlated with the blocking of COX immunoprecipitation. Thus, in 1:0.5 (lane 2) and 1:1 (lane 3) COX-1/Nuc mixtures, little free Nuc was observed, whereas a concentration-dependent (COX-1/Nuc) reduction of immunoprecipitable COX-1 was observed in these same mixtures. In the 1:5 (lane 4) COX-1/Nuc mixture, little additional blocking of immunoprecipitation occurred relative to the 1:1 (lane 3) COX-1/Nuc mixture.

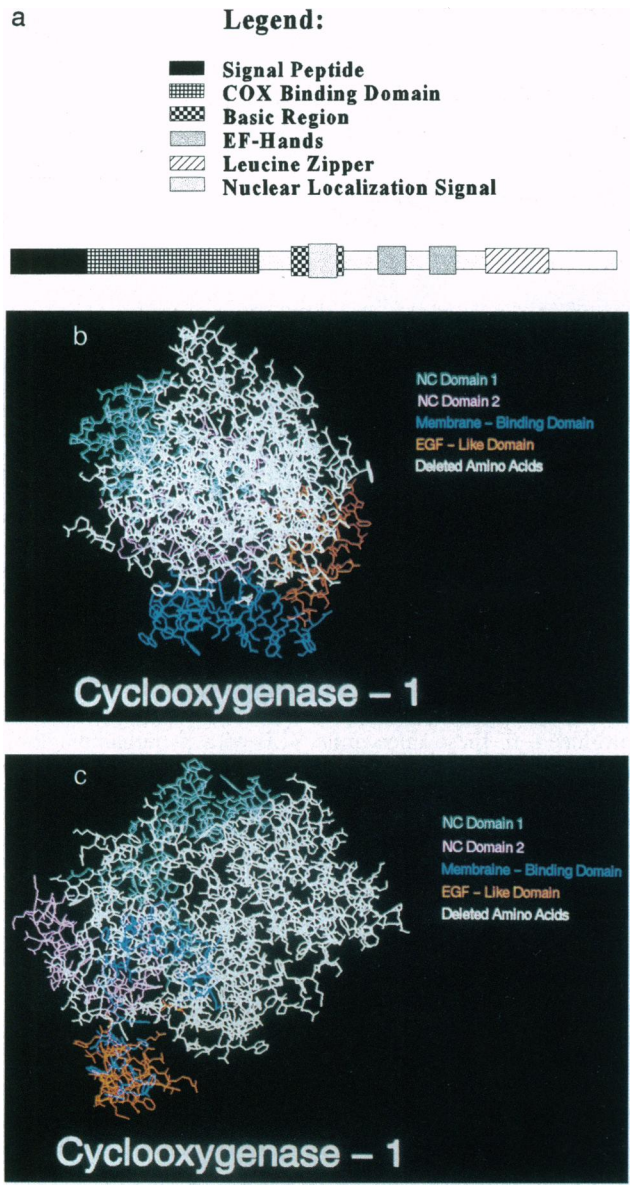

FIG. 2. (*A*) Functional domains of murine Nuc. Nuc is made as a 455-amino acid precursor protein with a hydrophobic amino terminal region and a predicted signal peptide cleavage site between amino acids 25 and 26. Known domains of Nuc are indicated. The COX-binding domain as defined in the two-hybrid system is Met^{1}–Glu^{123}. Nuc's basic, DNA-binding region is at His^{171}–Lys^{217} and contains a predicted bipartite nuclear localization signal at Arg^{198}–Arg^{213}. Two calcium-binding EF-hands (Asp^{252}–Glu^{263} and Asp^{304}–Glu^{315}) are operative (13, 26). However, Nuc's binding to DNA (14) and COX enzymes (see Fig. 3) is not regulated by calcium *in vitro*. A leucine zipper begins at Leu^{346} and extends through Leu^{398}. Not shown is the binding site for Gα proteins, which has been determined to be carboxyl-terminal to amino acid 139 of Nuc (30). (*B*) Computer-aided structural analysis of two-hybrid bait COX-1 NC. The computer-modeled COX-1 crystal structure (ref. 21; Brookhaven accession code 1PRH) is used to display the spacial location of COX-1 NC, the smallest COX fragment we have found to bind Nuc. One-half of the COX dimer is viewed in profile as originally done by Picot and coworkers (21). The NC domain corresponds to Met^{381}–Gly^{498} of murine COX-1 and is divided into two regions, NC1 and NC2, by color coding. The catalytic domain, except for the NC domains, is white. NC1 is at the lower lip of the peroxidase cleft at the top of the catalytic domain. NC2 is posterior in this photograph. (*C*) Top view of domains NC-1 and NC-2. Viewed from the top (i.e., lumenal) side of the enzyme, both Nuc-binding domains project into solvent. Amino acids 481–498 of NC-2 are seen in this orientation as protruding into solvent at the far left of the monomer.

However, substantial free Nuc was present in this mixture, suggesting that COX-1 was maximally saturated with Nuc. This correlation was also observed for COX-2, where Nuc produced little reduction of immunoprecipitation and where concomitant high levels of free Nuc were observed. The fact that Nuc binding was not out-competed by anti-COX antibody, and that epitope blocking occurred with COX and Nuc at sub-nanogram amounts in high concentrations of heterologous lysate proteins, suggested a strong COX–Nuc interaction, particularly for COX-1. The above results were reproducible in the presence or absence of 7 mM calcium chloride (data not shown).

COX Prevents Nuc Secretion *in Vivo*. Given the *in vitro* data showing high-affinity binding of Nuc with COX-1, and to a lesser extent with COX-2, we wished to determine (*i*) whether COX-1 and COX-2 interacted *in vivo* with Nuc and (*ii*) whether Nuc, in the absence of binding to COX, would be extracellularly released from cells. COS-1 cells, which contain low basal levels of COX-1 and COX-2 (unpublished data), were cotransfected with Nuc-expression plasmid and empty pSG5 expression vector, or with Nuc cotransfected with COX-1 or COX-2 expression vectors. Anti-Nuc sera were used to immunoprecipitate Nuc from the growth media of these cells and percent reduction in Nuc's extracellular release was measured by autoradiography and densitometry. Cells expressing Nuc in the absence of coexpressed COX released low but detectable amounts of Nuc into the growth media (Fig. 4*A*). In contrast, cells overexpressing both Nuc and COX enzymes showed a reduction of 86% and 81% (COX-1 and COX-2, respectively) in released Nuc (Fig. 4*A*). This reduction in Nuc synthesis was not caused by competition for transcription factors between the simian virus 40 promoters in the pSG5-COX and pSG5-Nuc expression vectors, because a 12-fold molar excess of competing pSG5 vector was cotransfected with pSG5-Nuc (lane 4, Fig. 4*A*), whereas only a 10-fold molar excess of pSG5-COX constructs was cotransfected with pSG5-Nuc (lanes 5 and 6, Fig. 4*A*).

Nuc has been found extracellularly without its signal peptide in SLE-prone mice and in bones of normal mice, suggesting that Nuc has been processed within the ER (12, 13). However, it has not been directly demonstrated that Nuc is directed into the ER compartment. Translation of Nuc cRNA with canine microsomes showed that the signal peptide of nascently translated Nuc is >99% cleaved by intralumenal microsomal peptidase, localizing Nuc into the lumen of the ER, where it could interact with COX isoenzymes (Fig. 4*B*).

DISCUSSION

The use of the two-hybrid system to identify COX-interacting proteins in the ER presented several obstacles not found when searching for cytosolic proteins by this method. Of particular difficulty were the facts that COX-1 and COX-2 contain a monotopic membrane-binding domain and that both enzymes require disulfide-bond formation, N-linked glycosylation, and heme-binding for activity (25). Clearly, all of these features are not present in COX bait constructs used in these studies. It is perhaps significant, therefore, that we were successful in identifying Nuc using construct COX-1 AE (Fig. 1), which encodes most of the catalytic region of the protein but not the membrane-binding and dimerizaton domains, the latter of which requires disulfide-bond formation for proper folding.

The pVP16 library-encoded Nuc fragment heterodimerized with COX constructs AE, NC, and CT, which restricted the COX–Nuc interaction domains to Met^{381}–Gly^{498} of the catalytic region of COX and a domain located within the first 123 amino acids of Nuc. It is important to note that pairing of Nuc with construct S9 (Fig. 1), which contains Pro^{11}–Gln^{481} of COX-1, failed to transactivate reporter genes in the two-hybrid assay. However, as noted above, large COX constructs tended to be unstable, and the lack of Nuc heterodimerization with S9

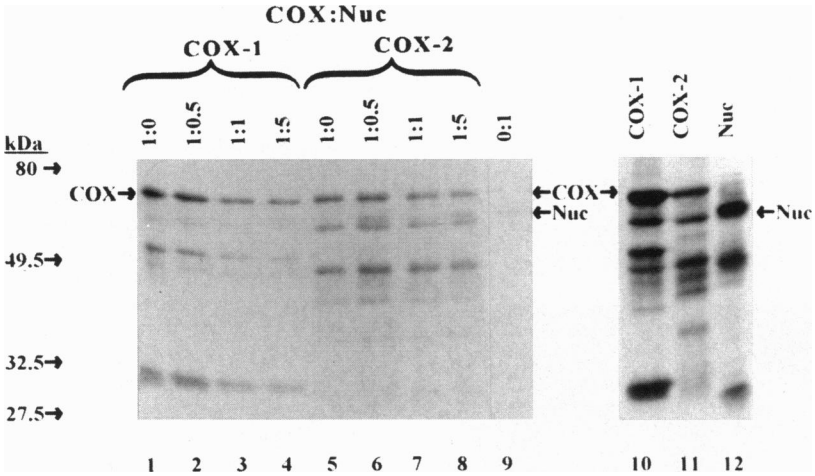

FIG. 3. Nuc inhibition of COX immunoprecipitation. *In vitro* translation products of COX-1 (lane 10) or COX-2 (lane 11) were mixed with *in vitro* translation products of Nuc (lane 12) in volumetric ratios (COX/Nuc) as indicated. Immunoprecipitations with polyclonal anti-COX-1 antibodies were done on the mixtures, and the precipitates were measured by autoradiography. Free Nuc was found to bind weakly to Sepharose protein A as shown in lane 9. This binding was qualitatively used to measure the amount of uncomplexed Nuc in lanes 1–8. Exposure of autoradiogram was 3 days.

may be due to this problem. If protein instability is not responsible for lack of COX–Nuc binding in this pairing, this finding further restricts the Nuc-interaction site to the small region of Gln^{481}–Gly^{498} of COX-1. The location of this peptide is in NC domain 2, where its amino acids project into solvent as seen in Fig. 2*C*. This peptide sequence is of interest in light of the recent studies of Ren and coworkers (31), who used epitope-specific antibodies to map the topology of COX-1 in the ER membrane. Only one of their tested antibodies was directed against an epitope that was entirely contained in Met^{381}–Gly^{498}, and this probe, antibody 5, was directed against residues 485–498 of COX-1. Although antibody 5 bound to purified COX-1 in enzyme immunoassays and Western blots, it was unique among the antibodies they tested in that it failed to bind COX-1 *in situ*, either with the ER membrane intact or removed by saponin treatment. This finding led the authors to conclude that this epitope was "screened" from antibody recognition by "membrane components" (31). Our data suggest Nuc as a candidate for this component.

The finding that COX-1 and COX-2 bound Nuc *in vitro* must be viewed with the understanding that in this assay, COX-1 and COX-2 were non-glycosylated and, with Nuc, contained their signal peptides. The catalytic activities of *in vitro* translated COX-1 and COX-2 are not known, but it is probable that the enzymes were not catalytically functional. Yet, when expressed in COS-1 cells, where the enzymes are fully processed and are active, both COX enzymes largely prevent release of Nuc into the extracellular space (Figs. 1, 3, and 4*A*). In this assay, COX-1 was better than COX-2 at preventing Nuc secretion, although the difference was not as marked as that seen between COX-1 and COX-2 in the *in vitro* assay. We propose that binding of Nuc to COX enzymes, which are anchored to the lumenal surface of the ER, prevents Nuc's secretion. The fact that only partial inhibition of Nuc release was achieved in COS-1 cells may indicate a lower COX–Nuc affinity *in vivo* (Fig. 4*A*) than *in vitro* (Fig. 3). Lowered affinity could be due to COX membrane binding, homodimerization, glycosylation, or other COX modifications *in vivo*.

Nuc was first identified bound to nucleosomal-laddered DNA released into the growth media of a lymphocyte cell line from SLE-prone MRL/lpr mice during apoptosis (14, 15). It is also present in the sera of MRL/lpr mice yet is absent from the sera of normal MRL/n mice (12) despite the wide-spread expression of its mRNA in tissues (12, 13). Exogenously administered Nuc appears to promote autoimmunity and apoptosis. Within 15 hr after injecting Nuc into 6-week-old normal BALB/c mice, animals apoptotically released thymic mononucleosomal DNA (16). Extended Nuc administration produced, in these mice, mild SLE with hypergammaglobulinemia, hypercellularity, and anti-nuclear antibody production, which included the production of IgG anti-double stranded DNA antibodies, anti-U1RNP antibodies, and IgG class rheumatoid factor (17, 18).

Although strongly associated with apoptosis, Nuc has also been shown to be released from osteocytes, where it is found at low levels in extracellular bone matrix (13). At present, this is the only example in which Nuc may be secreted under normal physiological conditions, although an apoptotic release of Nuc in bone has not been investigated. If release of Nuc in bone is through secretion, it would be of interest to determine how regulation of its secretion involves COX.

Nuc is unusual in that even though it is found in the ER lumen or extracellular space, it has many features of a transcription factor, including a leucine zipper, a predicted nuclear localization signal, and a physiologically functional DNA-binding domain (ref. 14; Fig. 2*A*). However, the only known DNA-binding function of Nuc is during apoptosis, when it presumably must be released from the ER to bind nuclear DNA. The COX-binding domain we have identified lies well outside Nuc's DNA-binding domain. The results of our library screens indicate the importance of this, since numerous false positive clones encoding intrinsic transcriptional transactivators were identified. All of these artifactual positives were found to contain the DNA-binding domains of known transcription factors (unpublished results). In addition to having DNA-binding activity, Nuc recently has been reported to bind Gα proteins in the two-hybrid system (30) via a domain that is carboxyl-distal to that involved in binding COX. It is possible that COX and Nuc are in a multimeric signal-transducing protein complex containing a Gα protein. Unanswered at present and problematic is the identity of an intralumenal Nuc-binding Gα protein, since these polypeptides are in heterotrimeric complexes on the cytosolic surface of membranes, where they are involved in signal transduction and the control of membrane trafficking (32). The unusual intralumenal compartmentalization of COX and Nuc would suggest that any interacting Gα proteins would be similarly compartmentalized.

Nuc–COX binding could allow calcium to regulate the complex via calcium-binding domains in Nuc. Such regulation would probably be through steric changes that alter COX or

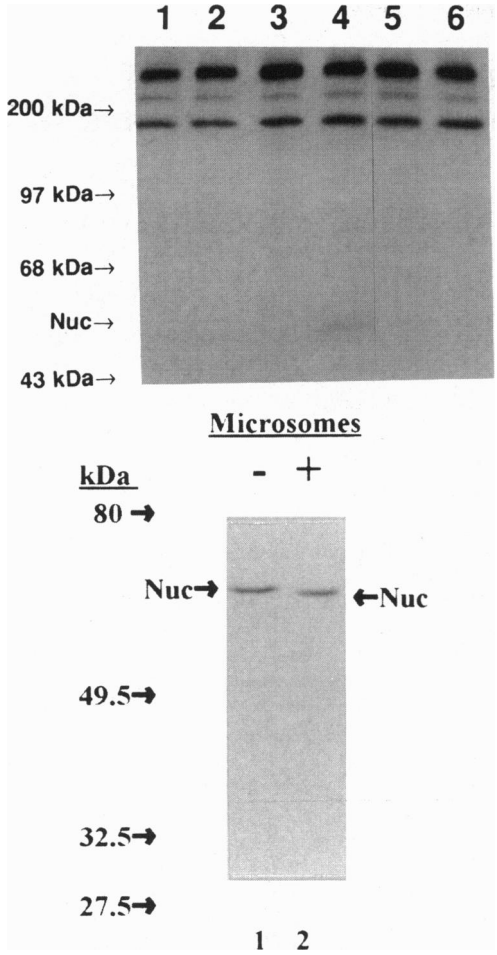

FIG. 4. (*A*) Inhibition of Nuc secretion upon coexpression with COXs. Anti-Nuc antibodies were used to immunoprecipitate Nuc from the growth media of COS-1 cells transfected with 10 μg of pSG5 plasmid (lane 1), 9 μg of pSG5-COX-1 and 1 μg of pSG5 (lane 2), 9 μg of pSG5-COX-2 and 1 μg of pSG5 (lane 3), 9 μg of pSG5 and 1 μg of pSG5-Nuc (lane 4), 9 μg of pSG5-COX-1 and 1 μg of pSG5-Nuc (lane 5), and 9 μg of pSG5-COX-2 and 1 μg of pSG5-Nuc (lane 6). High-molecular-weight proteins of more than 180 kDa (present in all lanes) are not Nuc-related and result from nonspecific binding to protein A Sepharose beads. Densitometric analysis showed secretion of Nuc to be reduced 86% and 81% by COX-1 and COX-2, respectively. Autoradiogram exposure was 18 days. (*B*) Cleavage of Nuc's signal peptide (Met^1–Ala^{25}) by microsomal signal peptidase *in vitro*. Transcription and translation *in vitro* were performed using full-length Nuc cDNA in both the absence (lane 1) and presence (lane 2) of canine pancreatic microsomes. When microsomes were present, >99% of Nuc was processed to a lower-molecular-weight protein, indicating the removal of the signal peptide.

Nuc activity rather than by regulating COX–Nuc binding *per se*, since calcium had no effect on COX–Nuc binding *in vitro*. Calcium binding is known to cause significant conformational changes in Nuc (26) that could sterically influence the conformation of Nuc-associated proteins such as COX.

We thank P. Bartel, S. Fields, S. Hollenberg, and O. Yuan for providing invaluable assistance and materials relating to the two-hybrid system, as well as L. Marnett for the anti-sheep COX-1 antisera. This work was supported by National Institutes of Health Grant CA55585.

1. Smith, W. L. & Marnett, L. J. (1991) *Biochim. Biophys. Acta* **1083,** 1–17.
2. Xie, W., Chipman, J. G., Robertson, D. L. & Simmons, D. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2692–2696.
3. Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W. & Herschman, H. R. (1991) *J. Biol. Chem.* **266,** 12866–12872.
4. O'Banion, M. K., Sadowski, H. B., Winn, V. & Young, D. A. (1991) *J. Biol. Chem.* **266,** 23261–23267.
5. Barnett, J., Chow, J., Ives, D., Chiou, M., Mackenzie, R., Osen, E., Nguyen, B., Tsing, S., Bach, C., Sigal, E. & Ramesha, C. (1994) *Biochim. Biophys. Acta* **1209,** 130–139.
6. Langenbach, R., Morham, S. G., Tiano, H. F., Loftin, C. D., Ghanayem, B. I., Chylada, P. C., Mahler, J. F., Lee, C. A., Goulding, E. H., Kluckman, K. D., Kim, H. S. & Smithies, O. (1995) *Cell* **83,** 483–492.
7. Morham, S. G., Langenbach, R., Loftin, C. D., Tiano, T. F., Vouloumanos, N., Jennette, J. C., Mahler, J. F., Kluckman, K. D., Ledford, A., Lee, C. A. & Smithies, O. (1995) *Cell* **83,** 473–482.
8. Reddy, S. T. & Herschman, H. R. (1994) *J. Biol. Chem.* **269,** 15473–15480.
9. Roshak, A., Ganesh, S. & Marshal, L. A. (1994) *J. Biol. Chem.* **269,** 2599–26005.
10. Grossman, C. J., Wiseman, J., Lucas, F. S., Trevethick, M. A. & Birch, P. J. (1995) *Inflamm. Res.* **44,** 253–257.
11. Simmons, D. L., Xie, W., Evett, G., Merrill, J., Robertson, D. L. & Bradshaw, W. S. (1993) *J. Lipid Mediators* **6,** 113–117.
12. Kanai, Y., Miura, K., Uehara, T., Amagai, M., Takeda, O., Tanuma, S. & Kurosawa, Y. (1993) *Biochem. Biophys. Res. Commun.* **196,** 729–736.
13. Wendel, M., Sommarin, Y., Bergman, T. & Heinegard, D. (1995) *J. Biol. Chem.* **270,** 6125–6133.
14. Miura, K., Kurosawa, Y. & Kanai, Y. (1992) *Biochem. Biophys. Res. Commun.* **187,** 375–380.
15. Kanai, Y., Katagari, T., Mori, S. & Kubota, T. (1986) *Int. Arch. Allergy Appl. Immunol.* **81,** 92–94.
16. Kanai, Y., Kyuwa, S., Miura, K. & Kurosawa, Y. (1995) *Immunol. Lett.* **46,** 207–214.
17. Kanai, Y., Takeda, O., Kanai, Y., Miura, K. & Kurosawa, Y. (1994) *Immunol. Lett.* **39,** 83–89.
18. Kanai, Y., Takeda, O., Miura, K., Amagai, M., Kaneko, T., Kubuta, T., Kanai, Y., Tanuma, S. & Kurosawa, Y. (1995) *Immunol. Lett.* **45,** 35–42.
19. Fields, S. & Song, O. (1989) *Nature (London)* **340,** 245–246.
20. Vojtec, A. B., Hollenberg, S. M. & Cooper, J. A. (1993) *Cell* **74,** 205–214.
21. Picot, D., Loll, P. J. & Garavito, R. M. (1994) *Nature (London)* **267,** 243–249.
22. Schiestl, R. H. & Gietz, R. D. (1989) *Curr. Genet.* **16,** 339–346.
23. Ward, A. C. (1990) *Nucleic Acids Res.* **18,** 5319.
24. Bartel, P., Chien, C. T., Sternglanz, R. & Fields, S. (1993) *Biotechniques* **14,** 920–924.
25. Simmons, D. L., Levy, D. B., Yannoni, Y. & Erikson, R. L. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1178–1182.
26. Miura, K., Kurosawa, Y. & Kanai, Y. (1994) *Biochem. Biophys. Res. Commun.* **199,** 1388–1393.
27. Lu, X., Xie, W., Reed, D., Bradshaw, W. S. & Simmons, D. L. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7961–7965.
28. Xie, W., Merrill, J. R., Bradshaw, W. S. & Simmons, D. L. (1993) *Arch. Biochem. Biophys.* **300,** 247–252.
29. Bartel, P. L. & Fields, S. (1995) *Methods Enzymol.* **254,** 241–263.
30. Mochizuki, N., Hibi, M., Kanai, Y. & Insel, P. A. (1995) *FEBS Lett.* **373,** 155–158.
31. Ren, Y., Walker, C., Loose-Mitchell, D. S., Ruan, K.-H. & Kulmacz, R. J. (1995) *Arch. Biochem. Biophys.* **323,** 205–214.
32. Colombo, M. I., Inglese, J., D'Souza-Schorey, C., Beron, W. & Stahl, P. D. (1995) *J. Biol. Chem.* **270,** 24564–24571.