The FASEB Journal express article 10.1096/fj.04-3274fje. Published online June 9, 2005.

Targeting the beta-catenin/APC pathway: a novel mechanism to explain the cyclooxygenase-2-independent anticarcinogenic effects of celecoxib in human colon carcinoma cells

Thorsten Jürgen Maier, Astrid Janssen, Ronald Schmidt, Gerd Geisslinger, and Sabine Grösch

Institut für Klinische Pharmakologie, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt, Frankfurt/Main, Germany

Corresponding author: Sabine Grösch, Institut für Klinische Pharmakologie Klinikum der Johann Wolfgang Goethe Universität Theodor Stern Kai 7 60590 Frankfurt/Main, Germany. E-mail: groesch@em.uni-frankfurt.de

ABSTRACT

Celecoxib, a cyclooxygenase-2 (COX-2) selective nonsteroidal anti-inflammatory drug, is a new anticarcinogenic agent. Its antitumor effects depend on the one hand on its COX-2-inhibiting potency, but on the other hand on COX-2-independent mechanisms, which until now have not been fully understood. Here, we investigated whether celecoxib has an impact on the APC/β-catenin pathway, which has been shown to play a pivotal role in the development of various cancers, especially of the colon. After only 2 h of treatment of human Caco-2 colon carcinoma cells with 100 μM celecoxib, we observed a rapid translocation of β-catenin from its predominant membrane localization to the cytoplasm. Inhibition of the glycogen-synthase-kinase-3β (GSK-3β) by LiCl prevented this celecoxib-induced translocation, suggesting that phosphorylation of β-catenin by the GSK-3β kinase was essential for this release. Furthermore, the cytosolic accumulation was accompanied by a rapid increase of β-catenin in the nuclei, starting already 30 min after celecoxib treatment. The DNA binding activity of β-catenin time dependently decreased 2 h after celecoxib treatment. After this cellular reorganization, we observed a caspase- and proteasome-dependent degradation of β-catenin after 8 h of drug incubation. Celecoxib-induced β-catenin degradation was also observed in various other tumor cell lines (HCT-116, MCF-7, and LNCAP) but was not seen after treatment of Caco-2 cells with either the anticarcinogenic nonsteroidal anti-inflammatory drug R-flurbiprofen or the highly COX-2-selective inhibitor rofecoxib. These findings indicate that the anticarcinogenic effects of celecoxib can be explained, at least partly, by an extensive degradation of β-catenin in human colon carcinoma cells.

Key words: colon cancer • nonsteroidal anti-inflammatory drugs • apoptosis

Today, nonsteroidal anti-inflammatory drugs (NSAIDs) are, more than ever before, under extensive investigation with respect to their anticarcinogenic efficacy. An interesting candidate is the COX-2-selective inhibitor celecoxib, which has been shown to prevent

the formation of pretumorous adenomatous polyps in patients with familial adenomatous polyposis (FAP), a disease owing to a dysfunction of the β-catenin/ adenomatous polyposis coli (APC) signaling pathway (1). Currently, celecoxib is the only NSAID, which was approved by the FDA and EMEA for the treatment of these FAP patients. The ability of celecoxib to primarily inhibit the initial stages of colorectal cancer progression prompted us to speculate that the β-catenin/APC pathway could be a possible target of this drug.

Beta-catenin expression is regulated by the APC protein, which is mutated in ~80% of all sporadic colon cancer diseases. As a consequence of APC-mutation, nearly all colon carcinoma tissues show significant overexpression of β-catenin, which has been attributed to be crucial for the early stages of colorectal carcinogenesis (2). Also, the hereditary FAP disease is provoked by mutations of the APC-gene. FAP is characterized by multiple formations of β-catenin-enriched adenomas and is an obligate precursor for colorectal cancer (CRC). Moreover, a variety of mutations in the β-catenin gene were determined in various colon cancer cell lines (3), which may be of importance for tumor development.

Under physiological conditions, a large fraction of β-catenin is bound to the membrane cell-cell adhesion protein e-cadherin thereby linking the cell membrane via α-catenin to the actin cytoskeleton (4). Furthermore, β-catenin underlies permanent turnover. The newly synthesized β-catenin is incorporated into a large protein complex, including the APC tumor suppressor protein, the glycogen synthase kinase (GSK-3β), and other proteins such as axin and conductin, both primarily responsible for complex stabilization (5). In this complex, GSK-3β efficiently phosphorylates β-catenin, APC, and axin. Phosphorylation of β-catenin by the GSK-3β kinase is required for its ubiquitination and degradation by the ubiquitin-proteasome pathway (5). However, recent studies have provided evidence for the existence of APC/axin-independent pathways to facilitate phosphorylation of β-catenin by GSK-3β and subsequent proteasomal degradation (6).

Inhibition of the GSK-3β by various stimuli leads to dissociation of the APC/axin/β-catenin complex and cytosolic β-catenin accumulation (7). Free unphosphorylated β-catenin was described to translocate into the nucleus. This nuclear translocation is regulated by several transcription factors of the TCF (T cell-factor) family such as TCF-4 and lef-1 (lymphoid-enhancer-factor-1), which were shown to interact with β-catenin (8). The β-catenin/TCF complex recruits further chromatin-remodeling proteins to responsive promoters, thereby activating the transcription of specific target genes, including c-myc, c-jun, fra-1, cyclin D1 and cyclooxygenase-2 (COX-2) (9, 10).

Next to deregulation of the β-catenin/APC pathway, ~80% of all colorectal tumors were shown to overexpress cyclooxygenase-2 (COX-2), which is the key enzyme in the conversion of arachidonic acid to prostaglandins. Because prostaglandins were shown to promote cell proliferation, angiogenesis and metastasis, and to inhibit the induction of apoptosis (11), the anticarcinogenic effects of NSAIDs were primarily attributed to their COX-2 inhibitory activity. However, recent studies have suggested also COX-2-independent mechanisms to explain the anticarcinogenic effects of NSAIDs. For example, it has been shown that celecoxib has an antitumorigenic effect in COX-2-deficient tumors in the nude mice model and induces apoptosis in cells which do not express COX-2 (12–14). Furthermore, the highly selective and potent COX-2 inhibitor rofecoxib showed significant lower anticarcinogenic effects in human

cholangiocarcinoma cells as compared with similar concentrations of celecoxib (15). These observations support the hypothesis that some of the antiproliferative and antineoplastic effects of celecoxib are independent of COX-2-inhibition.

Therefore, we address here the question of whether interference with the β-catenin/APC pathway may contribute to the COX-2-independent anticarcinogenic effects of celecoxib in human colon carcinoma cells.

MATERIALS AND METHODS

Cells and reagents

The human colon cancer cell line Caco-2 (ACC-169), the prostate cancer cell line LNCAP, and the breast cancer cell line MCF-7 were purchased from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). HCT-116 cells were purchased from the American Type Culture Collection (ATCC, Wesel, Germany). Caco-2 cells were cultured in Dulbecco's MEM (modified Eagle's medium) with Glutamax-I medium containing 10% FCS (fetal calf serum), HCT-116 cells in McCoy's 5A medium with Glutamax-I containing 10% FCS and MCF-7 and LNCAP cells in RPMI 1640 medium with Glutamax-I containing 10% FCS. All cell lines were cultured at 37°C in an atmosphere containing 5% CO_2. Berlin-Adlershof GmbH (Berlin, Germany) synthesized celecoxib and rofecoxib. The identity and purity of celecoxib and rofecoxib were determined using HPLC (16) and mass spectrometry and was >99%. Lactacystine was purchased from Biomol (Hamburg, Germany), actinomycin D from Carl Roth GmbH (Karlsruhe, Germany), cycloheximide from Calbiochem (La Jolla, CA), the caspase inhibitor III (Boc-D-FMC) from Calbiochem (La Jolla, CA), and lithium chloride from Riedel-de-Haën (Seelze, Germany).

Concentrations of celecoxib used for cell culture experiments

As a prerequisite for all experiments, we determined the concentrations of celecoxib required to induce apoptosis in our cell culture assays, as determined by PARP cleavage (data not shown). Antiproliferative effects were already observed after treatment of the cells with 40 μM celecoxib (17). This concentration is ~10-fold higher than the plasma concentrations found in subjects taking celecoxib (18). However, it is widely accepted that cell culture experiments with tumor cell lines require higher concentrations than those found in plasma samples of patients or animals under antitumor therapy. For instance, the medium concentrations of the widely used chemotherapeutic cisplatin required to induce apoptosis in human cervical carcinoma cells in vitro (up to 100 μM; Ref. 19) also clearly exceed the maximum cisplatin plasma concentrations in patients achieved after intravenous infusion (13.5±9.3 μM at a dose of 50 mg/m^2 daily for two days) and sufficient to cause tumor regression (20). Furthermore, cisplatin concentrations in the range of the in vivo plasma concentrations (<15 μM) were almost ineffective in causing the classical cisplatin-associated anticarcinogenic effects in tumor cell lines, such as the induction of the stress-activated protein kinase (SAPK) after cisplatin-mediated DNA-damage (19). This discrepancy of the concentrations needed to cause anticarcinogenic effects in vivo and in vitro becomes apparent when you consider that tumor regression in patients or animals requires weeks or months of drug treatment, whereas in cell culture experiments, antiproliferative effects are already observed a few hours after incubation with celecoxib. In vivo, this would suggest that

celecoxib may accumulate in the tumor cells over time. However, this hypothesis needs further elucidation in patient studies. Furthermore, proliferation of cultured cells occurred under conditions that cannot be compared with the situation in vivo. With these issues in mind and because of published results, we used 100 μM celecoxib in most experiments.

Determination of free celecoxib concentrations in medium containing 10% FCS

10 ml Dulbecco's MEM (modified Eagle´s medium) with Glutamax-I medium containing 10% FCS and increasing concentrations of celecoxib (0, 20, 40, 60, 80, and 100 μM) were centrifuged at 37,500 rpm and 37°C for 15 h using a Sorvall Discovery 90 SE ultracentrifuge (Kendro Laboratory Products, Asheville, NC). The supernatant containing free (nonprotein-bound) celecoxib was diluted 1:10 and 1:100 in acetonitrile/H_2O/ammonia solution 25% (50:50:0.1). Celecoxib concentrations were measured using liquid chromatography tandem mass spectrometry (LC/MS-MS), as described previously (16).

Western blot analysis

Cells treated for definite time periods with the respective drugs or substances were washed with 10 ml PBS, scraped in 1 ml PBS, and centrifuged for 1 min at 10,000 rpm. For preparation of soluble extracts, cells were resuspended in sonification buffer (10 mM Tris/HCl, pH 7.2, 1 mM EDTA (ethylenediaminetetraacetate), 1 mM mercaptoethanol, 5% glycine, 1 mM PMSF (phenyl-methylsulfonylfluoride), 5 mM DTT (dithiothreitol)), sonicated and centrifuged at 14,000 rpm for 10 min. For Western blot analysis of phosphoproteins, a commercially available phosphosafe extraction buffer (Novagen, Darmstadt, Germany) was used instead. The supernatant containing cytoplasmatic and nuclear proteins was then applied to Western blot analysis of soluble extracts. The remaining pellet predominantly containing membrane cell constituents was washed with PBS, solubilized in running buffer in the presence of sodiumdodecylsulfate and mercaptoethanol and used for the analysis of the membrane extracts.

To obtain nuclear protein extracts, cell pellets were resuspended in 500-μl lysis-buffer I (10 mM Tris-HCl (ph 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40), incubated for 5 min on ice and centrifuged at 3200 rpm for 5 min. Nuclei were then washed with washing buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$), resuspended in lysis-buffer II (20 mM Tris-HCl (pH 7.4), 40 mM Na-pyrophosphate, 50 mM NaF, 5 mM $MgCl_2$, 20 mM EDTA, 1% Triton-X-100, and 0.5% SDS, 0.1 mM PMSF), sonicated and centrifuged at 14,000 rpm for 10 min. The supernatant was diluted by the addition of one vol. lysis buffer III (20 mM HEPES-KOH, pH 7.4, 0.2 mM EDTA, 0.5 mM PMSF, and 2 mM DTT). Glycerol was added to obtain a final concentration of 20%, and aliquots were stored at –80°C. The protein content of the respective extracts was determined using the Bradford method. Fifty μg of soluble extracts and 30 μg of nuclear extracts were separated electrophoretically on 12% SDS-PAGE (sodiumdodecylsulfate-PAGE), and proteins were electroblotted onto a nitrocellulose membrane (Hybond–C, Amersham, Freiburg, Germany). Membranes were stained with 0.5% Ponceau in 1% acetic acid to confirm equal loading. After overnight incubation of the membranes in blocking buffer (5% nonfat dried milk in PBS) or Odyssey blocking-reagent (LI-COR Biosciences, Bad Homburg, Germany), diluted 1:1 in PBS, they were incubated for 2 h with the respective primary antibody directed against β-catenin (1:100 goat polyclonal), PARP (poly (ADP)-ribose polymerase, 1:100, rabbit polyclonal), ERK-2 (1:1000, mouse monoclonal), p-Ser-9-GSK-3β (1:100, goat

polyclonal), GSK-3β (1:100, rabbit polyclonal), diluted in blocking buffer in presence of 0.1% Tween 20. All antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Membranes were then washed three times with 0.1% Tween 20 in PBS and incubated with an IRDye800 or IRDye700 conjugated secondary antibody (Biotrend Chemikalien GmbH) in blocking buffer or with a peroxidase-conjugated secondary antibody (Santa Cruz, Heidelberg, Germany) for 1 h. After extensive rinsing in 0.1% Tween 20 in PBS, protein-antibody complexes conjugated with peroxidase were treated with ECL (enhanced chemoluminiscence, Amersham Pharmacia Biotech), according to the manufacturer's protocol and exposed to a chemoluminescence film. Protein-antibody complexes conjugated with IRDye800/700 were visualized on the Odyssey Infrared Imaging System (LI-COR, Biosciences).

Electrophoretic mobility shift assay

Nuclear extracts (4 μg, see Materials and Methods: Western blot analysis) from cells were incubated in 10% glycerol, 10 mM HEPES (4-(2-Hydroxyethyl)-1-piperazineethabesulfonic acid)-KOH, pH 7.9, 50 mM KCl, 4 mM $MgCl_2$, 4 mM Tris-HCl, 0.5 mM DTT (1,4 dithiothreitol), 0.5 mM EDTA, 1 μg/ml BSA (bovine serum albumin), 1 μg/ml poly (dI-dC) together with 25 fmol of [^{32}P]-ATP-labeled oligonucleotide (MWG-Biotech GmbH, Ebersberg, Germany) for 30 min at room temperature. The oligonucleotide sequence corresponded to the β-catenin/TCF/lef binding site (5′-AGTGGCCTTTGATCTAGT-3′). For supershift experiments, 4 μg of untreated nuclear protein extract were incubated together with 25 fmol of [^{32}P]-ATP-labeled oligonucleotide for 30 min at room temperature. Then, 2 μg of each supershift-antibody (lef-1 goat polyclonal, TCF-1 and TCF-4 rabbit polyclonal, p-c-jun mouse monoclonal) were added and the mixture incubated for 1 h at 4°C. All supershift antibodies were purchased from Santa Cruz (Heidelberg, Germany). For unlabeled probe competition, 4 μg of untreated nuclear protein extract were incubated together with 25 fmol of [^{32}P]-ATP-labeled oligonucleotide and increasing amounts of unlabeled probe (1-fold, 10-fold, and 100-fold excess compared with labeled probe) for 30 min at room temperature. The nucleotide/protein complexes were separated on a 5% native polyacrylamide gel in 0.25 × TBE buffer at 100 V and room temperature for 2 h. The radioactive gels were dried, and bands were detected using autoradiography.

Immunocytochemistry

Approximately 2×10^5 cells were seeded in 5-cm-diameter dishes on thin cover glasses and incubated for 24 h at 37°C in an atmosphere containing 5% CO_2. Cells were then treated with 100 μM of celecoxib for different time periods (0, 0.5, 1, 1.5, 2, 2.5, 3 h), washed with PBS, and fixated for 5 min with 4% paraformaldehyde at 4°C. After washing with PBS, cells were then permeabilized using 0.1% triton-X 100 for 5 min and washed with PBS again. Cells were blocked overnight with 3% BSA (bovine serum albumin) in PBS and subsequently incubated with the primary β-catenin antibody (diluted 1:100 in PBS/1% BSA) for 1 h. After washing the cover glass 3 times with PBS, cells were treated with a Cy3-labeled secondary antibody (diluted 1:1000 in PBS/1% BSA) for 1 h, subsequently 5 min with 1 μg/ml DAPI in PBS for nuclear staining, and finally washed 5 times with PBS. Cover glasses were fixated on microscope slides using clear nail polish solution. Cells were viewed using an eclipse 600 fluorescence microscope (Nikon, Tokyo, Japan).

RESULTS

30% of celecoxib are protein-unbound in medium containing 10% FCS

We found ~30% of the added celecoxib (0–100 μM) to be nonprotein-bound and hence to be available for affecting the cells (17). Thus, the free and therefore effective celecoxib concentrations in our cellular systems are lower than the total concentrations of drug in the medium. To finally answer the question of celecoxib concentrations needed, further experiments should be aimed at determination of intracellular concentrations both, in the in vitro and in vivo situation.

Celecoxib treatment of human Caco-2 colon carcinoma cells affected the protein levels of β-catenin and e-cadherin

To assess the pathways involved in induction of apoptosis and cell cycle arrest caused by celecoxib, human Caco-2 cells were treated with 100 μM celecoxib for various time periods (0, 2, 4, 6, 8, and 16 h) and the protein levels of β-catenin were analyzed in different cellular fractions. Western blot analysis of soluble extracts (Fig. 1*A*) revealed that treatment of Caco-2 cells with 100 μM celecoxib caused a time-dependent accumulation of β-catenin between 0 and 6 h, whereas longer incubation for 8 h and 16 h resulted in strong degradation of the protein. To rule out that these effects arise from physiological time-dependent fluctuation of β-catenin expression, the protein levels of β-catenin in Caco-2 cells were analyzed at identical time periods but without prior addition of celecoxib. No time-dependent changes in β-catenin expression were observed (data not shown), indicating that β-catenin degradation is specifically caused by celecoxib treatment. Because both β-catenin and e-cadherin are components of the cell adherence complex in the cell membrane, we determined the expression levels of both proteins in the membrane extract after celecoxib treatment. Beta-catenin levels decreased already 2 h after celecoxib treatment, whereas a reduction in membrane e-cadherin signals was observed not before 16 h of drug incubation (Fig. 1*B*). To confirm the specificity of celecoxib-induced β-catenin degradation, the cells were treated with increasing concentrations of celecoxib (0–100 μM) for 20 h (Fig. 1*C*). Moderately reduced β-catenin protein levels were observed already at medium concentrations of 60 μM celecoxib, whereas clear degradation of β-catenin was seen at concentrations of 80 μM and 100 μM celecoxib. These celecoxib concentrations are in the range of those sufficient to cause COX-2-independent effects in cell culture assays, such as the inhibition of PDK-1 (IC_{50}= 48 μM, in vitro assay; Ref. 21) or the inhibition of ER Ca^{2+}-ATPases (IC_{50}= 35 μM, in vitro assay; Ref. 22). Western blot analysis of nuclear β-catenin levels (Fig. 1*D*) showed that after treatment of the cells with 100 μM celecoxib β-catenin translocates into the nucleus within 0.5 h and remains there up to 4 h. Thereafter, β-catenin protein levels decreased to control levels. As a loading control, ERK-2-expression was assessed in all experiments, since membrane, nuclear, and cytoplasmatic ERK-2 protein levels were found to be unaffected by celecoxib treatment.

Celecoxib treatment decreased the β-catenin/TCF/lef DNA binding activity

As a second function, β-catenin regulates gene expression via binding as a transcription factor in complex with TCF or lef to the promoter region of different target genes. To investigate whether the early nuclear β-catenin translocation has an impact on β-catenin/TCF/lef-DNA binding

activity after celecoxib treatment, we used the technique of electromobility shift assay (EMSA). Surprisingly, the nuclear translocation of β-catenin was not associated with increased DNA binding activity. By contrast, DNA binding activity clearly decreased just 2 h after celecoxib treatment (Fig. 2*A*). To identify the components of the β-catenin DNA binding complex, we performed supershift experiments using antibodies directed against TCF-1, TCF-4, and lef-1 (Fig. 2*B*). Among others, these factors interact with β-catenin, thereby facilitating its binding to DNA and the regulatory activity on gene expression. Supershift signals were obtained using the anti-lef-1 and anti-TCF-1 antibodies, whereas the anti-TCF-4 antibody and the p-c-jun antibody as an unspecific control did not supershift any binding signal. The specificity of the β-catenin/TCF/lef signals was confirmed by performing unlabeled probe competition experiments (Fig. 2*B*).

Initial accumulation of β-catenin protein levels in the soluble cell extracts is not due to increased protein biosynthesis

To investigate whether new protein biosynthesis is the cause of the celecoxib-initiated (2–6 h) increase of β-catenin protein levels in soluble cell extracts, we preincubated the cells with 10 μg/ml cycloheximide and 5 μg/ml actinomycin D for 30 min. Cycloheximide blocks protein translation by inhibiting the peptidyltransferase activity of the eukaryotic ribosome, and actinomycin D is a potent suppressor of DNA-transcription. Even in the presence of these agents, the celecoxib-induced initial increase in β-catenin protein level was not inhibited (Fig. 3*A*). As a control experiment, cells were separately treated with both agents without addition of celecoxib (Fig. 3*B*). Cycloheximide slightly diminished the expression of β-catenin, whereas actinomycin D had no effect. This might be explained by the fact that cycloheximide immediately blocks complete protein expression by interfering with the last step of protein synthesis, whereas actinomycin D blocks transcription but is not able to prevent the translation of remaining RNA.

Celecoxib treatment affected the cellular distribution of β-catenin as determined by immunocytochemistry

Immunocytochemical analysis of untreated control Caco-2 cells showed β-catenin to be mainly localized in the outer cell membrane (Fig. 4*A*). Furthermore, low expression was observed in the cytoplasm, whereas the nuclei showed nearly no β-catenin staining. These findings are in accordance with previously published data (23). Interestingly, the cellular distribution of β-catenin changed after treatment of Caco-2 cells with 100 μM celecoxib for 1.5 and 3 h (Fig. 4*B* and *C*). Membrane-bound β-catenin diminished whereas β-catenin accumulation was observed in the cytoplasm, which is mainly localized around the nuclear envelope. In addition, a slight increase in nuclear β-catenin protein staining was observed after 1.5–3 h. These results confirm the data of Western blot analysis (Fig. 1*A–D*), which revealed an increase of β-catenin protein levels both in nuclear and soluble extracts and a simultaneous decrease in the membrane extracts. The initial increase of β-catenin levels in the soluble extract might be explained by translocation of β-catenin from the outer cell membrane into the cytoplasm and the nuclei.

Celecoxib-induced degradation of β-catenin is caused by both the proteasomal pathway and caspases

Treatment of Caco-2 cells with 100 μM celecoxib for 8, 10, and 16 h resulted in time-dependent degradation of β-catenin (Fig. 1*A* and 5*A*). Simultaneously, also PARP (poly (ADP)-ribose polymerase)-cleavage occurred, indicating the induction of apoptosis (Fig. 5*A*). Beta-catenin was described to be degraded by either the proteasomal pathway or by caspases because of the induction of apoptosis (24). To investigate the mechanism by which β-catenin was degraded after celecoxib treatment, we preincubated the cells for 30 min with the irreversible broad range caspase inhibitor Boc-D–FMK (100 μM) or the selective proteasome inhibitor lactacystine (10 μM). Lactacystine, and to a greater extent, the caspase inhibitor partly blocked the celecoxib-induced degradation of β-catenin (Fig. 5*A*). In parallel, as expected, the caspase inhibitor prevented the cleavage of PARP, whereas lactacystine did not retard the progression of apoptosis. As a control experiment, cells were separately treated with both agents without the addition of celecoxib (Fig. 5*B*). Incubation with lactacystine slightly increased the expression of β-catenin presumably due to the blockade of β-catenin´s basal turnover rate, whereas the caspase inhibitor alone had no effect.

One precondition for the proteasomal degradation of β-catenin is its phosphorylation by the GSK-3β-kinase (5). The activity of this enzyme is regulated by its phosphorylation status, whereas GSK-3β phosphorylated at Ser-9 by the Akt kinase represents the inactive form (25). To find out whether celecoxib treatment has an impact on the GSK-3β activity, we analyzed the phospho-ser-9-GSK-3β and total GSK-3β levels in soluble extracts using the Western blot method (Fig. 5*C*). Phospho-GSK-3β levels declined in Caco-2 cells starting just 2 h after celecoxib treatment, whereas the total-GSK-3β protein levels remained unchanged. To assess the role of GSK-3β for the membrane degradation of β-catenin, Caco-2 cells were pretreated for 30 min with the selective GSK-3β inhibitor lithium chloride (26) using a concentration of 20 mM and, subsequently, with 100 μM celecoxib for 4 and 6 h. The presence of LiCl prevented the early membrane β-catenin degradation after celecoxib treatment, whereas alone, it had no effect on membrane β-catenin levels (Fig. 5*D*).

To get more insight into the role of GSK-3β and the proteasomal pathway in the celecoxib-induced degradation of β-catenin, we used the human colon carcinoma cell line HCT-116 expressing mutated β-catenin. The three-base-pair deletion in a highly conserved region results in the loss of a serine[45] residue, which was shown to be essential for the correct binding and, consequently, for the phosphorylation of ^{33}Ser, ^{37}Ser, and ^{41}Thr by GSK-3β (3, 27, 28). The use of these cells enables us to determine the extent to which β-catenin degradation depends on phosphorylation by GSK-3β. Treatment of HCT-116 cells with 100 μM celecoxib for various time periods (0, 2, 4, 6, 8, 10, 16, and 24 h) led to moderate reduction in β-catenin protein levels after 10 h in soluble extracts, whereas clear degradation was not observed both in soluble and membrane extracts before 16 h of incubation (Fig. 6*A* and 6*B*). Also, the early increase of β-catenin levels in soluble extracts, observed in Caco-2 cells after celecoxib treatment, did not occur in HCT-116 cells.

The caspases, responsible for exerting β-catenin degradation, were already identified to be members of the caspase-3 family. Beta-catenin is directly cleaved by these enzymes into several fragments with a molecular size in the range of 76 to 70 kDa. Whereas caspase-3 itself was

found to play the major role, caspase-6 and 7 showed no or only weak cleavage activity (24). Therefore, we used MCF-7 breast cancer cells that were caspase-3-depleted owing to a 47-base pair deletion within exon 3 of the *CASP-3* gene (29). The use of this cell line provides the opportunity to determine the extent to which β-catenin degradation after celecoxib treatment is due to caspase-3-dependent mechanisms.

We treated the human cell line MCF-7 with 100 μM celecoxib for various time periods (0, 2, 4, 6, 8, 16, and 24 h; Fig. 7*A*) and observed moderately decreased protein levels of β-catenin in soluble extracts between 6 and 24 h of treatment. An early increase of β-catenin protein level in soluble extracts after 2-4 h was not that pronounced as compared with Caco-2 cells. In parallel, we determined PARP cleavage, which did not occur in MCF-7 cells due to the lack of caspase-3, -6, and –7 cleavage activities (Fig. 7*A*). Similar to the results obtained from Caco-2 cells, Western blot analysis of the respective membrane extracts showed clear decrease of β-catenin levels already 4 h after celecoxib treatment (Fig. 7*B*).

It can be concluded that caspases and the proteasomal pathway functionally independently mediate the degradation of β-catenin after celecoxib treatment, since degradation was observed in both HCT-116 and MCF-7 cells.

To additionally demonstrate that β-catenin degradation is not cell-type-restricted, we treated human LNCAP prostate cancer cells with 100 μM celecoxib for various time periods. Beta-catenin degradation was seen after 24 h of drug incubation, but this effect was less pronounced as compared with Caco-2 cells (data not shown). Thus, β-catenin degradation was observed in four different tumor cell types (Caco-2, HCT-116, MCF-7, and LNCAP). Therefore, a cell-type-specific celecoxib-induced degradation of β-catenin can be excluded.

Rofecoxib and R-flurbiprofen had no effect on β-catenin expression in Caco-2 cells

To finally answer the question of celecoxib's specificity to cause β-catenin degradation, we treated Caco-2 cells with two other NSAIDs differing in their ability to induce apoptosis or to inhibit cyclooxygenase-2. The NSAID R-flurbiprofen was described to show strong antiproliferative effects in vivo and in vitro (30) but lacks COX-2 inhibiting activity. Treatment of Caco-2 cells with 1000 μM R-flurbiprofen for up to 24 h (Fig. 8*A*) did not affect the protein level of β-catenin but caused PARP-cleavage after 16 and 24 h of incubation. Rofecoxib was shown to have only a weak antiproliferative effect in various cell lines (14) but is one of the most selective and most potent COX-2 inhibitors available. In line with these findings, no induction of apoptosis, as determined by the lack of PARP-cleavage, or down-regulation of β-catenin levels was observed in Caco-2 cells after rofecoxib treatment, even at concentrations up to 100 μM, which are far higher than the IC_{50} required for COX-2 inhibition. These results clearly indicate that celecoxib-mediated β-catenin degradation is neither an unspecific effect owing to induction of apoptosis, in general, nor a consequence of COX-2 inhibition, but seems to be specific for celecoxib. Moreover, these experiments provide additional evidence that, in absence of celecoxib, β-catenin protein levels remain time-dependently unchanged.

DISCUSSION

Numerous studies over the last few years focused on determining cellular targets of celecoxib to explain its COX-2-independent anticarcinogenic effects. However, the molecular mechanisms responsible for the anticarcinogenic effects of celecoxib are still not fully understood.

In the present study, we have obtained evidence that after celecoxib treatment, β-catenin was released from its predominant membrane localization into the cytoplasm where its protein levels time-dependently increased. This removal was found to be triggered by glycogen synthase kinase-3β (GSK-3β) activity, suggesting that phosphorylation of β-catenin by the GSK-3β kinase was essential for this release. The cytoplasmatic β-catenin accumulation was accompanied by a rapid translocation of β-catenin into the nuclei. Interestingly, the DNA binding activity of β-catenin simultaneously and time-dependently decreased after celecoxib treatment. After this cellular reorganization, we observed a strong degradation of β-catenin, which depends on both proteasomal and caspase activity. Both pathways were found to functionally independently cause this degradation. Unfortunately, we were not able to show β-catenin phosphorylation using Western blot analysis, probably because of a low specificity of a commercially available phospho-β-catenin ($^{33/37}$Ser/^{41}Thr) antibody. However, β-catenin degradation was not observed after treatment of Caco-2 cells either with the anticarcinogenic NSAID R-flurbiprofen or with the highly selective COX-2 inhibitor rofecoxib, indicating that this effect is not a general one of all NSAIDs and is therefore independent of COX-2 inhibition.

These data are supported by recent studies describing reduced β-catenin protein levels in human colon carcinoma cells after treatment with various NSAIDs, including sulindac-sulfide and -sulfone, whereas the sulfone lacks COX-2 inhibitory activity. Furthermore, also no effects on β-catenin expression were seen after treatment of human colon cancer cells with the highly COX-2 selective inhibitor rofecoxib (31–33).

The APC/β-catenin/TCF pathway is known to play a central role in cancer development. Although APC mutations are almost exclusively found in colorectal cancers, deregulation of APC/β-catenin/TCF signaling is also common in other gastrointestinal and extra-gastrointestinal human cancers (9, 34). For example, such deregulations are also frequently observed in advanced stages of non-small cell lung cancer and have been correlated with poor prognosis (35). Dysregulation of the APC/β-catenin/TCF pathway was described to result in up-regulation of unphosphorylated β-catenin, which binds to the transcription factors TCF/lef and activates transcription of specific target genes such as c-Myc, cyclin D1, VEGF, and MMP7 (36–39). The products of these genes are involved in cell-proliferation and promote cancer development (27). Recently, it has been shown that down-regulation of β-catenin expression by accelerating its proteasomal degradation led to impaired cell proliferation and abolished the tumorigenic potential of these cells in the nude mice model (40). These observations provide additional evidence, that β-catenin overexpression is crucial for the growth of colorectal tumor cells. Interestingly, celecoxib was described to have antineoplastic effects, especially on tumors arising from dysregulation of this APC/β-catenin pathway, such as those found in patients with the FAP-disease (1), in lung cancer patients (41, 42), and patients with breast cancer (42, 43). This observation suggests a possible link between the anticarcinogenic effects of celecoxib and the APC/β-catenin pathway as a potential target. To answer the question of celecoxib's impact on this pathway, we treated several human carcinoma cell lines with celecoxib.

We were able to show that celecoxib treatment truly decreased the DNA binding-activity of β-catenin/TCF/lef transcription complexes in human Caco-2 colon cancer cells. Similar effects were reported by Dihlmann et al., who observed a reduced β-catenin/TCF transcription factor binding signal after treatment of human colon carcinoma cells with aspirin and indomethacin due to an increased cytoplasmatic stabilization of phosphorylated β-catenin (44). Interestingly, such phosphorylated β-catenin, when present in the nucleus, was shown to bind to nuclear lef-1 proteins, but the resulting complexes lacked any DNA-binding activity (45). Consequences arising from such decreased β-catenin-mediated gene expression were reported by Heinen et al., who observed decreased β catenin protein levels and β-catenin-mediated transcription in human colorectal cancer cells by concerted overexpression of the wild-type APC protein. The reduced β-catenin activity immediately lead to a G1-phase block in these cells (46). The studies mentioned above are in accordance with the present findings and with our previous studies in which we have demonstrated that celecoxib induces a G1-phase cell cycle block in various human colon cancer cell lines (12, 17).

However, not only tumor-promoting genes are under the transcriptional control of the β-catenin/TCF transcription factor, but also the expression levels of caspases-3, -7, and -9 are regulated by β-catenin/TCF. In $Apc^{+/min}$ mice, as well as in human colon cancer specimens known to harbor APC mutations, expression of caspase-3, -7, and -9 was clearly diminished in comparison to normal mucosa controls. This effect was accompanied by a resistance of these cells to apoptosis-inducing agents (47). These findings were further supported by the observation that transient overexpression of a dominant negative TCF/lef construct resulted in significant increase in caspase protein expression, indicating that the β-catenin/TCF/lef transcription factor represses transcription of these caspases (47). Next to the repression of caspase expression on transcriptional level, also free nuclear β-catenin was reported to regulate apoptosis independently of its function as a transcription factor. Kim et al. were able to show that high nuclear levels of β-catenin lacking DNA binding activity were capable to trigger the induction of apoptosis in several colon carcinoma cells (48). Interestingly, such high nuclear β-catenin levels lacking DNA binding activity were also observed in our Caco-2 cells after celecoxib treatment. Moreover, celecoxib-induced apoptosis in human colon carcinoma cell lines was accompanied by a strong activation of caspase-3 and –9, as mentioned above (17)

With respect to these data, it can be assumed that the decrease in DNA-binding activity of the β-catenin/TCF/Lef transcription complex after celecoxib treatment contributes to both, induction of cell cycle arrest by inhibiting the transcription of cell proliferation promoting genes and induction of apoptosis by transcriptional activation of caspase-3, -7, and -9. Furthermore, the celecoxib-induced high nuclear levels of β-catenin lacking transcriptional activity may raise additional proapoptotic signals.

Next to the inhibition of β-catenin nuclear DNA binding activity, we were able to show that celecoxib caused a strong degradation of β-catenin protein levels only 8 h after drug incubation. This action was shown to depend on both, GSK-3β-mediated proteasomal degradation and caspase activity. Recent studies were able to provide approaches to explain GSK-3β-mediated β-catenin degradation after celecoxib treatment. Cross et al. reported that GSK-3β kinase activity is inhibited through phosphorylation by the PKB/Akt kinase (49). Interestingly, Hsu et al. were able to show that celecoxib potently inhibits PKB kinase activity in prostate carcinoma cells, resulting in induction of apoptosis (50). Furthermore, Arico et al. demonstrated that celecoxib

inhibits the 3-phosphoinositide-dependent protein kinase-1 (PDK1), an upstream activator of Akt kinase, and this inhibition was accompanied by an induction of apoptosis in HT-29 colon cancer cells (51). Therefore, we put forward the hypothesis that celecoxib induces degradation of β-catenin partly by inhibition of PDK-1, which results in inactivation of Akt kinase and in subsequent activation of GSK-3β. This hypothesis is supported by our findings that celecoxib treatment truly reduced GSK-3β– phosphorylation at ^{9}Ser, the specific phosphorylation site of Akt, leading to activation of the GSK-3β enzyme. The active GSK-3β kinase could then phosphorylate membrane-associated β-catenin, which translocates into the cytoplasm, where it is degraded by the proteasomal pathway. However, recent studies investigating the structure and functions of the cadherin-catenin complexes showed that the integrity of this complex is regulated by the balance of tyrosine kinases and phosphatases (6). Therefore, additional kinase activities next to GSK-3β might be implicated in the celecoxib-induced release of β-catenin from the cell membrane. Further studies are necessary to elucidate the precise mechanism of this β-catenin translocation.

Furthermore, using the caspase inhibitor BOC-D-FMK we were able to show that, in addition to proteasomal activity, celecoxib-induced β-catenin degradation is also mediated by caspases. These findings are corroborated by the study of Steinhusen et al., who reported that β-catenin can directly be cleaved by caspase-3 (24), and our own previous studies revealed caspase-3 to be a major executor of celecoxib-induced apoptosis in Caco-2 cells (17).

The relevance of our data for the situation in vivo can be derived from studies in nude mice showing that suppression of β-catenin expression caused by administration of antisense-oligonucleotides clearly inhibits the tumorigenic growth of colon cancer xenografts (52). Similar effects were reported by Yamada et al., who observed a suppression of β-catenin-accumulated crypts in rats treated with celecoxib (53). In line with these findings, Brown et al. described reduced nuclear β-catenin levels in tumors of rats treated with various NSAIDs, including celecoxib (54). Furthermore, reduced levels of β-catenin were also seen in polyps of FAP patients treated with sulindac (32). Recently, celecoxib was reported to reduce a neointimal hyperplasia in rats through inhibition of Akt-signaling, indicating that modulation of Akt-kinase activity is a physiologically relevant COX-2-independent mechanism of celecoxib (55). Taken together, these results clearly point out that the reduction of β-catenin expression is an antiproliferative mechanism that is of relevance in vivo and contributes to the anticarcinogenic effects of celecoxib.

In conclusion, we have obtained evidence that targeting the β-catenin/APC signaling pathway might be a novel approach to explain COX-2 independent anticarcinogenic effects of celecoxib. The importance of these findings for the in vivo use of celecoxib becomes apparent, considering that strong nuclear or cytoplasmatic β-catenin staining in colorectal cancer tissues correlates with more invasive tumor growth, a higher susceptibility of disease recurrence after surgery, and a lower survival rate (56–58).

ACKNOWLEDGMENTS

The authors would like to thank Dr. Dietmar Falke for critically reading this manuscript. The work was supported by the Deutsche Forschungsgemeinschaft (DFG GR2011/1-1 and the European Graduate School “Roles of Eicosanoids in Biology and Medicine,” DFG GRK 757/1).

REFERENCES:

1. Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., et al. (2000) The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N. Engl. J. Med.* **342,** 1946–1952

2. Moon, R. T., Kohn, A. D., De Ferrari, G. V., and Kaykas, A. (2004) WNT and beta-catenin signalling: diseases and therapies. *Nat. Rev. Genet.* **5,** 691–701

3. Ilyas, M., Tomlinson, I. P., Rowan, A., Pignatelli, M., and Bodmer, W. F. (1997) Beta-catenin mutations in cell lines established from human colorectal cancers. *Proc. Natl. Acad. Sci. USA* **94,** 10,330–10,334

4. Takeichi, M. (1991) Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251,** 1451–1455

5. Kikuchi, A. (2000) Regulation of beta-catenin signaling in the Wnt pathway. *Biochem. Biophys. Res. Commun.* **268,** 243–248

6. Nelson, W. J., and Nusse, R. (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303,** 1483–1487

7. Papkoff, J., and Aikawa, M. (1998) WNT-1 and HGF regulate GSK3 beta activity and beta-catenin signaling in mammary epithelial cells. *Biochem. Biophys. Res. Commun.* **247,** 851–858

8. Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G., and Kemler, R. (1996) Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. *Mech. Dev.* **59,** 3–10

9. Kolligs, F. T., Bommer, G., and Goke, B. (2002) Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis. *Digestion* **66,** 131–144

10. Mann, B., Gelos, M., Siedow, A., Hanski, M. L., Gratchev, A., Ilyas, M., Bodmer, W. F., Moyer, M. P., Riecken, E. O., Buhr, H. J., et al. (1999) Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc. Natl. Acad. Sci. USA* **96,** 1603–1608

11. Trifan, O. C., and Hla, T. (2003) Cyclooxygenase-2 modulates cellular growth and promotes tumorigenesis. *J. Cell. Mol. Med.* **7,** 207–222

12. Grosch, S., Tegeder, I., Niederberger, E., Brautigam, L., and Geisslinger, G. (2001) COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. *FASEB J.* **15,** 2742–2744

13. Tegeder, I., Pfeilschifter, J., and Geisslinger, G. (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J.* **15,** 2057–2072

14. Yamazaki, R., Kusunoki, N., Matsuzaki, T., Hashimoto, S., and Kawai, S. (2002) Selective cyclooxygenase-2 inhibitors show a differential ability to inhibit proliferation and induce apoptosis of colon adenocarcinoma cells. *FEBS Lett.* **531,** 278–284

15. Zhang, Z., Lai, G. H., and Sirica, A. E. (2004) Celecoxib-induced apoptosis in rat cholangiocarcinoma cells mediated by Akt inactivation and Bax translocation. *Hepatology* **39,** 1028–1037

16. Bräutigam, L., Vetter, G., Tegeder, I., Heinkele, G., and Geisslinger, G. (2001) Determination of Celecoxib in human plasma and rat microdialysis samples by liquid chromatography tandem mass spectrometry. *J. Chromatogr. Biomed. Appl.* **261,** 203–212

17. Maier, T. J., Schilling, K., Schmidt, R., Geisslinger, G., and Grosch, S. (2004) Cyclooxygenase-2 (COX-2)-dependent and -independent anticarcinogenic effects of celecoxib in human colon carcinoma cells. *Biochem. Pharmacol.* **67,** 1469–1478

18. Niederberger, E., Tegeder, I., Vetter, G., Schmidtko, A., Schmidt, H., Euchenhofer, C., Brautigam, L., Grosch, S., and Geisslinger, G. (2001) Celecoxib loses its anti-inflammatory efficacy at high doses through activation of NF-κB. *FASEB J.* **15,** 1622–1624

19. Brozovic, A., Fritz, G., Christmann, M., Zisowsky, J., Jaehde, U., Osmak, M., and Kaina, B. (2004) Long-term activation of SAPK/JNK, p38 kinase and fas-L expression by cisplatin is attenuated in human carcinoma cells that acquired drug resistance. *Int. J. Cancer* **112,** 974–985

20. Sileni, V. C., Fosser, V., Maggian, P., Padula, E., Beltrame, M., Nicolini, M., and Arslan, P. (1992) Pharmacokinetics and tumor concentration of intraarterial and intravenous cisplatin in patients with head and neck squamous cancer. *Cancer Chemother. Pharmacol.* **30,** 221–225

21. Zhu, J., Huang, J. W., Tseng, P. H., Yang, Y. T., Fowble, J., Shiau, C. W., Shaw, Y. J., Kulp, S. K., and Chen, C. S. (2004) From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors. *Cancer Res.* **64,** 4309–4318

22. Johnson, A. J., Hsu, A. L., Lin, H. P., Song, X., and Chen, C. S. (2002) The cyclo-oxygenase-2 inhibitor celecoxib perturbs intracellular calcium by inhibiting endoplasmic reticulum Ca2+-ATPases: a plausible link with its anti-tumour effect and cardiovascular risks. *Biochem. J.* **366,** 831–837

23. Wong, N. A., and Pignatelli, M. (2002) Beta-catenin–a linchpin in colorectal carcinogenesis? *Am. J. Pathol.* **160,** 389–401

24. Steinhusen, U., Badock, V., Bauer, A., Behrens, J., Wittman-Liebold, B., Dorken, B., and Bommert, K. (2000) Apoptosis-induced cleavage of beta-catenin by caspase-3 results in proteolytic fragments with reduced transactivation potential. *J. Biol. Chem.* **275,** 16,345–16,353

25. Porter, A. C., and Vaillancourt, R. R. (1998) Tyrosine kinase receptor-activated signal transduction pathways which lead to oncogenesis. *Oncogene* **17,** 1343–1352

26. Stambolic, V., Ruel, L., and Woodgett, J. R. (1996) Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signaling in intact cells. *Curr. Biol.* **6,** 1664–1668

27. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275,** 1787–1790

28. Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G. H., Tan, Y., Zhang, Z., Lin, X., and He, X. (2002) Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* **108,** 837–847

29. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J. Biol. Chem.* **273,** 9357–9360

30. Grosch, S., Tegeder, I., Schilling, K., Maier, T. J., Niederberger, E., and Geisslinger, G. (2003) Activation of c-Jun-N-terminal-kinase is crucial for the induction of a cell cycle arrest in human colon carcinoma cells caused by flurbiprofen enantiomers. *FASEB J.* **17,** 1316–1318

31. Gardner, S. H., Hawcroft, G., and Hull, M. A. (2004) Effect of nonsteroidal anti-inflammatory drugs on beta-catenin protein levels and catenin-related transcription in human colorectal cancer cells. *Br. J. Cancer* **91,** 153–163

32. Boon, E. M., Keller, J. J., Wormhoudt, T. A., Giardiello, F. M., Offerhaus, G. J., van der Neut, R., and Pals, S. T. (2004) Sulindac targets nuclear beta-catenin accumulation and Wnt signaling in adenomas of patients with familial adenomatous polyposis and in human colorectal cancer cell lines. *Br. J. Cancer* **90,** 224–229

33. Rice, P. L., Kelloff, J., Sullivan, H., Driggers, L. J., Beard, K. S., Kuwada, S., Piazza, G., and Ahnen, D. J. (2003) Sulindac metabolites induce caspase- and proteasome-dependent degradation of beta-catenin protein in human colon cancer cells. *Mol. Cancer Ther.* **2,** 885–892

34. Rask, K., Nilsson, A., Brannstrom, M., Carlsson, P., Hellberg, P., Janson, P. O., Hedin, L., and Sundfeldt, K. (2003) Wnt-signalling pathway in ovarian epithelial tumours: increased expression of beta-catenin and GSK3beta. *Br. J. Cancer* **89,** 1298–1304

35. Sanz-Ortega, J., Bryant, B., Sanz-Esponera, J., Asenjo, J. A., Saez, M. C., Torres, A., Balibrea, J. L., Sobel, M. E., and Merino, M. J. (1999) LOH at the APC/MCC gene (5Q21) is frequent in early stages of non-small cell lung cancer. *Pathol. Res. Pract.* **195,** 677–680

36. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) Identification of c-MYC as a target of the APC pathway. *Science* **281,** 1509–1512

37. Tetsu, O., and McCormick, F. (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398,** 422–426

38. Easwaran, V., Lee, S. H., Inge, L., Guo, L., Goldbeck, C., Garrett, E., Wiesmann, M., Garcia, P. D., Fuller, J. H., Chan, V., et al. (2003) beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. *Cancer Res.* **63,** 3145–3153

39. Crawford, H. C., Fingleton, B. M., Rudolph-Owen, L. A., Goss, K. J., Rubinfeld, B., Polakis, P., and Matrisian, L. M. (1999) The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. *Oncogene* **18,** 2883–2891

40. Cong, F., Zhang, J., Pao, W., Zhou, P., and Varmus, H. (2003) A protein knockdown strategy to study the function of beta-catenin in tumorigenesis. *BMC Mol. Biol.* **4,** 10

41. Abou-Issa, H., and Alshafie, G. (2004) Celecoxib: a novel treatment for lung cancer. *Expert Rev. Anticancer Ther.* **4,** 725–734

42. Virmani, A. K., Rathi, A., Sathyanarayana, U. G., Padar, A., Huang, C. X., Cunnigham, H. T., Farinas, A. J., Milchgrub, S., Euhus, D. M., Gilcrease, M., et al. (2001) Aberrant methylation of the adenomatous polyposis coli (APC) gene promoter 1A in breast and lung carcinomas. *Clin. Cancer Res.* **7,** 1998–2004

43. Basu, G. D., Pathangey, L. B., Tinder, T. L., Lagioia, M., Gendler, S. J., and Mukherjee, P. (2004) Cyclooxygenase-2 inhibitor induces apoptosis in breast cancer cells in an in vivo model of spontaneous metastatic breast cancer. *Mol. Cancer Res.* **2,** 632–642

44. Dihlmann, S., Klein, S., and Doeberitz Mv, M. K. (2003) Reduction of beta-catenin/T-cell transcription factor signaling by aspirin and indomethacin is caused by an increased stabilization of phosphorylated beta-catenin. *Mol. Cancer Ther.* **2,** 509–516

45. Sadot, E., Conacci-Sorrell, M., Zhurinsky, J., Shnizer, D., Lando, Z., Zharhary, D., Kam, Z., Ben-Ze'ev, A., and Geiger, B. (2002) Regulation of S33/S37 phosphorylated beta-catenin in normal and transformed cells. *J. Cell Sci.* **115,** 2771–2780

46. Heinen, C. D., Goss, K. H., Cornelius, J. R., Babcock, G. F., Knudsen, E. S., Kowalik, T., and Groden, J. (2002) The APC tumor suppressor controls entry into S-phase through its ability to regulate the cyclin D/RB pathway. *Gastroenterology* **123,** 751–763

47. Chen, T., Yang, I., Irby, R., Shain, K. H., Wang, H. G., Quackenbush, J., Coppola, D., Cheng, J. Q., and Yeatman, T. J. (2003) Regulation of caspase expression and apoptosis by adenomatous polyposis coli. *Cancer Res.* **63,** 4368–4374

48. Kim, K., Pang, K. M., Evans, M., and Hay, E. D. (2000) Overexpression of beta-catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. *Mol. Biol. Cell* **11,** 3509–3523

49. Cross, D. A., Alessi, D. R., Vandenheede, J. R., McDowell, H. E., Hundal, H. S., and Cohen, P. (1994) The inhibition of glycogen synthase kinase-3 by insulin or insulin-like

growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochem. J.* **303,** 21–26

50. Hsu, A. L., Ching, T. T., Wang, D. S., Song, X., Rangnekar, V. M., and Chen, C. S. (2000) The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. *J. Biol. Chem.* **275,** 11397–11403

51. Arico, S., Pattingre, S., Bauvy, C., Gane, P., Barbat, A., Codogno, P., and Ogier-Denis, E. (2002) Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line. *J. Biol. Chem.* **277,** 27,613–27,621

52. Roh, H., Green, D. W., Boswell, C. B., Pippin, J. A., and Drebin, J. A. (2001) Suppression of beta-catenin inhibits the neoplastic growth of APC-mutant colon cancer cells. *Cancer Res.* **61,** 6563–6568

53. Yamada, Y., Yoshimi, N., Hirose, Y., Hara, A., Shimizu, M., Kuno, T., Katayama, M., Qiao, Z., and Mori, H. (2001) Suppression of Occurrence and Advancement of beta-catenin-accumulated crypts, possible premalignant lesions of colon cancer, by selective cyclooxygenase-2 inhibitor, celecoxib. *Jpn. J. Cancer Res.* **92,** 617–623

54. Brown, W. A., Skinner, S. A., Vogiagis, D., and O'Brien, P. E. (2001) Inhibition of beta-catenin translocation in rodent colorectal tumors: a novel explanation for the protective effect of nonsteroidal antiinflammatory drugs in colorectal cancer. *Dig. Dis. Sci.* **46,** 2314–2321

55. Yang, H. M., Kim, H. S., Park, K. W., You, H. J., Jeon, S. I., Youn, S. W., Kim, S. H., Oh, B. H., Lee, M. M., Park, Y. B., et al. (2004) Celecoxib, a cyclooxygenase-2 inhibitor, reduces neointimal hyperplasia through inhibition of Akt signaling. *Circulation* **110,** 301–308

56. Hugh, T. J., Dillon, S. A., Taylor, B. A., Pignatelli, M., Poston, G. J., and Kinsella, A. R. (1999) Cadherin-catenin expression in primary colorectal cancer: a survival analysis. *Br. J. Cancer* **80,** 1046–1051

57. Maruyama, K., Ochiai, A., Akimoto, S., Nakamura, S., Baba, S., Moriya, Y., and Hirohashi, S. (2000) Cytoplasmic beta-catenin accumulation as a predictor of hematogenous metastasis in human colorectal cancer. *Oncology* **59,** 302–309

58. Ougolkov, A. V., Yamashita, K., Mai, M., and Minamoto, T. (2002) Oncogenic beta-catenin and MMP-7 (matrilysin) cosegregate in late-stage clinical colon cancer. *Gastroenterology* **122,** 60–71

Received October 25, 2004; accepted April 15, 2005.

Fig. 1

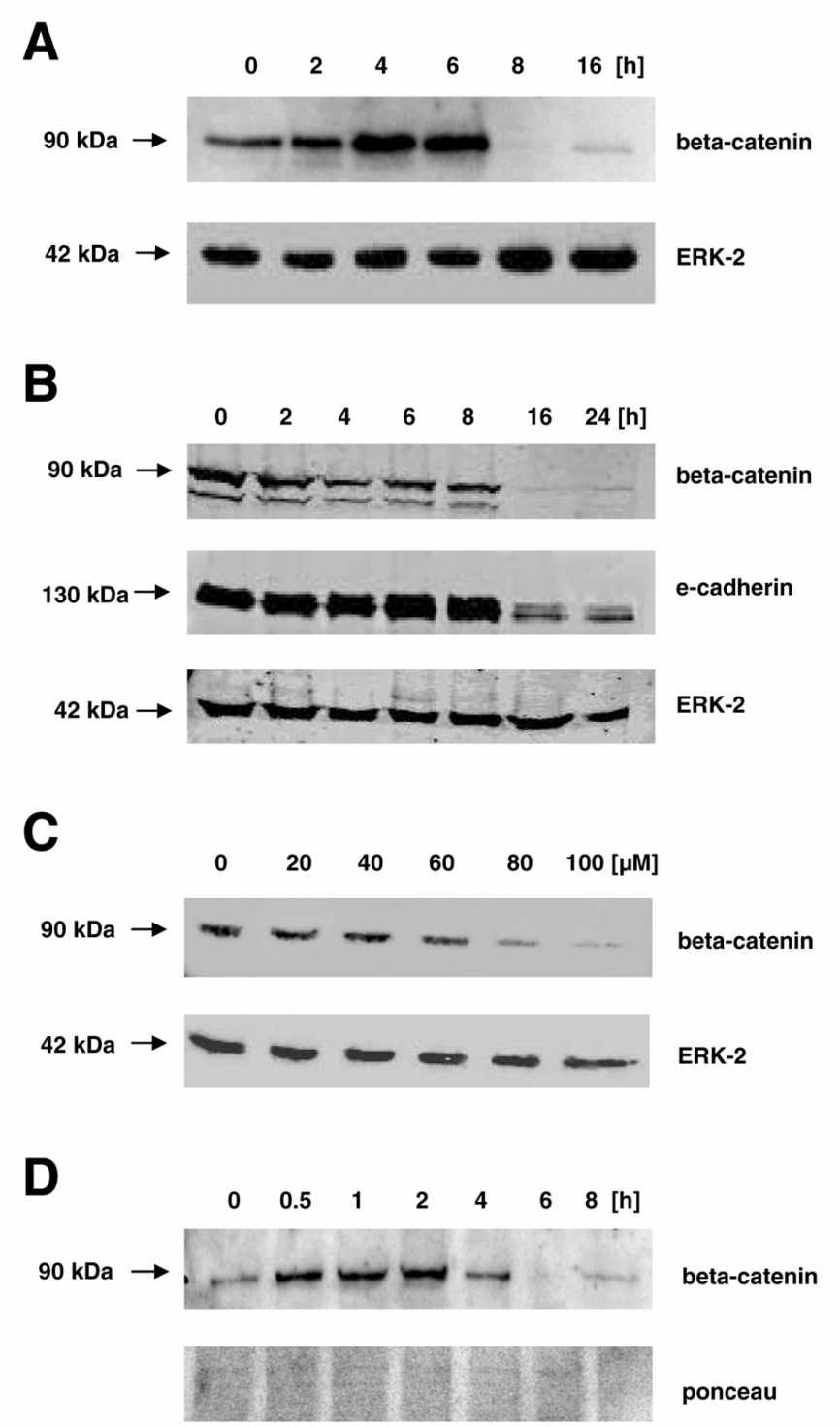

Figure 1. Western blot analysis of β-catenin and e-cadherin expression in (***A***) soluble protein extracts or (***B***) membrane extracts after treatment of Caco-2 cells with 100-μM celecoxib for the indicated time periods. ***C*)** Western blot analysis of β-catenin expression in soluble protein extracts after treatment of Caco-2 cells with increasing concentrations of celecoxib (0-100 μM) for 20 h. (***D***) Western blot analysis of β-catenin expression in nuclear extracts after treatment of the cells with 100 μM celecoxib for the indicated time periods. For Western blot analysis, 50 μg of soluble (***A–C***) or 30 μg of nuclear protein extracts (***D***) were separated onto 12% SDS-polyacrylamide gels and electroblotted onto a nitrocellulose membranes. The membranes were then incubated with antibodies recognizing the human β-catenin or e-cadherin protein. Equal loading of the gels was checked by reprobing the blot with an anti-ERK-2 antibody or by Ponceau S staining. Representative experiments from 3 separate experiments each are shown.

Fig. 2

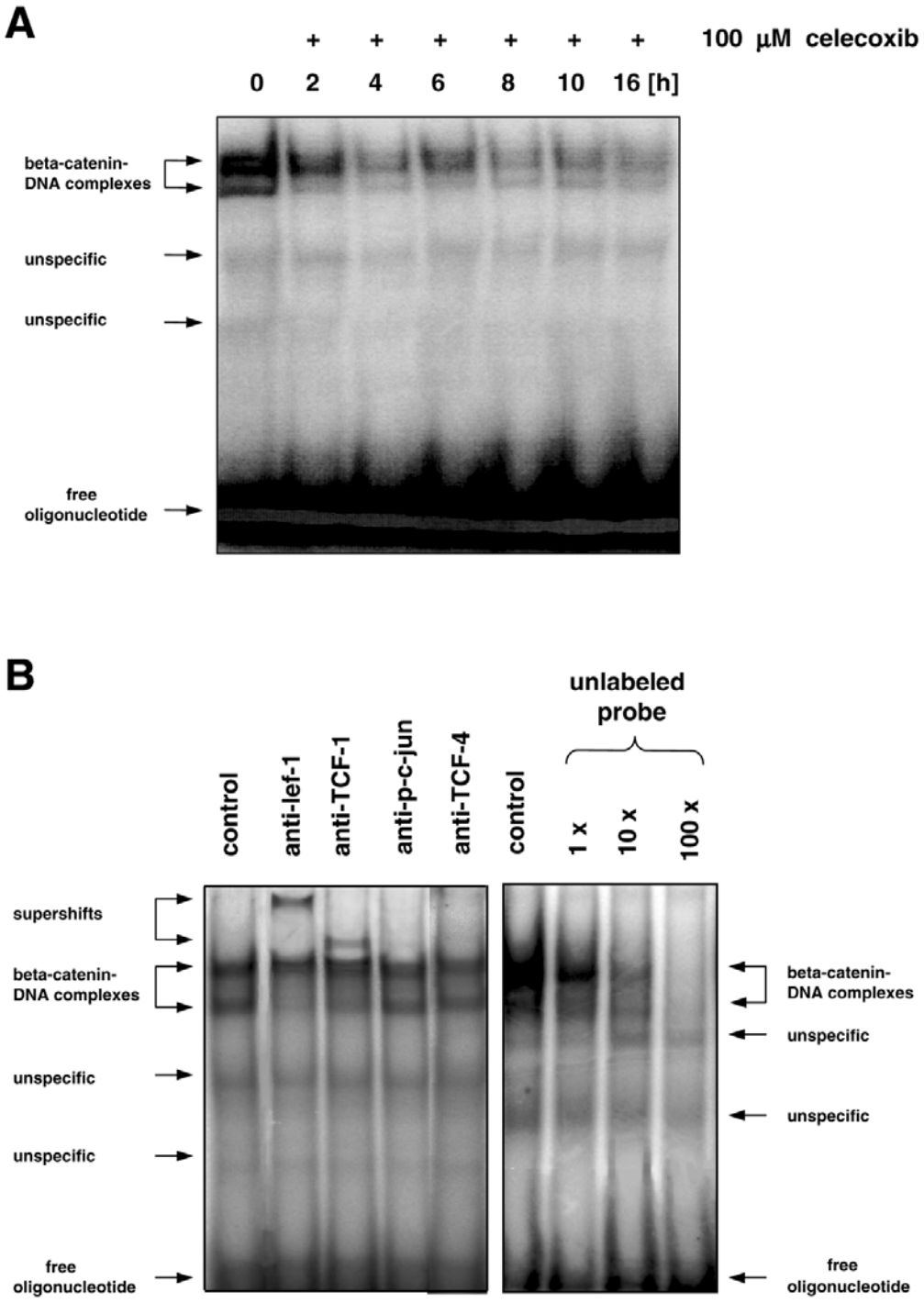

Figure 2. ***A)*** Determination of β-catenin DNA binding activity after celecoxib treatment using electrophoretic mobility shift assay. Caco-2 cells were treated with 100 µM celecoxib for the times indicated. Then, nuclear extracts were prepared. For electrophoretic mobility shift assay, 4 µg of the nuclear extract were incubated with a radioactive labeled DNA oligonucleotide containing the corresponding sequence for β-catenin. A representative experiment from 3 separate experiments is shown. ***B)*** For supershift experiments, 4 µg of untreated nuclear protein extract were incubated together with the radioactive labeled oligonucleotide for 30 min at room temperature. Then, 2 µg of each supershift-antibody (lef-1 goat polyclonal, TCF-1 and TCF-4 rabbit polyclonal, p-c-jun mouse monoclonal) were added and the mixture incubated for 1 h at 4°C. For unlabeled probe competition experiments, 4 µg of untreated nuclear protein extract were incubated together with the radioactive labeled oligonucleotide, and increasing amounts of unlabeled probe (one-fold, 10-fold, and 100-fold excess) for 30 min at room temperature.

Fig. 3

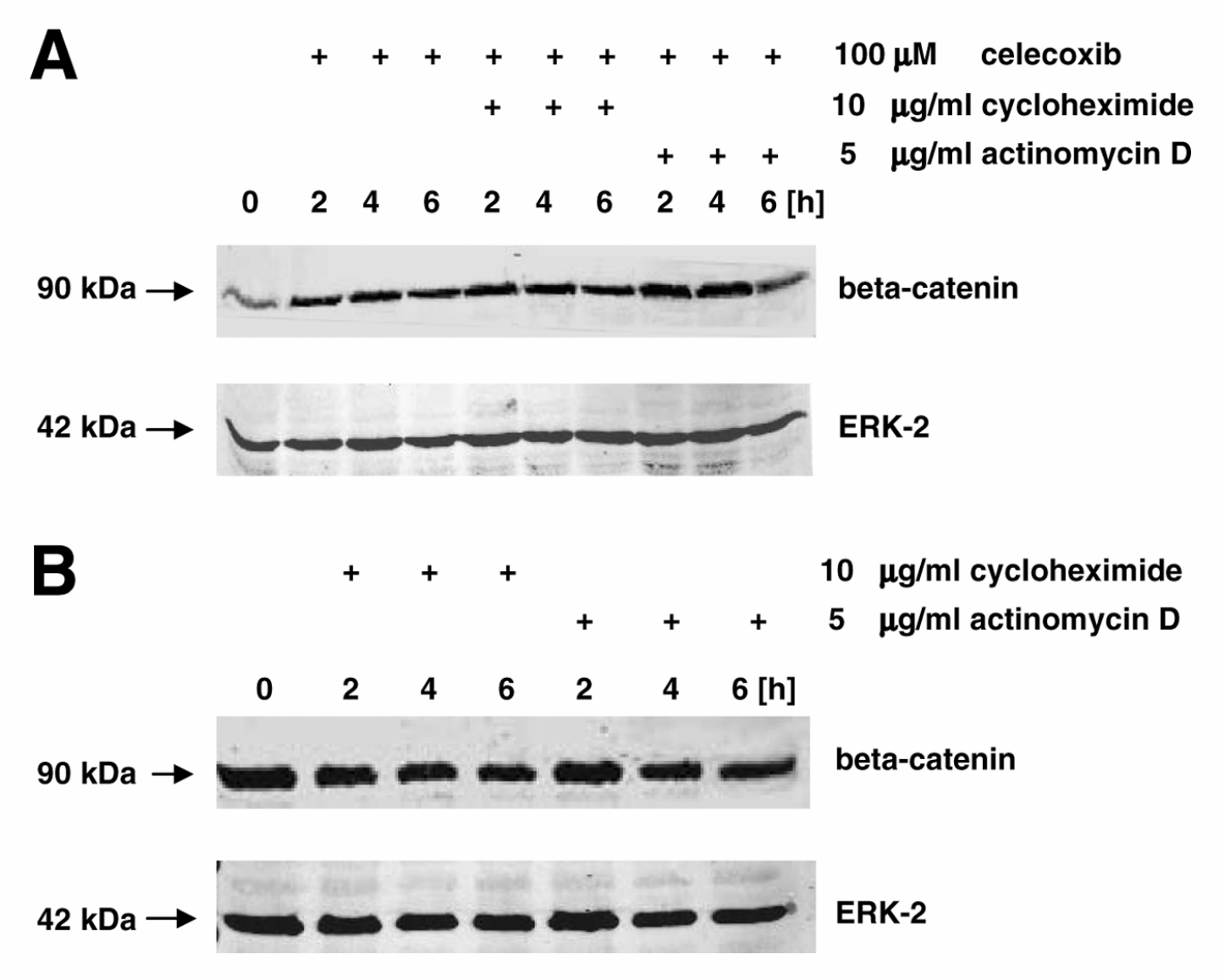

Figure 3. ***A)*** Western blot analysis of β-catenin expression after treatment of Caco-2 cells for the indicated time periods with the combination of either 100 µM celecoxib and 10 µg/ml cycloheximide, 100 µM celecoxib, and 5 µg/ml actinomycin D or 100 µM celecoxib alone. For Western blot analysis, 50 µg of total soluble protein extract were separated onto a 12% SDS-polyacrylamide gel and electro-blotted onto a nitrocellulose membrane. The membrane was then incubated with antibodies directed against human β-catenin. Equal loading of the gel was checked by staining the membrane with Ponceau S solution and reprobing the blot with an anti-ERK-2 antibody. A representative experiment from two separate experiments is shown. ***B)*** In a control experiment, Caco-2 cells were separately treated with 10 µg/ml cycloheximide and 5 µg/ml actinomycin D without addition of celecoxib. Western blot analysis of β-catenin expression in soluble extracts was then performed as described above. Staining the membrane with Ponceau S solution and reprobing the blot with an anti-ERK-2 antibody checked equal loading of the gel. A representative experiment from two separate experiments is shown.

Fig. 4

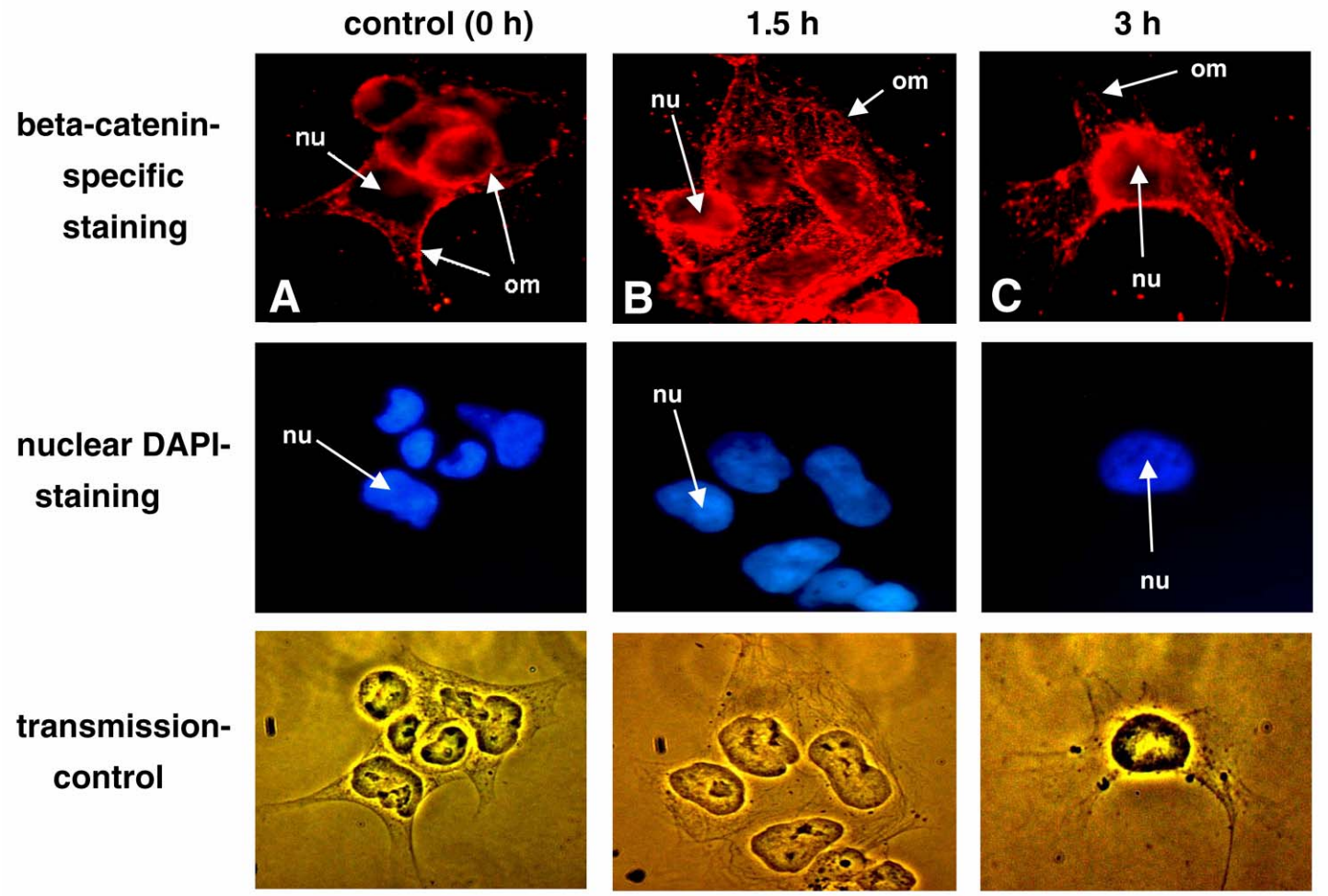

Figure 4. Determination of cellular β-catenin distribution using immunocytochemistry. Cells were treated with 100-μM celecoxib for 1.5 h (***B***), and 3 h (***C***), while the control cells were left untreated (***A***). Cells were then fixated with paraformaldehyde and then permeabilized using triton-X 100. Cells were blocked overnight with BSA/PBS, subsequently incubated with the primary β-catenin-specific antibody, and then treated with the Cy3-labeled secondary antibody. Cells were viewed using an eclipse 600 fluorescence microscope (Nikon, Tokyo, Japan). A representative experiment from 3 separate experiments is shown. Abbreviations: om = outer membrane, nu = nucleus.

Fig. 5

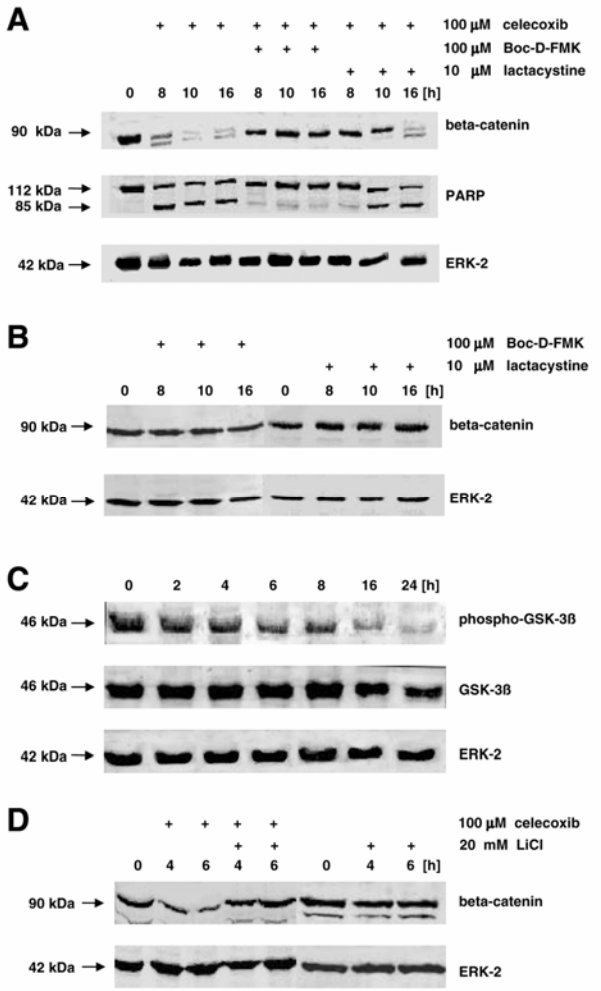

Figure 5. ***A)*** Western blot analysis of β-catenin expression and PARP cleavage after treatment of Caco-2 cells for the indicated time periods with the combination of either 100 µM celecoxib and 100 µM caspase inhibitor, 100 µM celecoxib and 10 µM lactacystine or 100 µM celecoxib alone. For Western blot analysis, 50 µg of total soluble protein extract were separated onto a 12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was then incubated with an antibody directed against human β-catenin. PARP (112 kDa), and the apoptotic cleavage product of PARP (85 kDa) was detected using a rabbit polyclonal anti-PARP antibody. Staining the membrane with Ponceau S solution and reprobing the blot with an anti-ERK-2 antibody checked equal loading of the gel. A representative experiment from three separate experiments is shown. ***B)*** In a control experiment, Caco-2 cells were separately treated with 100 µM caspase inhibitor and 10 µM lactacystine without addition of celecoxib. Western blot analysis of β-catenin expression in soluble extracts was then performed as described above. Staining the membrane with Ponceau S solution and reprobing the blot with an anti-ERK-2 antibody checked equal loading of the gel. A representative experiment from two separate experiments is shown. ***C)*** Western blot analysis of phospho-GSK-3β and total GSK-3β protein levels after treatment of Caco-2 cells for the indicated time periods with 100 µM celecoxib. For Western blot analysis, the soluble extracts were separated onto a 12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was then incubated with antibodies raised against human GSK-3β or phospho-GSK-3β protein. Equal loading of the gel was checked by staining the membrane with Ponceau S solution and reprobing the blot with an anti-ERK-2 antibody. A representative experiment from two separate experiments is shown. ***D)*** Western blot analysis of membrane β-catenin expression after preincubation of Caco-2 cells with 20 mM LiCl for 30 min and subsequent treatment with 100 µM celecoxib. For Western blot analysis, the membrane extract was separated onto a 12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was then incubated with antibodies raised against the human β-catenin protein. Equal loading of the gel was checked by staining the membrane with Ponceau S solution and reprobing the blot with an anti-ERK-2 antibody. A representative experiment from 3 separate experiments is shown.

Fig. 6

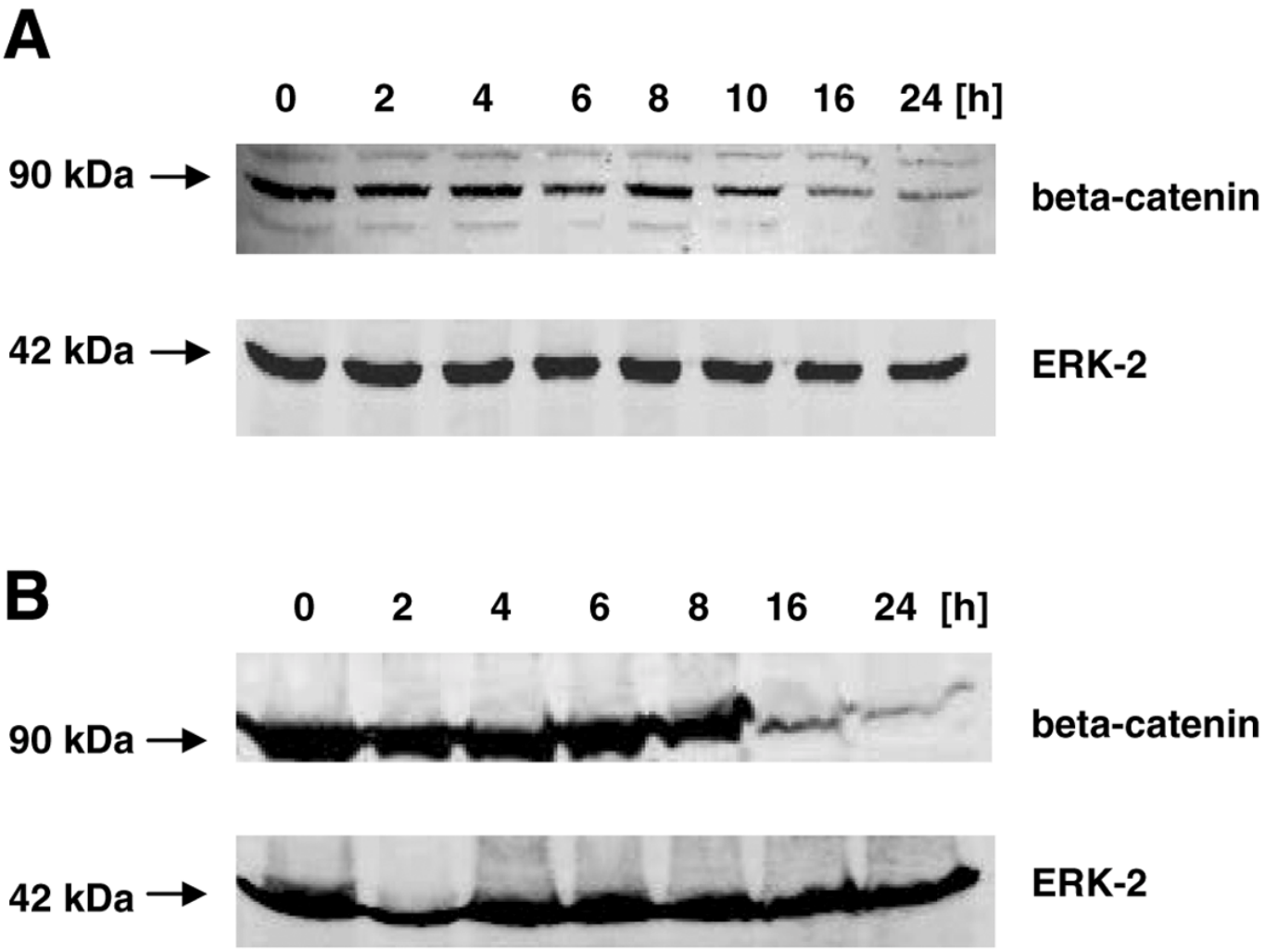

Figure 6. Western blot analysis of β-catenin expression after treatment of HCT-116 cells with 100 μM celecoxib for the indicated times. For Western blot analysis, 50 μg of total soluble protein extracts (***A***) or membrane extracts (***B***) were separated onto a 12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membranes were then incubated with antibodies recognizing the human β-catenin protein. Staining the membranes with Ponceau S solution and reprobing the blots with an anti-ERK-2 antibody checked equal loading of the gels. Representative experiments from three separate experiments each are shown.

Fig. 7

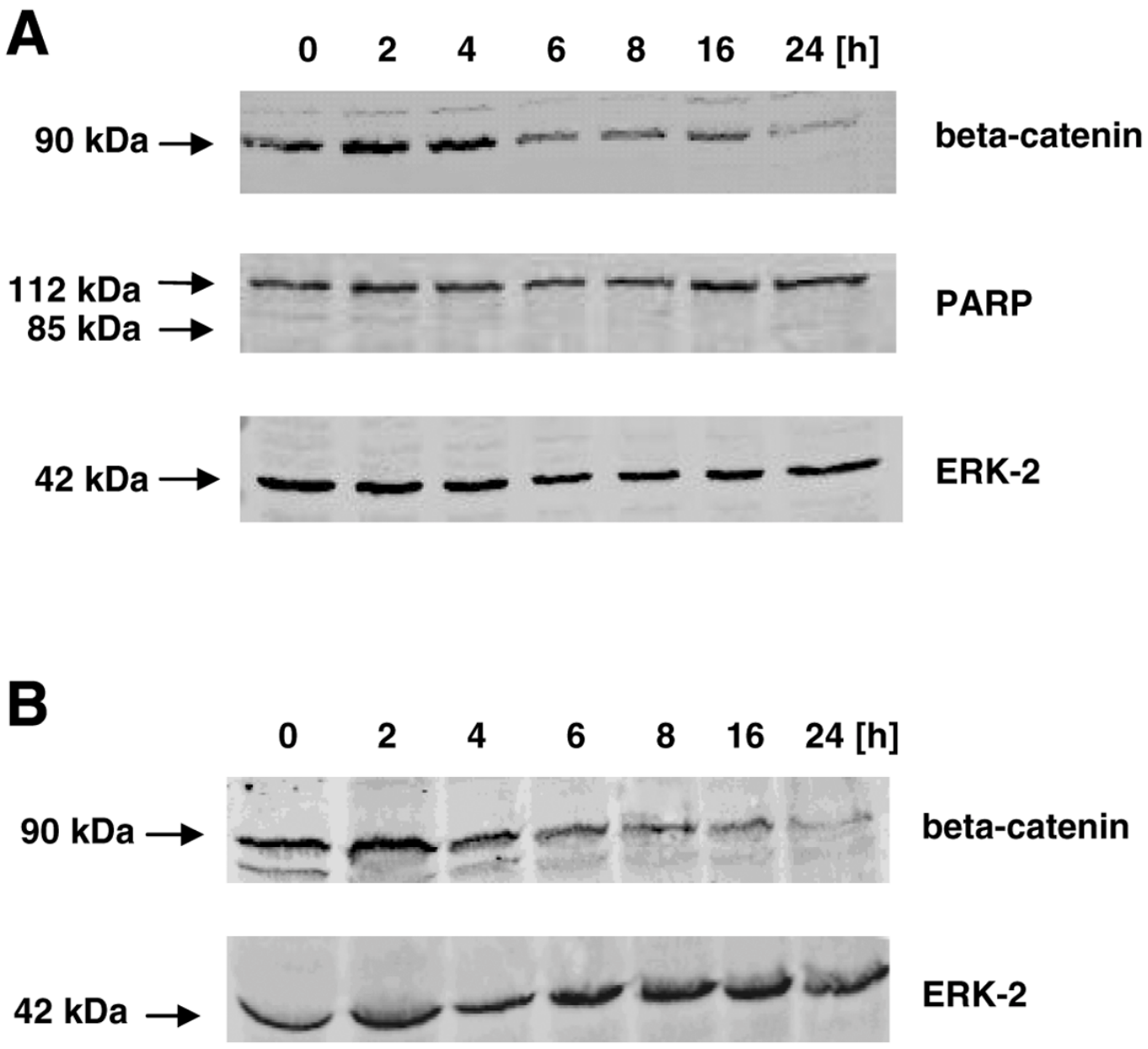

Figure 7. Western blot analysis of β-catenin expression and PARP cleavage after treatment of MCF-7 cells with 100 μM celecoxib for the indicated times. For Western blot analysis, 50 μg of total soluble protein extracts (***A***) or membrane extracts (***B***) were separated onto 12% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. The membranes were then incubated with antibodies recognizing the human β-catenin protein. PARP (112 kDa) and the apoptotic cleavage product of PARP (85 kDa) were detected using a rabbit polyclonal anti-PARP antibody. Equal loading of the gel was checked by staining the membrane with Ponceau S solution and reprobing the blot with an anti-ERK-2 antibody. Representative experiments from 3 separate experiments each are shown.

Fig. 8

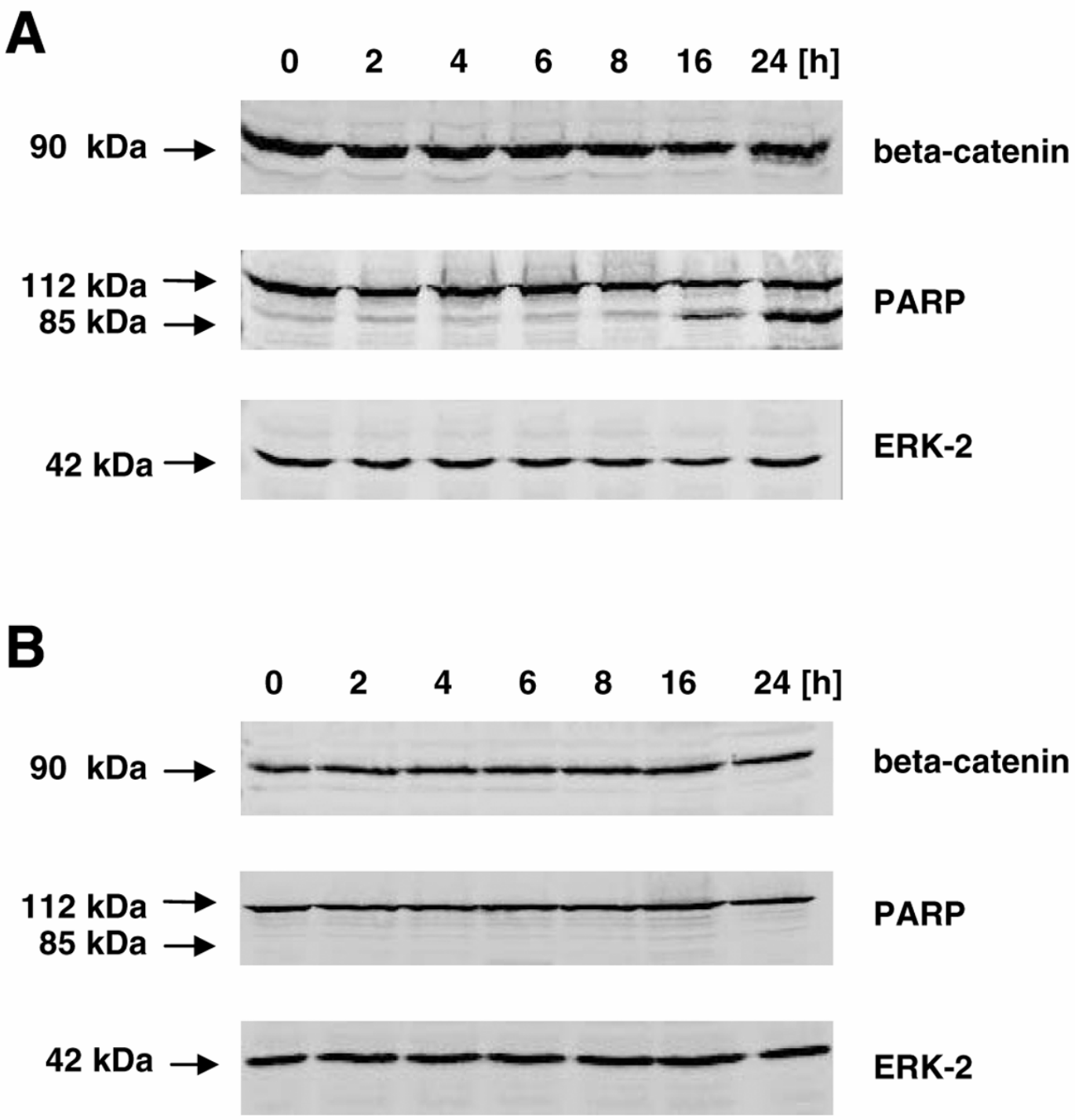

Figure 8. Western blot analysis of β-catenin expression and PARP cleavage after treatment of Caco-2 cells for the indicated times with 1000 μM R-flurbiprofen (***A***) and 100 μM rofecoxib (***B***). For Western blot analysis, 50 μg of total soluble protein extracts were separated onto 12% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. The membranes were then incubated with antibodies directed against human β-catenin. PARP (112 kDa), and the apoptotic cleavage product of PARP (85 kDa) were detected using a rabbit polyclonal anti-PARP antibody. Staining the membrane with Ponceau S solution and reprobing the blots with an anti-ERK-2 antibody checked equal loading of the gel. Representative experiments from two separate experiments each are shown.