Adenomatous polyposis coli protein (APC)-independent regulation of β -catenin/Tcf-4 mediated transcription in intestinal cells

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Alterations in the transcriptional activity of the β -catenin–Tcf complex have been associated with the earlier stages of colonic transformation. We show here that the activation of protein kinase C by the phorbol ester PMA in several intestinal cell lines increases the levels of β -catenin detected in the nucleus and augments the transcriptional activity mediated by β -catenin. The response to PMA was not related to modifications in the cytosolic levels of β -catenin and was observed not only in cells with wild-

type adenomatous polyposis coli protein (APC) but also in APC-deficient cells. Binding assays *in vitro* revealed that PMA facilitates the interaction of the β -catenin with the nuclear structure. Our results therefore show that β -catenin-mediated transcription can be regulated independently of the presence of APC.

Key words: β -catenin, protein kinase C, Tcf-4.

INTRODUCTION

The cellular mechanisms responsible for spontaneous and genetically transmitted colonic carcinogenesis are poorly understood. During the past few years several reports have revealed that β -catenin, a known component of the cell–cell contact complexes, also behaves as a signalling molecule in colonic carcinogenesis and embryonic development. Thus β -catenin can be found either associated with cellular contacts or in the nucleus, where it interacts with and modulates the activity of a Lef/Tcf family of transcription factors [1–3]. Although several genes, including those for c-Myc, cyclin D1, c-Jun, Fra-1 and uPA receptor have been described as being activated by the β -catenin–Tcf complex [4–6], the characterization of the genes targeted by β -catenin is still an open issue required for a full understanding of the initial steps in colonic carcinogenesis.

Genetic studies on *Xenopus laevis* and *Caenorhabditis elegans*, as well as biochemical approaches on mammalian cells, have revealed an accurate mechanism for controlling cytosolic levels of free β -catenin protein, involving phosphorylation and degradation through the proteasome (reviewed in [7–9]). Cytosolic complexes of β -catenin, adenomatous polyposis coli protein (APC) and glycogen synthase kinase 3 β (GSK-3 β) are necessary for serine phosphorylation and the subsequent degradation of the β -catenin. GSK-3 β acts as a kinase that phosphorylates β -catenin and probably APC as well; the presence of APC is required because this protein seems to act as an anchor between the kinase and the substrate. Recently another protein, axin, has been described to form part of the complex β -catenin–GSK-3 β –APC [10–12]. Physiological stimulus, i.e. Wnt signalling, increases β -catenin degradation by inhibiting GSK-3 β activity, whereas in colonic carcinogenesis, mutations mainly in APC allow β -catenin to avoid degradation [9].

A model relating the activation of protein kinase C (PKC) to colonic carcinogenesis has been discussed [13]. Addition of PMA promotes a scattered phenotype and blocks differentiation of epithelial intestinal cells [14,15], phenomena related to progression of colonic transformation. Kinases as src and FAK have been related to the early PMA-induced disorganization of

the adherent junctions [15,16]. However, molecular mechanisms responsible for the late responses to this phorbol ester, like loss of epithelial cell markers [14], have not been characterized yet. In this report we show that PKC enhances transcription mediated by β -catenin/Tcf-4.

EXPERIMENTAL

Cell culture and DNA constructions

All cell lines were grown in Dulbecco's modified Eagle's medium plus 10% (v/v) fetal bovine serum under standard conditions of temperature and humidity. Clone SW-480 209-1 was isolated from the heterogeneous colonic-carcinoma-derived cell line SW-480 by limit dilution. Intestinal HT-29 M6 cells have been broadly characterized [17]. The generation and properties of HT-29 M6 A4 clone have been reported previously [18]. The lung cancer cells SKLC-16 were provided by Dr. F. X. Real (Institut Municipal d'Investigació Mèdica, Barcelona, Spain). DNA coding for an N-terminal truncated form of human Tcf-4 (Δ Tcf-4) that cannot bind β -catenin [2] was amplified by PCR from two overlapping expressed sequence tags (Image Clones ye89d06 and y188b10) and cloned in pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). The primers used were 5'-GGCGATCCACC-ATGGAAACTCCTC-3' and 5'-GGACTATGGAGTGAGC-CG-3' for ye89d06, and 5'-CCGGATATCCTTCTAAAGAC-TTGGTGA-3' and 5'-ATCCTTCTAAAGACTTGGTGACG-AG-3' for y188b10. The two PCR products obtained were annealed through the overlapped region and extended to obtain a DNA fragment containing a *Bam*HI restriction site and a Kozak sequence at the 5' end and an *Eco*RV restriction site at the 3' end. This fragment was directionally cloned into pcDNA3 with the enzymes mentioned.

Immunofluorescence

Cellular β -catenin localization was analysed by immunofluorescence, as described previously [14], with an anti-(β -catenin) monoclonal antibody (Transduction Laboratories, Lexington,

Abbreviations used: APC, adenomatous polyposis coli protein; BMT, β -catenin-mediated transcription; Gf, PKC inhibitor GF109203X; GSK-3 β , glycogen synthase kinase 3 β ; GST, glutathione S-transferase; PKC, protein kinase C.

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KY, U.S.A.). In brief, cells, grown in Dulbecco's modified Eagle's medium plus fetal bovine serum on a glass coverslip, were treated or not with 100 nM PMA and fixed with paraformaldehyde. After being blocked with PBS supplemented with 5% (v/v) horse serum, coverslips were incubated with anti- β -catenin monoclonal antibody and subsequently with a goat anti-mouse IgG conjugated with rhodamine (Pierce, Rockford, IL, U.S.A.) or to FITC (Dako A/S, Copenhagen, Denmark). Coverslips were mounted with Mowiol and analysed under a fluorescence microscope. To locate the nucleus, cells were stained with propidium iodide. As a control, the cellular distributions of other catenins (α -catenin and p120) were analysed with specific monoclonal antibodies (Transduction Laboratories).

Tcf-4 transcriptional activity

Approx. 6×10^4 cells per point were transiently transfected by using a Lipofectamine-based protocol (Gibco BRL; Life Technologies, Cergy Pontoise, France) with equal quantities of two plasmids containing luciferase as reporter gene. Reporter plasmid TOP (a gift from Dr. H. Clevers, University of Utrecht, Utrecht, The Netherlands) contained three copies of the Tcf-4 binding site upstream of the c-Fos minimal promoter, and the firefly luciferase gene as reporter; the control plasmid contained the *Renilla* luciferase gene under the control of the constitutive thymidine kinase promoter (pRL-TK, Promega). Firefly and *Renilla* luciferase activities were determined by following a dual-luciferase reporter assay system protocol (Promega) 48 h after transfection. Luciferase activities were measured with an FB 12 luminometer (Berthold Detection Systems, Pforzheim, Germany) and expressed as firefly luciferase activity/*Renilla* luciferase activity for each sample. Typically, treatment with PMA was initialized 24 h before cell lysis. Controls for specificity were performed with a reporter gene containing mutated Tcf-4-binding sites (plasmid FOP) (provided by Dr. H. Clevers); values lower than 5% were subtracted from TOP values. When indicated, an expression plasmid containing APC (kindly provided by Dr. K. Kinzler, Johns Hopkins University, Baltimore, MD, U.S.A.) or Δ Tcf-4 under the control of a cytomegalovirus promoter was included in the transfection. Controls were performed in parallel and always carried equal quantities of empty plasmid.

β -Catenin binding assay

Recombinant β -catenin was expressed in *Escherichia coli* as a glutathione S-transferase (GST) fusion protein in pGEX-6P3 plasmid (Pharmacia, Uppsala, Sweden); it was purified by chromatography on glutathione-Sepharose columns and the GST domain was removed by cleaving with PreScission protease (Pharmacia). After a second chromatography on the same column the purity of the preparation was more than 95%, as estimated by SDS/PAGE and Coomassie staining. β -Catenin or an irrelevant protein used as control (GST) was labelled with Alexis 488 dye as recommended the manufacturer (Molecular Probes, Eugene, OR, U.S.A.). SW-480 cells were grown over a glass coverslip up to 20–30% confluence and incubated with or without 100 nM PMA for 24 h. Subsequently, cells were permeabilized for 5 min at 4 °C with digitonin (35 μ g/ml) in NI buffer [250 mM sucrose/100 mM potassium acetate/20 mM Hepes/KOH (pH 7.4)/2 mM magnesium acetate/2 mM dithiothreitol/1 mM EGTA/10 μ g/ml leupeptin/10 μ g/ml aprotinin]. Binding reactions were performed for 30 min at room temperature on a 20 μ l drop containing 2–10 μ l of labelled β -catenin (approx. 0.6–3 ng) or GST in NI buffer plus 10 mg/ml BSA. As control, the binding of labelled β -catenin was competed for by a

50-fold excess of unlabelled protein. After binding cells were fixed with 4% (w/v) paraformaldehyde, coverslips were mounted with Mowiol and analysed under the fluorescence microscope (Zeiss Axioskop). In parallel, cells were stained with propidium iodide to reveal nuclei. The amount of nuclear staining from the images obtained with a Sony 3CCD colour video camera was determined with Adobe PhotoShop. Values expressing the number of pixels per cell were measured. A total of 100–150 cells per coverslip were analysed.

RESULTS

Treatment with PMA and activation of PKC α trigger β -catenin localization to the nucleus

The effects of PKC activation on the morphology of intestinal epithelial cells have been well described [14,15]. Under standard conditions, HT-29 M6 cells, a well-differentiated cell line that forms an epithelium-like monolayer [17], grow as compact colonies with well-defined intercellular contacts. When analysed by immunofluorescence these cells showed a localization of β -catenin exclusively at the plasma membrane, indicating a cell–cell contact role for β -catenin (Figure 1A). However, when cells were treated for long periods (24 h) with PMA (Figure 1B), β -catenin staining was preferentially cytoplasmic and nuclear. The effect of PMA was blocked by the addition of GF109203X (Gf), a PKC-specific inhibitor [19] (results not shown). A4 cells, an HT-29 M6 transfected clone that expresses a constitutively active form of PKC α and consequently does not establish cell–cell contacts [18], also presented cytoplasmic and nuclear β -catenin staining (Figure 1C). This nuclear signal was increased by treatment with PMA (Figure 1D) because, although partly active, the PKC α form expressed by A4 cells can be further activated by PMA [18]. Catenin translocation to the nucleus was specific for β -catenin because nuclear staining was not observed when cells were analysed with anti- α -catenin or anti-(p120-catenin) (results not shown).

A similar response to PMA was detected in SW-480, an intestinal cell line used in the initial studies on β -catenin signalling [2]. In this cell line, β -catenin staining was not detected at the plasma membrane, which is consistent with the lack of cell–cell contacts observed under the microscope (Figure 2A). β -Catenin was found in the cytosol and, in less extension, in the nucleus. After treatment with PMA it was detected almost exclusively in the nucleus (Figure 2B). Thus the activation of PKC in intestinal cell lines triggers the localization of β -catenin to the nucleus.

We next analysed whether the effect of PMA was related to its ability to disrupt cell–cell contacts. Two different types of experiment were performed. HT-29 M6 cells were treated with PMA under different conditions of confluence and with additions that hampered or favoured cell contacts. When HT-29 M6 cells were plated at low dilutions, β -catenin was detected in single cells, diffusely distributed throughout the cytosol but not in the nucleus (Figure 1E). When these cells began to form cell–cell contacts, the presence of β -catenin was observed predominantly in these areas (Figure 1E). To block the disruption of cell contacts, HT-29 M6 cells were incubated with high extracellular concentrations of Ca^{2+} , a condition that blocks cell scattering [14]. In contrast with cells incubated in standard medium (Figure 1B), under these conditions the presence of PMA did not induce the appearance of β -catenin staining in the nucleus (Figure 1F).

Similar results were obtained when the effect of PMA was studied in a subclone of SW-480 cells (209-1), which has a spherical phenotype and does not establish cell–cell contacts. β -Catenin was not localized in the nucleus under standard con-

Figure 1 PMA increases β -catenin staining in HT-29 M6 cell nuclei

Non-transfected HT-29 M6 cells (**A, B, E, F**) or transfected with an active PKC α form (**C, D**) were grown on collagen-coated coverslips. The distribution of β -catenin from non-treated cells (left panels) or cells treated with 100 nM PMA for 24 h (right panels) was assayed by immunofluorescence as described. The HT-29 M6 cells in (**E**) were plated at low density and examined 24 h after seeding. In (**F**), cell culture medium was supplemented with 10 mM CaCl₂ during treatment with PMA. To reveal nuclear structures, samples were treated with RNase and stained with propidium iodide (results not shown).

ditions of growth (Figure 2C) but was translocated there when these cells were treated with PMA (Figure 2D). These experiments suggest that the translocation of β -catenin to the nucleus requires the disruption of contacts; however, additional signals are also needed.

PMA specifically increases Tcf-4/ β -catenin-mediated transcription in epithelial cells

To analyse whether the PMA-induced nuclear translocation of β -catenin has functional relevance, we determined β -catenin-mediated transcription (BMT) with a luciferase reporter assay, as described in the Experimental section. SW-480 cells were chosen for these studies because this cell line is more efficiently transfected than HT-29 M6; however, transcriptional responses to PMA were also observed in the latter cell line (results not shown). As shown in Table 1, PMA consistently and reproducibly

increased the BMT of a reporter gene placed under the control of a promoter constructed by fusing three copies of the Tcf-4 consensus responsive sequence and a c-Fos minimal promoter (TOP). As a control, a mutated form of the TOP plasmid (FOP) was used; the levels of transcription of this plasmid were lower than 5% of the control and were not sensitive to PMA (results not shown). These same constructions have previously been used to determine BMT [2,20]. Basal BMT in SW-480 cells was comparable to that from the thymidine kinase promoter, a low-to-moderate promoter used to normalize the efficiency of transfection. SW-480 cells were co-transfected during the reporter experiments with an expression plasmid containing an N-terminal truncated form of Tcf-4 that cannot bind β -catenin (Δ Tcf-4) [2]. The presence of Δ Tcf-4 decreased the basal levels of BMT and abolished the PMA-induced response (Table 1) indicating that at least 65% of basal and PMA-induced TOP transcription requires the interaction of β -catenin with Tcf-4.

Figure 2 β -Catenin translocates to the nucleus similarly in SW-480 and in SW-480 (209-1) cells

SW-480 (**A, B**) and SW-480 (209-1) (**C, D**) cells were grown directly on glass coverslips and treated (right panels) or not (left panels) with 100 nM PMA for 24 h. The localization of β -catenin was determined by immunofluorescence as described for Figure 1.

Table 1 PMA stimulates transcription mediated by β -catenin in SW-480 cells

Luciferase reporter assays were performed on SW-480 cells as described in the Experimental section. Results are means $\pm$ S.D. for three or four experiments performed in duplicate or in triplicate. Treatment with PMA was performed with 100 nM for 24 h.

Cell type	PMA	Relative luciferase activity (fold compared with control)
SW-480 transfected with pcDNA3	—	1
	+	3.0 ± 1.20
SW-480 transfected with Δ TCF-4	—	0.35 ± 0.05
	+	0.36 ± 0.05
SW-480 transfected with APC	—	0.25 ± 0.05
	+	0.50 ± 0.05

The involvement of PKC activity in basal BMT was also analysed with the use of the PKC-specific inhibitor Gf. The addition of low concentrations ($1 \mu\text{M}$) of Gf for 24 h significantly decreased BMT in SW-480 cells (by $43 \pm 2\%$, mean $\pm$ S.D.) and totally abolished the effect of PMA on BMT. This concentration of Gf did not affect the proliferation of these cells, estimated by measuring the incorporation of thymidine to DNA (results not shown). These results suggest that PKC controls BMT in SW-480 cells under standard conditions of growth.

Stimulation of BMT by PMA was also observed in SW-480 (209-1) cells (2.5 ± 0.5 -fold, average $\pm$ S.D. of three experiments). This value was not significantly different from that in control SW-480 cells (3.3 ± 1.1 -fold). As mentioned above, SW-480 (209-1) cells lack cell–cell contacts and also present very low contacts with the culture plate. The effect of PMA on these cells suggests that stimulation of BMT by this phorbol ester is not directly associated with changes in cell contacts with the matrix. To confirm this conclusion, BMT was determined in SW-480 cells that had been trypsin-treated and left suspended for the duration of treatment. In these cells the stimulation by PMA (2.5 ± 0.4 -fold) was not significantly different from that in control conditions.

Stimulation of BMT by PMA is also observed in cells containing wild-type APC

In intestinal cells, BMT is dependent on the cellular stability of β -catenin, a process regulated by APC (see the Introduction section). Like most intestinal epithelial cell lines, SW-480 and HT-29 M6 contain a truncated form of APC [21–23] that is unable to participate in the degradation of β -catenin. For this reason the levels of this protein in these and other intestinal cell lines are high in comparison with other epithelial cells. Although our results indicate that the regulation of BMT by PMA is not dependent on the presence of a functional APC, we examined whether the phorbol ester was increasing the stability and cellular levels of β -catenin. No modifications in the cellular levels of

Figure 3 β -Catenin docks more effectively to nuclei from SW-480 cells treated with PMA than to nuclei from control cells

Untreated SW-480 cells (A) or SW-480 cells treated with 100 nM PMA for 24 h (B) were permeabilized and incubated with labelled β -catenin as described in the text. (C) Quantification was performed by determining the number of pixels per cell in ten different fields. Results are means $\pm$ S.D. for a representative experiment of three performed. Abbreviation: TPA, PMA.

β -catenin were observed after incubation of the cells with PMA (results not shown). The addition of this phorbol ester did not modify the amount of phosphate incorporated in either Ser or Thr residues in this molecule (results not shown).

We also examined whether the effects of PMA were observed in cells containing wild-type APC. As shown in Table 1, co-transfection of APC decreased by 75 % the basal levels of BMT, indicating that wild-type APC was functional in our experiments. Transfection of APC also induced a similar decrease (83 %) in BMT determined in the presence of PMA. However, although the absolute values of BMT were lower in the presence of APC, PMA caused a significant stimulation of BMT in these conditions (Table 1).

Consistently, PMA responses were also obtained in an APC wild-type epithelial cell line, SKLC-16. As expected, basal levels of BMT were lower than in APC-deficient SW-480 cells (one-tenth of the thymidine kinase promoter activity). A treatment with PMA similar to that described in Table 1 increased BMT 3.1 ± 0.8 -fold.

These results indicate that PMA stimulates BMT in the presence of APC but that the effect is more evident in the absence of this protein. This is probably due to the higher availability of β -catenin in APC mutant cells.

PMA facilitates the binding of β -catenin to the nuclear envelope

β -Catenin has been described to behave as an importin because it docks by itself to the nuclear pore in binding assays *in vitro* [24]. We therefore assayed the ability of labelled β -catenin to associate with the nuclear envelope from PMA-treated or non-treated SW-480 cells. Cells were permeabilized with digitonin and incubated with recombinant, fluorescein-labelled β -catenin. As shown in Figure 3(A), β -catenin bound exclusively to a nucleus-related structure. The binding contained all the properties for it to be considered specific: it was not observed with

irrelevant proteins (GST), it was competed for by an excess of unlabelled β -catenin, and it was saturable. In untreated cells, binding saturated at concentrations of β -catenin of approx. 6 ng per 20 μ l drop. Treatment with PMA increased the amount of nuclear staining 3-fold as well as modifying the staining pattern (Figures 3B and 3C). Because this effect was detected in the absence of cytosol, the result suggests that PMA could increase the β -catenin docking to the nuclear envelope by modifying a component or regulator of the nuclear pore complexes. Increasing amounts of labelled β -catenin used in the assay enhanced the basal luminance and decreased the effect of PMA (results not shown). Our results indicate that PMA increases the β -catenin accumulation in the nucleus at least in part by modifying a nucleus-related component.

DISCUSSION

Changes in PKC activity have been related to the progress of colon carcinogenesis [13]. At present it is not known which PKC isoform is involved in this process. However, a transgenic model has recently shown that the overexpression of PKC β II induces colonic hyperproliferation and increased sensitivity to colon carcinogenesis [25]; these results suggest that this isoform might be positively involved in tumorigenesis *in vivo*. Our results indicate that PKC activation triggers the translocation of β -catenin to the nucleus and enhances BMT. Although the mechanism is currently unknown, this effect might explain the proposed action of PKC as the target of tumour promoters. It has been shown that these agents accelerate the process of carcinogenesis in animals with mutations in the gene for APC [26].

Several results provide evidence for the existence of a mechanism of control of BMT not based on interaction with APC. It has been shown that the presence of β -catenin in the nucleus varies in tumours of different grades, being higher in carcinomas

than in adenomas, although APC is mutated in both types of tumour [27]. We have described here the existence of such a mechanism in intestinal cells. Experiments aiming at a further characterization of the mechanism triggered by PKC are in progress in our laboratory.

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