Localization of Protein Kinase C Isozymes in Rat Colon

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Abstract

We have demonstrated previously the presence of classical (α), novel (δ and ε), and atypical (ζ) protein kinase C (PKC) isozymes in human and rat colonic mucosa (L. A. Davidson et al., Arch. Biochem. Biophys., 312: 547–553, 1994). To gain insight into the functions of individual PKC isozymes in colonic epithelium in situ, we determined the localization of the major PKC isozymes expressed in normal rat colonic epithelial cells using in situ reverse transcription (RT)-PCR and immunohistochemistry (IH). Cytokeratin, a positive biological control known to be expressed in epithelial cells, was shown by in situ RT-PCR and IH to be expressed only in epithelial cells within the colonic crypt. PKC γ, a negative control for the colonic since it is expressed only in the central nervous system, was not detectable in colonic sections by either methodology. In situ RT-PCR analysis revealed that PKC α, δ, ε, and ζ mRNAs are expressed in epithelial cells along the entire colonic crypt. In addition, PKC δ and ζ mRNA are expressed in the stromal layer. All four PKC isozymes in the colonic epithelial cells were also detected by IH. However, in general, isozyme protein expression was greater at the top of the crypt axis, associated primarily with cells having acquired a differentiated phenotype. These results suggest that PKC isozyme protein expression may be localized to mature differentiated cells at the top of the colonic crypt. Therefore, PKC isozyme-dependent signal transduction may play a role in colonic epithelial cell ontogeny along the colonic crypt axis.

Introduction

Colon cancer is the second most common cause of death from cancer in the United States (1, 2). However, the mechanisms underlying control of cell growth and differentiation in colonic epithelial tissues are poorly understood, and the treatment of colon cancer has not resulted in a significant reduction in mortality over the past 30 years (3). There is considerable evidence that PKC isozymes, members of a large family of serine/threonine kinases, regulate cell growth, differentiation, apoptosis, and function (4–10). PKC is unique in that it is activated by two classes of lipids, PS and DAG (reviewed in Refs. 4 and 5). In general, PS, a membrane phospholipid, activates the kinases in a Ca2+-dependent manner, whereas DAG, a product of phospholipid turnover, stimulates kinase activation in the presence of PS and Ca2+. Twelve PKC isozymes with distinct enzymological functions, differential tissue expression, and intracellular localization have been identified to date (4, 11–13). Based on their structure and biochemical characteristics, these isozymes can be classified into three major groups (4, 11). Classical PKCs (α, βII, and γ) are characterized enzymatically by their requirement for Ca2+, phospholipids, and DAG for activation. The novel PKC isozymes (δ, ε, η, θ, and μ) do not require Ca2+ for activation. Atypical PKCs (ζ, λ, and υ) do not respond to either Ca2+ or DAG but require PS for activation. Differences in cofactor requirements and substrate specificity, together with the varied consequences of PKC activation in the same cell, suggest that individual isozymes have distinct and specialized functions in cell signaling (4, 5).

Changes in PKC isozyme expression and subcellular distribution in a variety of epithelial cell subpopulations have been evaluated previously (14, 15). Activation of specific PKC isozymes may be involved in the mediation of increased colonic mucosal proliferation (16), which has been shown to be associated with enhanced incidence of colon cancer (17). A recent study by Craven and DeRuebertis (16) in rats showed that PKC isozyme expression in colonic mucosa can be altered by carcinogen treatment, resulting in a dramatic decrease in PKC α with a corresponding increase in PKC β expression. In addition, we have demonstrated recently that dietary fats and fibers are capable of altering colonic PKC activity (18), cell proliferation (18), and PKC isozyme expression (19).

To further define the role of PKC with respect to colonic cell ontogeny, we have determined the localization of the major PKC isozymes expressed in the rat colon model system using in situ RT-PCR in conjunction with IH. Results from this study demonstrate that four major PKC isozymes, α, δ, ε, and ζ, are expressed in rat colonic epithelial cells in situ, consistent with previous reports using colonic extracts (19, 20). Our results also show that PKC isozyme mRNAs, in general, are expressed along the entire colonic crypt axis, whereas the proteins are expressed to a greater degree at the top of the crypts. These results suggest that PKC isozyme protein expression is regulated during epithelial cell ascension along the crypt axis.

Results

In situ RT-PCR was performed using isozyme-specific primers to determine PKC isozyme mRNA localization in rat colonic epithelium. The primers used are shown in Table 1. An important advantage of this approach is that it provides evidence of PKC isozyme mRNA localization at the individual cell level. Primers were designed to select unique regions of each isozyme and were initially evaluated by standard RT-PCR and sequenced as described previously (20).
Table 1: Primers for in situ RT-PCR

| PKC α | Forward 5'-TGAACCTCAGTTGAGAT-3' | Reverse 5'-GGCTCTGGTGTCACTCGT-3' |
| PKC γ | Forward 5'-TGATTGGAAATGAGAGG-3' | Reverse 5'-GGAATCACTCGTGTGCT-3' |
| PKC δ | Forward 5'-CACATTCTCCAAGAGAAGG-3' | Reverse 5'-CTTCTCATAGGCTCCCTTG-3' |
| PKC ε | Forward 5'-CCGAGACCTTGGTTGATCC-3' | Reverse 5'-CAGTTCATCGGGACATCGT-3' |
| PKC ζ | Forward 5'-GATGTTGACATGCTTTGACT-3' | Reverse 5'-GATGTTGACCTGCTTTGACT-3' |
| Cytokeratin 8 | Forward 5'-TGACTGTGGAAGGGAGG-3' | Reverse 5'-ACACAGTCCCACACATGG-3' |
| Vimentin | Forward 5'-GGAGTGGAGAAGAGG-3' | Reverse 5'-TTGACTCTGTGCTTGG-3' |

The expression of mRNA for cytokeratin 8 (positive biological control), a characteristic marker of colonic epithelium (21, 22), was localized to colonic crypts (Fig. 1A), as visualized by the blue alkaline phosphatase reaction product. Eosin counterstaining can be seen as a faint pink color. Stromal and muscle layers did not express detectable cytokeratin transcripts, which is consistent with the IH staining patterns (Fig. 1B) and previous reports indicating that cytokeratin is expressed only in epithelial cells (21, 22). In contrast, no detectable signal by in situ RT-PCR or IH (Fig. 1, C and D) was observed for the negative biological control, PKC γ, which we have shown previously is not expressed in colonic mucosa (20). As an additional nonepithelial cell control, vimentin mRNA was detected only in colonic stroma and muscularis mucosa (data not shown), confirming the specificity of amplification and the lack of product diffusion during the PCR process.

Using serial sections, the fidelity of in situ mRNA amplification was monitored on each slide by evaluating a negative control (DNase treated, no reverse transcription), a positive control (no DNase treatment, no reverse transcription), and the sample (DNase treated and reverse transcribed). As a representative example, PKC ε is shown in Fig. 2, A-C. No detectable signal was observed for the negative control (Fig. 2A). For the positive control, in which DNA is detected and therefore all primers should amplify product in every cell, staining was evident in all cells along the crypt axis, stroma, and muscularis mucosa, indicating adequate fixation and digestion of the tissue (Fig. 2B). Fig. 2C shows the sample, PKC ε, with staining in epithelial cells along the entire crypt. Immunohistochemical staining for PKC ε protein expression is shown in Fig. 2D. Staining was more prevalent in the cells at the top of the colonic crypts, seen as a brown reaction product. Inhibition of PKC ε staining is shown in Fig. 2E, in which preincubation of the antibody with PKC ε inhibitory peptide completely eliminated epithelial staining.

Detection of PKC ζ mRNA and protein are shown in Figs. 3, A and B. In situ RT-PCR using PKC ζ primers resulted in amplification of message in epithelial cells along the entire crypt, with some staining of the stromal layer near the top of the crypts. Detection of PKC ζ protein with its antibody showed a pattern of staining similar to that for PKC ε, with more intense staining at the top of the crypts. IH staining of PKC ζ was completely abolished when preincubated with PKC ζ inhibitory peptide (data not shown). PKC α and δ gave similar patterns of staining, with mRNA detected in epithelial cells throughout the crypt and protein staining more prevalent in the cells at the top of the crypts. PKC α is shown in Fig. 3, C and D, and PKC δ, with in situ RT-PCR results shown at a higher magnification, can be seen in Fig. 3, E and F.

Discussion

Members of the PKC family are present in all tissues of the body, an indication of their biological importance. In the present study, we demonstrate for the first time using in situ RT-PCR and IH methodologies that multiple PKC isozymes (α, δ, ε, and ζ) are present in colonic epithelium and that they exhibit similar patterns of expression and localization along the colonic crypt axis. PKC α and ε, as well as cytokeratin mRNAs, were localized exclusively to epithelial cells, with no detectable staining seen in the stroma or muscle layers. Staining was fairly uniform throughout the entire crypt. In contrast, PKC δ and ζ mRNAs were not confined to epithelial cells, with light stromal staining detected following in situ RT-PCR. IH analyses showed that, in general, PKC isozyme protein expression was greater at the top relative to the base of the crypt.

Colonic epithelial cells are derived from a stem cell population at the base of the colonic crypt and migrate from a region of active cell proliferation in the bottom two-thirds of the crypt towards the top of the crypt, obtaining an increasingly differentiated phenotype (23). This would indicate, therefore, that PKC message, although present throughout the crypt column, is not translated to a detectable degree until colonocytes begin to differentiate and move up the crypt. Interestingly, apoptotic cells are also seen in the terminally differentiated phenotypes near the top of the crypt axis (24). Therefore, the level of protein expression for PKC α, δ, ε, and ζ is increased in differentiating/differentiated or apoptotic colonic epithelial cells, rather than proliferating basal cells. These data are consistent with previous reports indicating that the pathway of a cell toward death or differentiation may, in part, be modulated by alterations within the PKC signal transduction pathway (9, 10, 15).

Little is known regarding the mechanisms involved in the regulation of colonic cell growth, differentiation, and apoptosis. Studies have shown that various PKC isozymes may regulate colonic epithelial cytokinetics since alterations in indices of colonocyte differentiation and proliferation are correlated with PKC activity (18, 25, 26) and the level of isozyme expression (19). In addition, PKC isozyme profiles in colonic mucosa are altered by carcinogen treatment in rats (16) and tumor development in humans (20, 27, 28). Observed differences in the localization of individual PKC isozymes suggest that this signaling pathway may play a distinct and specialized role in the function of colonic epithelial cells. Compartmentalization of PKC isozymes within specific anatomical regions may enable these kinases to participate in colonic cell growth, differentiation, and programmed cell death (9, 10, 14).

In conclusion, our data demonstrate that the translation of PKC isozymes is related to growth inhibition, differentiation, and apoptosis in colonic epithelial cells in situ. Although the regulation of colonic PKC isozyme expression is unknown, specific PKCs could play different roles regulating coloniccyte ontogeny. These studies provide a cadre for
Fig. 1. *In situ* RT-PCR and immunohistochemical detection of cytokeratin (positive biological control) and PKC γ (negative biological control) in colonic mucosa. A, expression of mRNA for cytokeratin 8, visualized by the blue alkaline phosphatase reaction product. Eosin counterstaining can be seen as a faint pink color. B, immunohistochemical staining of cytokeratin, visualized by the brown diaminobenzidine product. Hematoxylin counterstaining can be seen as a blue color. For A and B, positive staining is localized to the crypts. Stromal and muscle layers are not stained. C, PKC γ mRNA is not present, as shown by the lack of a blue alkaline phosphatase reaction product. Eosin counterstaining can be seen as a pink color. D, No PKC γ protein is evident, as shown by lack of brown reaction product. × 160.

Fig. 2. *In situ* RT-PCR and immunohistochemical detection of PKC ε in colonic mucosa. Using serial sections, the fidelity of *in situ* mRNA amplification was evaluated using: a negative control (A) in which tissue was DNase pretreated and no reverse transcription performed, as described in "Materials and Methods"; positive control (B) in which tissue was neither DNase treated nor reverse transcribed; and sample (C), in which tissue was DNase pretreated and reverse transcribed. Note: for the negative control (A), no detectable PKC ε signal (blue reaction product) is present, indicating the complete destruction of tissue PKC ε DNA. Eosin counterstaining can be seen as a pink color. For the positive control (B), PKC ε DNA was amplified (blue reaction product) in every cell, indicating adequate fixation and digestion of tissue. For the experimental sample (C), PKC ε mRNA is present (blue reaction product) along the entire crypt axis. D, immunohistochemical detection of PKC ε protein expression. Note: staining is more prevalent at the top of the colonic crypt axis (brown reaction product). Hematoxylin counterstaining can be seen as a blue reaction product. Inhibition of PKC ε staining is shown in E, in which PKC ε antibody was preincubated with PKC ε antigenic peptide. × 160.
ongoing experiments designed to determine how environmental factors, including diet and carcinogen, influence colonic epithelial PKC localization in situ in relation to patterns of cell proliferation, differentiation, and apoptosis. These studies also provide the methodological framework for ongoing experiments designed to rapidly and sensitively detect mRNAs in intestinal sections by sequential RT and PCR amplification using intact cells. Adoption of the in situ detection of PCR-amplified cDNAs allows for the rapid amplification of any message of interest. Overall, this procedure can easily be accomplished within 2 days.

Materials and Methods

Materials. Male Sprague-Dawley rats weighing 200–250 g were obtained from Harlan Sprague-Dawley (Houston, TX). SuperScript II reverse transcriptase, Taq DNA polymerase, and affinity-purified antibodies to PKC α, γ, δ, ε, and ζ were from GIBCO-BRL (Gaithersburg, MD). Polyclonal antibodies to protein kinase C δ and ζ were also obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cytokeratin antibody was from Biomedical Technologies, Inc. (Stoughton, MA). Inhibitory peptides to the PKC
antibodies were obtained from Gibco-BRL and Oxford Biomedical Technologies (Oxford, MI). Keratin protein from Dako Corporation (Carpinteria, CA) was used to inhibit the cytokeratin antibody. Peroxidase conjugated goat anti-rabbit IgG was from Kirkegaard and Perry (Gaithersburg, MD). Diaminobenzidine and hematoxylin (Harris type) were purchased from Sigma Chemical Co. (St. Louis, MO). RNase-free DNase, oligo dT primers, and deoxynucleotides were from Promega (Madison, WI). Tween 20, Superfrost Plus slides, and Permount were from Fisher (Fair Lawn, NJ). Blocking reagent, RNase inhibitor, DIG-DUTP labeling mix, alkaline phosphatase-conjugated anti-DIG, nitroblue tetrazolium, and X-phosphate were from Boehringer Mannheim (Indianapolis, IN).

**Immunohistochemical Staining.** Rats were killed by CO₂ asphyxiation, and the colon was resected from the junction between the cecum and colon to immediately above the anus. The colon was rinsed with PBS (pH 7.5). A 1-cm tissue section was taken from the distal colon and fixed in ethanol overnight, rinsed in 70% ethanol, and paraffin embedded. Tissue sections (5 μm) were placed onto Superfrost Plus slides and stored at room temperature until use. Deparaffinized, rehydrated sections were incubated with 0.3% H₂O₂ in methanol for 30 min to inhibit endogenous peroxidase activity. Samples were then equilibrated with PBS (pH 7.5) and blocked with 10% goat serum in PBS for 30 min. Slides were then incubated in a humidified chamber with primary antibody specific for PKC isoforms or cytokeratin at 4°C for 20–24 h. Sections were washed four times in PBS containing 0.1% Tween 20. Peroxidase conjugated goat anti-rabbit IgG was added for 1 h, followed by four washes in PBS containing 0.1% Tween 20. Sections were then incubated with diaminobenzidine solution (0.5 mg/ml) and 30% H₂O₂ for 3–5 min. The slides were rinsed with distilled water, counterstained with hematoxylin, dehydrated, air dried, and mounted using Permount. Specificity of staining was monitored by the inclusion of negative controls pretreated with excess antigenic peptide.

**In Situ RT-PCR.** A 1-cm colonic tissue section as described above was fixed for 4 h in freshly prepared 4% paraformaldehyde in PBS. All tissues were then dehydrated in an ethyl alcohol series. Tissue blocks were treated with xylene, embedded in paraffin, and stored at −80°C prior to sectioning. Using a disposable blade and RNase-free water bath, 5-μm sections were cut, and three sections were mounted on Superfrost Plus slides and stored at −80°C with desiccant until use. Prior to in situ RT-PCR, the slides were processed as follows: (a) slides were deparaffinized and rehydrated; (b) sections on each slide were treated with pepsin (Research Genetics, Huntsville, AL) at room temperature for 3 min; (c) pepsin was removed, and the slides were washed in a large volume of RNase-free water followed by 100% ethanol; (d) two of three sections on each slide were treated with DNase (1 unit RNase-free DNase in 10 μl 40 mM Tris-HCl, pH 7.4, containing 6 mM MgCl₂ and 2 mM CaCl₂) for 18 h at 37°C in a humidified chamber. The third section (positive control) was treated with buffer alone under the same conditions; and (e) the DNase solution was removed and the slides were washed in a large volume of water followed by 100% ethanol. In situ RT-PCR was performed as described by Nuovo et al. (29) with several modifications. The RT mixture consisted of 2 μl buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 15 mM MgCl₂], 1 μl 0.1 M DTT, 1 μl deoxynucleotides (stock solution, 5 mM), 0.5 μl RNase inhibitor, 0.5 μl 3′ primer, 0.5 μl SuperScript II reverse transcriptase, and 4.5 μl RNase-free water and was placed onto a tissue section that was DNase treated as described above. The remaining sections on each slide were covered with buffer. The slides were placed in a humidified chamber and incubated at 42°C for 1 h. Following incubation, the RT mixture was carefully removed, and slides were washed in a large volume of RNase-free water followed by ethanol. The sections were air dried and covered with PCR solution consisting of 2 μl 10× Taq buffer, 3.6 μl MgCl₂ (25 mM stock), 0.8 μl DIG-11-DUTP labeling mixture, 0.4 μl each of forward and reverse primers, 0.8 μl Taq DNA polymerase, and 12 μl water. Coverslips were carefully placed as described by Vogel and Kell (30) and surrounded with mineral oil. Subsequently, the slides were placed on a PCR slide block (Hybaid; Omnigen, Woodbridge, NJ). The thermocycler was programmed for denaturation (92°C for 1 min), annealing (59°C for 1 min), and extension (74°C for 1 min) for 25 cycles. After the completion of PCR, slides were placed in xylene to remove the mineral oil, and the sections were rehydrated with serial ethanol washes. In situ DIG-labeled PCR products were detected using solutions from the Boehringer Mannheim Genius System: (a) slides were washed in Genius buffer #1 for 5 min, 3 times; (b) sections were blocked in Genius buffer #2 for 30 min and rinsed with Genius buffer #1; (c) sections were incubated with alkaline phosphatase-conjugated anti-DIG in Genius buffer #1 (1:500) for 1 h; (d) excess antibody was removed by washing in Genius buffer #3 for 5 min, 3 times; and (e) substrate solution containing nitroblue tetrazolium and X-phosphate was applied for 10 min to 1 h. The color development was monitored under a microscope to determine signal intensity for optimal staining. Sections were dehydrated in a series of graded ethanol washes, counterstained with eosin, and finally dehydrated with xylene followed by mounting with Permount. Specific in situ amplification of mRNA was monitored on each slide by using three serial sections consisting of a negative control (DNase treated, no reverse transcription), a positive control (no DNase treatment, no reverse transcription), and the sample tissue (DNase treated and reverse transcribed).

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**References.**


