Activation of Protein Kinase C Isozymes Is Associated with Post-mitotic Events in Intestinal Epithelial Cells In Situ

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Abstract. The mechanisms underlying control of cell growth and differentiation in epithelial tissues are poorly understood. Protein kinase C (PKC) isozymes, members of a large family of serine/threonine kinases of fundamental importance in signal transduction, have been increasingly implicated in the regulation of cell growth, differentiation, and function. Using the rat intestinal epithelium as a model system, we have examined PKC-specific activity as well as individual PKC isozyme expression and distribution (i.e., activation status) in epithelial cells in situ. Increased PKC activity was detected in differentiating and functional cells relative to immature proliferating crypt cells. Immunofluorescence and Western blot analysis using a panel of isozyme-specific antibodies revealed that PKC α, βII, δ, ε, and ζ are expressed in rat intestinal epithelial cells and exhibit distinct subcellular distribution patterns along the crypt-villus unit. The combined morphological and biochemical approach used permitted analysis of the activation status of specific PKC isozymes at the individual cell level. These studies showed that marked changes in membrane association and level of expression for PKC α, βII, δ, and ζ occur as cells cease division in the mid-crypt region and begin differentiation. Additional changes in PKC activation status are observed with acquisition of mature function on the villus. These studies clearly demonstrate naturally occurring alterations in PKC isozyme activation status at the individual cell level within the context of a developing tissue.

Direct activation of PKC in an immature intestinal crypt cell line was shown to result in growth inhibition and coincident translocation of PKC α from the cytosolic to the particulate subcellular fraction, paralleling observations made in situ and providing further support for a role of intestinal PKC isozymes in post-mitotic events. PKC isozymes were also found to be tightly associated with cytoskeletal elements, suggesting participation in control of the structural organization of the enterocyte. Taken together, the results presented strongly suggest an involvement of PKC isoforms in cellular processes related to growth cessation, differentiation, and function of intestinal epithelial cells in situ.

C OORDINATED control of cell growth and differentiation is essential for normal tissue development; however, little is known regarding the underlying mechanisms involved in these processes. The epithelial lining of the mammalian intestinal tract, which undergoes continuous and rapid renewal, provides a unique system in which the events involved in regulation of cell growth and differentiation can be studied. Proliferating, differentiating, and functional cells are organized into well defined regions in this polarized tissue (for review see Leblond, 1981; Potten and Hendry, 1983; Lipkin, 1987), and the entire sequence of developmental events is displayed at any moment in time. Mitosis is restricted to the lower regions of the crypts of Lieberkunh and functional cells are located on finger-like intestinal villi. Intestinal epithelial cells (IEC) migrate continuously from the proliferative to the functional compartment and are extruded into the intestinal lumen at the villus tip (Cheng and Leblond, 1974). The maintenance of mucosal integrity under conditions of continuous epithelial renewal suggests the existence of tightly regulated mechanisms to balance proliferative activity with functional capacity and loss of mature cells from the villus (Podolsky, 1993). However, although the growth kinetics of IEC as well as the structural and functional changes that occur with differentiation have been well characterized, the mechanisms underlying epithelial homeostasis in this tissue are still poorly understood.

Protein kinase C (PKC) consists of a family of serine/threonine kinases of fundamental importance in signal transduction (Nishizuka, 1989, 1992) which have been increasingly implicated in the regulation of cell growth (e.g., Hocevar and Fields, 1991; Hocevar et al., 1992), differentiation (e.g., Dlugosz and Yuspa, 1993) and function (e.g. Godson et al., 1990) in diverse biological systems. To date, twelve isozymes (α, βI, βII, γ, δ, ε, ζ, η, θ, λ, μ, and χ) with distinct enzymological characteristics and differential tissue expres-

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sion and intracellular localization have been identified (e.g., Coussens et al., 1986; Ono et al., 1986, 1989; Ohno et al., 1987; Osada et al., 1990, 1992; Mizuno et al., 1991; Koide et al., 1992; Wetsel et al., 1992; Selbie et al., 1993; for reviews see Dekker and Parker, 1994; Nishizuka, 1992). Differences in structure, substrate specificity, cofactor requirements, and localization of individual PKC isozymes have been reported (e.g., Parker et al., 1986; Hidaka et al., 1988; Huang et al., 1988; Hocevar and Fields, 1991; Hocevar et al., 1992; Liyanage et al., 1992). These differences, together with the varied consequences of PKC activation in the same cell, suggest that individual isozymes have distinct and specialized functions in cell signaling. Translocation of PKC activity from the cytosolic to the particulate fraction of the cell (Kraft and Anderson, 1983) is considered the first step in activation of the enzyme (Zidovetzki and Lester, 1992), and reflects PKC association with membrane and/or cytoskeletal elements (Kraft and Anderson, 1983; Wolf and Sahyoun, 1986; Jaken et al., 1989; Mohly-Rosen et al., 1990; Gregorio et al., 1992, 1994). Biochemical and morphological evidence for the redistribution of certain PKC isozymes from the cytosol to the cell periphery in response to specific activators (Ito et al., 1988) or physiological stimuli (Ganesan et al., 1990, 1992) has demonstrated a correlation between redistribution and cellular responses related to growth, differentiation, or functional behavior.

Several recent studies have suggested that PKC is involved in regulation of intestinal epithelial renewal, although the reported data are conflicting. Some studies show increased levels of PKC activity in small intestinal crypt cells relative to villus cells (e.g., Durr et al., 1986), suggesting that PKC participates in positive regulation of cell growth in the intestinal tract. In contrast, a role has been proposed for PKC in IEC growth cessation based on evidence that agonist-induced PKC activation in cultured jejunal crypt cells (Baliga and Borowitz, 1988) and colon adenocarcinoma cells (McBain et al., 1988a,b; Choi et al., 1990) results in inhibition of cell growth. In a survey of PKC isozyme expression in epithelial tissues, Osada et al. (1993) reported the presence of PKC α, δ, γ, and μ mRNA in epithelial scrapings of the mouse small intestine. The goal of the present study was to gain insight into the function(s) of individual PKC isozymes in intestinal epithelial renewal in situ by examining their expression and activation status in proliferating, differentiating, and functionally mature IEC. Results from these studies indicate that PKC isozyme activation is associated with post-mitotic events in intestinal epithelial cells in situ. A role for PKC in signal transduction pathways related to growth cessation is supported by studies in the IEC-18 immature crypt cell line, in which direct activation of PKC by phorbol ester results in inhibition of cell growth and coincident translocation of PKC α from the cytosol to the particulate subcellular compartment. Tight association of individual PKC isozymes with cytoskeletal elements suggests that they may also play a role in assembly and/or maintenance of the enterocyte cytoskeleton.

Materials and Methods

Antibodies

Monoclonal antibodies specific for the catalytic (Leach et al., 1988) and regulatory (Hidaka et al., 1988) domains of PKC α and for the regulatory domain of PKC γ were purchased from Upstate Biotechnology, Inc. (UBI; Lake Placid, NY). Monoclonal antibody specific for PKCβ (I-11) was obtained from Gibco BRL (Grand Island, NY). Polyclonal rabbit anti-PKC α (affinity purified), γ, δ, ε, and η anti-peptide antibodies were purchased from Gibco BRL. The antibodies which specifically recognize PKC α and γ were generated against unique peptides corresponding to the V5 region (amino acids 313-326 and 306-318, respectively) as described previously (Makowske et al., 1988). PKC ε, δ, and η anti-peptide antibodies were generated against peptides corresponding to the V5 region of PKC (amino acids 662-673, 726-737, and 577-592, respectively) (Ono et al., 1988, 1989). PKC γ anti-peptide antibody, generated against the same amino acid sequence described above, was also obtained from Research and Diagnostic Antibodies (R&D; Berkeley, CA). Antiserum against the V6 region of PKC α isoforms (amino acids 660-673) of PKC II (Hocevar and Fields, 1991) was a kind gift from Dr. Alan Fields (Case Western Reserve, Cleveland, OH). Other antibodies used include peptide antibodies against PKC β (I-11; Gibco BRL), II (affinity purified; Gibco BRL), ε (R&D), and γ (R&D), and peptide antiserum generated in this laboratory against the same amino acid sequence of PKC II described above. The antibodies used in this study have been extensively characterized for the absence of cross-reactivity with other PKC isoforms. To ensure reliability of results from immunofluorescence localization studies and Western blot analyses, at least two different antibodies were used for each isoform studied. The specificity of polyclonal antibody reactivity was confirmed in every case in competition assays using excess antigenic peptide (see below).

Rat Intestinal Epithelial Cell Isolation

200-300-g male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). Epithelial cells were isolated along the crypt-to-villus gradient of rat small intestine by timed incubations with EDTA-containing buffer (Weiser, 1973). Upper villus (fraction 1), low-to-mid villus (fraction 5), and crypt cells (fraction 9) were collected and washed twice with ice cold PBS (Gibco BRL) before further processing. Crypt cells were obtained as intact pouches of short columnar epithelium, consisting of both proliferating (lower crypt) and villus (upper crypt) cells. Isolated low-to-mid villus epithelium contained differentiating as well as some fully mature cells, and upper villus epithelial fractions contained only functionally mature cells. Each epithelial fraction was characterized as described previously (Weiser, 1973; Burgess et al., 1989).

For preparation of epithelial cell scrapings, which consist predominantly of villus cells, the small intestine was gently flushed with normal saline containing 1 mM DTT (BioRad Laboratories, Richmond, CA) at 4°C to remove contents, slit open with scissors to expose the epithelium, and cells were scraped into PBS (containing 25 μg/ml leupeptin, 25 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM PMSF, 0.5 mM benzamidine, and 1 mM EDTA) using a glass slide. Protease inhibitors were purchased from Sigma Chemical Co (St. Louis, MO) or Boehringer Mannheim (Indianapolis, IN) and, unless otherwise noted, were used at the above concentrations throughout the study. Cells were washed twice in PBS prior to further processing (see below).

PKC Activity Assay

PKC activity was measured in the cytosolic, membrane, and cytoskeletal subcellular fractions of isolated crypt, low-to-mid villus and upper villus cells using the PKC assay system obtained from Gibco BRL. [γ-32P]ATP was purchased from NEN Research Products (Boston, MA). To prepare the cytosolic subcellular fraction, isolated cells were suspended in digitonin buffer (0.5 mg/ml digitonin in 20 mM Tris, pH 7.5, 140 mM NaCl, 25 mM KCl, 5 mM MgCl2, 2 mM EDTA, and 2 mM EGTA) containing leupeptin, aprotinin, pepstatin, benzamidine, and PMSF for 5 min at 4°C (Pelech et al., 1986). Cytosolic and particulate fractions were separated by sedimentation at 100,000 g in a centrifuge (TL-100; Beckman Instruments, Fullerton, CA) for 30 min. The resulting pellet was extracted in 1% Triton buffer (1% Triton X-100, 10 mM Tris–HCl, pH 7.5, 140 mM NaCl, 25 mM KCl, 5 mM MgCl2, 2 mM EDTA, and 2 mM EGTA) containing protease inhibitors at room temperature (Mooschek and Tilney, 1975; Matsudaira and Burges, 1979), and the membrane subcellular fraction was recovered as the supernatant following centrifugation. After removal of membranous components, the second pellet (cytoskeletal fraction) was sonicated in 1% Triton buffer. Each sample was diluted 10 to 80 times with 20 mM Tris, pH 7.5, and the phosphorylation of an acetylated synthetic peptide from myelin basic protein (Yasuda et al., 1990) was measured using the assay kit. PKC specificity was determined by using a PKC pseudosubstrate inhibitor pep-
tide (PKC[19-36]) provided by the manufacturer, which acts as a potent in-
hibitor for this substrate.
PKC activity was also measured in cytosolic and particulate subcellular
fractions of cultured IEC-18 immature intestinal epithelial cells (Quaroni
and Isselbacher, 1981; see below).

Preparation of Brush Borders
A modification of the methods of Forster et al. (1968) and Bretscher
and Weber (1978) was used to isolate brush borders. All procedures were
performed at 4°C. Briefly, epithelial cell scrapings (see above) from the small
intestine of one rat were suspended in 200 ml of Buffer A (5 mM EDTA
and 1 mM Heps-Tris, pH 7.5) containing PMSF, benzamidine,
and 1 mM DTT and homogenized in a Waring blender for 25 s. After centrifugation
of the homogenate at 450 g for 15 min, the pellet was resuspended in Buffer
A and centrifuged again at 800 g for 15 min. The resulting brush border
pellet was resuspended in a small volume of Buffer B (0.09 M NaCl, 0.8
mM EDTA, 1 mM Heps-Tris, pH 7.5) containing PMSF, benzamidine,
and 1 mM DTT and filtered through a pad of glass wool and No. 25 bolting
silk to remove aggregates. Brush borders were recovered by centrifugation
for 10 min at 800 g, washed once with fresh Buffer B, and once with Buffer
c (75 mM KCl, 5 mM MgCl2, 1 mM EGTA, 1 mM EDTA, and 10 mM
imidazole, pH 7.3) containing leupeptin, aprotinin, pepstatin, benzamidine,
and PMSF. Following isolation, brush borders were boiled in Laemmli sam-
ple buffer or extracted in detergent-containing buffers for solubility studies.
The purity of the preparation and the presence of intact brush borders were
confirmed by light and electron microscopy.

Preparation of Rat Brain Homogenate
Rat brain homogenate was used as a control for PKC antibody specificity
in immunoblotting experiments and for comparison with intestinal PKC iso-
yzmes. Rat cerebral cortex was rinsed in PBS containing protease inhibi-
tors. Using a Dounce homogenizer with a type A pestle, the tissue was
homogenized in PBS with protease inhibitors and then boiled in Laemmli
sample buffer.

Western Blot Analysis
IEC or brain samples (40 μg) were subjected to 10% SDS-PAGE (Laemmli,
1970) and electrotransferred to nitrocellulose membrane by the method of
Towbin et al. (1979). Membranes were blocked with 5% dried milk in 20
mM Tris-HCl and 137 mM NaCl, pH 7.6 (TBS), for 1 h at room tempera-
ture and then overnight at 4°C. PKC isoforms were detected by incubating
the nitrocellulose membranes for 2 h with anti-PKC isoform antibodies at
1 μg/ml in TBS containing 0.1% Tween 20 (TBS/Tween; Sigma Chemical
Co.). After washing in 5% milk/TBS/Tween, membranes were incubated
with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat
anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN) diluted 1:1,000
in 5% milk/TBS/Tween. Following washes, immunoreactivity was detected
using the Enhanced Chemiluminescence detection system (Amersham
Corp., Arlington Heights, IL) according to the manufacturer's instructions.
To demonstrate specificity of these antibodies, the relevant antigenic peptide
was used in competition experiments at a 1:2 (wt/wt) peptide to antibody
ratio.

Immunofluorescence
Rat duodenum was gently flushed with ice cold PBS to remove contents.
The tissue was then immersed in 2% freshly depolymerized paraformalde-
yde fixative for 2 h at 4°C, washed in 50 mM NH4Cl/PBS for 45 min, and
cryoprotected in 30% sucrose/PBS. The tissue was embedded and frozen in
Tissue Tek OCT medium (ICN Biomedicals, Costa Mesa, CA), and 2-4
μm sections were obtained using a cryostat microtome (Reichert Jung,
Rochester, NY). Between sections were obtained using a cryostat microtome (Reichert Jung,
Rochester, NY). Frozen sections were allowed to air dry at room tempera-
ture for 15 min in Triton buffer (Mooseker and Tilney, 1975; Mat-
sudaira and Burgess, 1979) and at 4°C for 30 min in RIPA buffer, with inter-
mittent vortexing. Soluble and insoluble fractions were separated by
ultracentrifugation and prepared for SDS-PAGE and Western blotting to de-
termine relative levels of PKC isoforms as described above. Three
or more experiments were conducted for each solubility condition in intesti-
nal scrapings or brush borders.

Identification of Membrane and Cytoskeletal
Associations of PKC Isozymes
Detergent-containing buffers were used to examine membrane and
cytoskeletal associations of PKC isoforms in whole cells and isolated brush
borders. Epithelial cell scrapings or brush borders were resuspended in 10
vol of either Triton buffer (see above) or RIPA buffer (1% NP-40, 1% sodium
decylsulfate, 0.1% SDS, 10 mM Tris–HCl, pH 7.5, 140 mM NaCl, 25 mM
KCl, 5 mM MgCl2, and 5 mM EGTA) containing the protease inhibitors
listed above, and dispersed by vortexing. Cell scrapings were also passed
through 20-gauge needle three times. Incubations were carried out at room
temperature for 15 min in Triton buffer (Mooseker and Tilney, 1975; Mat-
sudaira and Burgess, 1979) and at 4°C for 30 min in RIPA buffer, with inter-
mittent vortexing. Soluble and insoluble fractions were separated by
ultracentrifugation and prepared for SDS-PAGE and Western blotting to de-
termine relative levels of PKC isoforms exactly as described above. Three
or more experiments were conducted for each solubility condition in intesti-
nal scrapings or brush borders.

Preparation of Membrane Fractions
Membranes were prepared by the method of Kajii et al. (1989). Isolated
crypt or villus cells were suspended in PBS containing the protease inhibi-
tors listed above and disrupted by freeze-thawing three times in alternating
dry ice/ethanol and 37°C baths. Particulate material was collected by cen-
trifugation for 30 min at 4°C in an Eppendorf microfuge at 12,000 rpm.
The resulting pellet was resuspended in Renex lysis buffer (2% Renex, 10
mM Tris–HCl, pH 8.5, 140 mM NaCl, 25 mM KCl, 1 mM CaCl2, 0.02% NaN3,
and all protease inhibitors), incubated for 1 h, and centrifuged as
above. Crypt and villus membranes were recovered as the supernatant,
diluted with an equal volume of 2× Laemmli sample buffer and boiled. Pro-
tein concentrations were determined using the BCA protein assay kit (Pierce
Chem. Co., Rockford, IL). Equivalent amounts of membrane protein for
each sample were subjected to SDS-PAGE. Levels of PKC isoforms in crypt,
low-to-mid villus, and upper villus epithelial cell membranes were
determined by Western blotting using isozyme-specific antibodies in three
or more experiments.

Saxon et al. Role of PKC Isozymes in Intestinal Epithelial Renewal
IEC-18 Cells

IEC-18 cells, derived from rat ileal crypt epithelium (Quaroni and Isselbacher, 1981), were obtained from the American Type Culture Collection (Rockville, MD) and maintained in DME containing 0.01 mg/ml insulin (GIBCO BRL), 4 mM glutamine (GIBCO BRL), and 5% fetal calf serum (Intergen, Purchase, NY) at 37°C in a humidified atmosphere of 5% CO2. The medium was replaced every two days and cultures were passaged before confluency.

For growth studies, 1 x 10^5 cells in serum-containing DME (as described above) were seeded in six-well plates, 24 h later the medium was removed, and the wells were washed three times with unsupplemented DME. Cells were then treated with either 0.01, 0.1, or 1 mM phorbol myristate 13-acetate (PMA; Sigma Chemical Co.) or ethanol for 15 min in DME supplemented with a mixture of insulin, transferrin, selenium, and albumen (serum-free DME; Collaborative Research, Becton Dickinson, Bedford, MA) at 37°C in 5% CO2. The wells were washed four times and cultures were maintained for 24 h. Cells in each well were then trypsinized and counted using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Each treatment condition was examined in triplicate and the data are representative of three experiments. Results are expressed as percent of control growth. DNA synthesis under each treatment condition was also measured by 3H-Thymidine incorporation (results are not shown for these experiments).

For immunofluorescence studies, cells were grown to subconfluency on sterile glass coverslips and treated with either 100 nM PMA or ethanol in serum-free DME at 37°C in 5% CO2. The coverslips were removed from the dishes, washed three times in PBS, and fixed in 2% formaldehyde for 15 min. After washing in PBS for 30 min, the cells were permeabilized with PBS/Triton for 5 min before staining for PKC α as described above. Immunofluorescence localization of PKC α in four separate experiments was analyzed using a BioRad confocal microscope (Boston, MA).

A modification of the method of Pelech et al. (1986) was used for preparation of cytosolic and particulate fractions of control and PMA-treated IEC-18 cells. Briefly, subconfluent cells growing in 150 x 25 mm^2 culture dishes were exposed to 100 nM PMA or an equal volume of ethanol in serum-free DME at 37°C in 5% CO2. Cells were washed several times with PBS and immediately incubated at 4°C with digitonin buffer for 5 min. The soluble (cytosolic) fraction was removed and the residue was scraped in digitonin buffer containing 0.5% Triton X-100. Cytosolic and particulate subcellular fractions from control and phorbol-ester-treated cells were analyzed for PKC activity as described above. Both fractions were also precipitated with 10% TCA and prepared for Western blot analysis as described for tissue IEC. The distribution of PKC α in each compartment was analyzed before and after activation of PKC with PMA in four separate experiments.

Results

Analysis of PKC Activity in Subcellular Fractions of Isolated Rat Intestinal Epithelial Cells

Table I. Analysis of PKC Activity in Subcellular Fractions of Isolated Rat IEC

| IEC Subpopulation | Cytosol | Membrane | Cytoskeleton |
|-------------------|---------|----------|-------------|
| Crypt             | 702 ± 61| 195 ± 29 | 137 ± 31    |
| Low-to-mid villus | 786 ± 66| 530 ± 40 | 394 ± 107   |
| Upper villus      | 901 ± 47| 583 ± 67 | 402 ± 60    |

Cells were isolated by the method of Weiser (1973). Cytosolic, membrane, and cytoskeletal subcellular fractions were prepared as described in Materials and Methods. PKC activity was measured using the GIBCO BRL Protein Kinase C Assay System. Values are representative of three experiments and are the mean ± SD of triplicate samples.

Individual PKC isozyme expression in isolated rat villus IEC was determined by Western blot analysis using a panel of isozyme-specific monoclonal or polyclonal antibodies and compared with that in rat brain. As shown in Fig. 1, PKC α, βII, δ, ε, and γ were detected in rat IEC. Although PKC βII was readily detected in brain samples, this isozyme does not appear to be expressed in the intestinal epithelium. PKC α was detected at ~80 kD in IEC in brain with both the anti-peptide and monochlonal antibodies. The relative level of expression of PKC α in IEC was found to be ~50% of that in brain. PKC βII immunoreactive bands were detected at ~72, 70, 63, and 58 kD in IEC and in brain. While brain PKC βII was detected primarily as a 72-kD band, intestinal PKC βII was usually detected as a 63-kD fragment, suggesting that this isozyme is particularly susceptible to degradation in this tissue. The relative level of PKC βII detected in villus IEC was significantly lower than that in brain. Antibodies specific for PKC δ detected bands of similar intensity at ~80 kD in both tissues. Immunoreactive bands of ~54 and 50 kD, which may correspond to the catalytic domain of the enzyme (Mizuno et al., 1991), were consistently seen in intestinal samples; peptide competition studies confirmed that these fragments are related to PKC δ. Antibodies specific for PKC ε reacted with a 90-kD band in both tissues, although the levels of this isozyme were markedly lower in IEC. Anti-PKC
pressed in villus cells of mouse small intestine (Osada et al., 1992). Interestingly, the lower molecular mass fragments were also of different sizes, suggesting that the isozyme is differentially processed in these tissues.

Figure 1. Western blot analysis of PKC isozyme expression in isolated intestinal villus cells. Samples (40 μg) from villus cells (I) and rat brain (B) were subjected to SDS-PAGE and Western blot analysis. Where indicated, specificity was confirmed by competition with the antigenic peptide (lanes labeled +). The immunoreagents used were all polyclonal anti-peptide antibodies except the α (Cat) reagent, which is a mouse monoclonal antibody specific for the catalytic domain (Kiley and Jaken, 1990). The higher molecular mass species detected in brain with this antibody has been observed by others (Kiley and Jaken, 1990) and remains to be characterized. Molecular mass standards are at the left. See text for details.

PKC isozyme subcellular distribution is detected in proliferating cells by the antibody to the catalytic domain (Kiley and Jaken, 1990). Although PKC ε was not examined in this study, this isozyme has been shown to be expressed in villus cells of mouse small intestine (Osada et al., 1993). The expression of PKC βI in intestinal tissue remains to be determined.

Immunofluorescence Analysis Reveals Changes in PKC Isozyme Subcellular Distribution as Cells Cease Division in the Mid-crypt Region

Immunofluorescence microscopy was performed using isozyme-specific antibodies to determine PKC isozyme localization in cells along the crypt-to-villus axis. An important advantage of this morphological approach is that it provides evidence of enzyme redistribution at the individual cell level. Since the cell positions along the crypt-to-villus axis at which such events as cell growth cessation, differentiation, appearance of the fully differentiated phenotype and senescence/extrusion are known, morphological techniques allow for the precise correlation of growth and differentiation status with the expression and behavior of individual PKC isozymes. PKC isozyme translocation is reflected morphologically in the appearance of discrete isozyme-specific immunostaining in membranous subcellular compartments (Ito et al., 1988; Ganesan et al., 1992) and/or in regions of the cell enriched in cytoskeletal proteins (Mochly-Rosen et al., 1990; Gregorio et al., 1992, 1994).

Diffuse cytoplasmic fluorescence for PKC α, βII, δ, and ε was detected in proliferating cells of the lower crypt, presumably reflecting the presence in these cells of the inactive form of these isozymes (Fig. 2 I, A–D). It is noteworthy that none of these PKC isozymes were detected at the plasma membrane of proliferating cells, although PKC βIII immunoreactivity was found to be accumulated in the terminal web region and PKC ε was observed in the apical junctional complexes (staining patterns which persist along the length of the crypt-villus unit). At cell position 14–18 from the crypt base, the region in which cells cease division and commit to differentiation (Cheng and Leblond, 1974), PKC α, βII, δ, and ε become clearly detectable in the lateral plasma membrane domains (Fig. 2 I, arrows). In addition, PKC α-specific immunofluorescence staining appears in the developing brush border microvilli (Fig. 2 IA, arrowhead). Thus, precisely in the region in which IEC undergo growth cessation, there is a marked redistribution of four PKC subspecies to the cell periphery in the classical indication of PKC activation. These results suggest that one or more of these isozymes is involved in signaling pathways related to cell growth inhibition in this tissue.

PKC ε immunolocalization studies revealed that, in contrast to PKC α, βII, δ, and ε, this isozyme does not undergo changes in subcellular distribution with cell growth cessation in the intestinal crypts (Fig. 2 II). Staining of the junctional complexes and lateral membranes was apparent in all cells along the crypt-to-villus axis. These results suggest that PKC ε plays a similar role in crypt and villus cells and that it is not directly involved in growth- or maturation-related events in this tissue. It is noteworthy that PKC ε appears to be expressed at significantly higher levels in Paneth cells (data not shown).

Differential Regulation of PKC Isozyme Subcellular Distribution Is Detected in Cells of the Intestinal Villus

The Association of PKC α and ε with the Cell Periphery Is Sustained Along the Length of the Villus. As described above, recruitment of PKC α and ε to the cell periphery correlates with cell growth cessation and commitment to differentiation in the mid-crypt region. As shown in Fig. 3 (A and B), the expression of PKC α along the lateral membranes and in the brush border microvilli is subsequently maintained along the entire length of the villus. Similarly, PKC ε is localized in the junctional complex region and in the lateral membrane domains of all differentiating functional IEC (Fig. 3, C and D). Based on evidence that sustained PKC activation is a prerequisite for long-term physiological responses such as cell differentiation (Aihara et al., 1991; Asaoka et al., 1991, 1992), it is tempting to speculate that the sustained activation of PKC α and ε along the length of the villus reflects an involvement of these PKC isozymes in such long-term physiological responses in intestinal tissue.

Acquisition of Functional Maturity Is Associated with Alterations in the Expression and Localization of PKC βII and δ. As shown in Fig. 4 I, lateral membrane staining for PKC βII was detected only in cells of the mid-to-upper crypt and lower villus, becoming markedly diminished as the cells...
become functionally mature. Since membrane staining was specifically associated with cells in the differentiation zone, it is possible that activation of this isozyme is linked to differentiation-related events.

Changes in the level of expression and subcellular distribution of PKC δ were detected in several regions along the crypt-villus unit (Fig. 4 II). Staining of the lateral membranes was found to become more intense as the cells exit the crypts and to increase further in cells of mid- and upper villus. Although PKC δ is absent from the developing microvilli of cells in the upper crypt and lower villus regions, it was found to accumulate in the brush border of mature enterocytes. Thus, as the epithelial cells become functionally mature, the level of PKC δ at the cell periphery increases markedly and the isozyme accumulates in a different subcellular compartment (Fig. 4 IIC).

Taken together, these data indicate that, in addition to undergoing redistribution with cell growth cessation in the
mid-crypt region, PKC βII and δ are subject to additional regulation as the cells become functionally mature on the villus. The recruitment of PKC βII to the lateral membranes of cells in the differentiation zone, together with its down-regulation in functional cells, is indicative of a transient role in IEC renewal, presumably in the differentiation process itself (i.e., acquisition of the mature phenotype). The appearance of PKC δ in the microvilli of mature cells, on the other hand, suggests an involvement in function of the fully developed brush border.

**Biochemical Analysis of the Subcellular Partitioning of PKC Isozymes in Isolated Crypt and Villus Cells Further Implicates PKC Isozyme Activation in Post-mitotic Events**

Changes in PKC isozyme expression and subcellular distribution in different intestinal epithelial cell subpopulations were also evaluated by Western blot analysis as described previously (e.g., Ganesan et al., 1990; Godson et al., 1990). For this analysis, cytosolic contents were released from iso-
lated crypt, low-to-mid villus and upper villus cells by perforation of the plasma membrane with digitonin (Pelech et al., 1986), and cytosolic and particulate fractions were separated by ultracentrifugation. The particulate fraction analyzed for PKC isozyme expression included both membrane and cytoskeletal elements; increased association of PKC isozymes with the particulate fraction was considered indicative of PKC activation. To examine changes in association of PKC isozymes with membranous elements alone, isolated membranes were prepared from IEC subpopulations and examined for specific PKC isozyme expression. Results from this analysis support and extend those obtained using morphological techniques and clearly demonstrate differential regulation of individual PKC isozyme expression and subcellular distribution along the crypt-to-villus axis. The expression of PKC α, ζ, and δ generally increases as cells migrate towards the villus tip (Fig. 5 A), while that of PKC βII decreases on the villus (Fig. 5 B) and that of PKC ε remains relatively constant along the length of the crypt-villus unit (Fig. 5 C).

Migration of cells from the crypts to the lower villus regions is accompanied by a 1.4-fold increase in total level of expression of PKC α, ζ, and δ (range 1.3–1.5, n = 3; Fig. 5 A). Total levels of PKC α and ζ subsequently remain relatively constant along the length of the villus. In keeping with morphological data, levels of PKC α and ζ associated with the particulate subcellular fraction are higher in lower villus cells than in crypt cells and undergo relatively minor changes on the villus. Analysis of membrane association of these isozymes parallels these results. In contrast, in the case of PKC δ, both total levels of expression and levels in the particulate fraction are markedly higher in upper villus cells relative to lower villus cells. The progressive increase in levels of PKC δ associated with the particulate subcellular fraction was also observed in isolated membranes and is consistent with morphological data. Thus, PKC α, ζ, and δ appear to be activated during cell migration from crypt to lower villus and PKC δ appears to undergo further activation as IEC acquire functional maturity on the villus. The increase in PKC δ in the particulate fraction of upper villus cells likely reflects the increased membrane and microvillus staining seen by immunofluorescence analysis (see above).

Regulation of the expression of PKC βII differs from that of PKC α, ζ, and δ (Fig. 5 B). Levels of this isozyme are highest in the crypt cell subpopulation and decrease markedly in cells of the villus. A similar trend was seen in isolated membranes. Since the immunofluorescence data shown above clearly indicated activation of PKC βII in cells of the mid- and upper crypt regions, the high levels of this isozyme detected in crypt cells can be attributed to its activation in the differentiating cells present in this fraction (see Materials and Methods). Consistent with morphological data, membrane association of PKC βII in functional cells of the upper villus is markedly lower than that in lower villus cells.

Analysis of PKC ε in this tissue revealed little change in the level of expression of this isozyme along the length of the crypt-villus unit (Fig. 5 C). More than 90% of detectable PKC ε immunoreactivity was observed in the particulate compartment of crypt and villus cells; in some experiments, lower molecular mass bands were detected in the soluble fraction (data not shown). No PKC ε was detected in association with isolated membranes, suggesting that this isoform is associated with cytoskeletal elements in these cells (see below).

**Activation of PKC in IEC-18 Immature Intestinal Crypt Cells Results in Cell Growth Inhibition Accompanied by Changes in the Subcellular Distribution of PKC α**

To investigate further the role of PKC isozymes in intestinal epithelial cell renewal, IEC-18 immature crypt cells derived from rat ileum were used as an in vitro model system since these cells are known to maintain many characteristics of their tissue counterpart (Quaroni and May, 1980; Quaroni and Iselfieber, 1981). PMA was used to activate PKC directly in these cells and changes in cell proliferation and the subcellular distribution of PKC α were determined. Growth of IEC-18 cells was inhibited in a dose-dependent manner after brief exposure (15 min) to PMA (Fig. 6 A). In-

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**Figure 4. Immunofluorescence localization of PKC βII and δ in duodenal villus cells.** (I) Immunofluorescence localization of PKC βII in villus IEC. (A) Longitudinal section through the lower and mid-villus region of rat duodenum. Although lateral membrane staining is clearly seen in cells of the lower villus (arrow), it becomes barely detectable in cells of the functional zone (F). (B) Enlargement of lower villus IEC. Note that lateral membrane staining is weak in these cells (arrow). (C) Enlargement of mid-villus IEC. Note that lateral membrane staining persists (arrowhead). PKC βII was not detected in the brush border microvilli. (D) Diagrammatic representation of the distribution of PKC βII in the crypt-villus unit of rat duodenum. The arrows indicate the cell positions at which the subcellular distribution of PKC βII changes. Note that PKC βII activation is associated with cells in the differentiation zone (D). (II) Immunofluorescence localization of PKC δ in rat duodenum showing transitions in subcellular distribution patterns. (A) Longitudinal section through the crypt and lower villus of rat duodenum showing PKC δ immunofluorescence diffusely distributed throughout the cytoplasm of lower crypt (LC) cells; lateral membrane staining (short arrow) becomes apparent in mid- and upper crypt cells and more distinct staining in this membrane domain (large arrow) is seen in lower villus cells. Note the absence of staining in the brush border (open arrow). (B) Low (L)-to-mid (M) villus region of the same crypt-villus unit shown in (A) stained for PKC δ. Although lateral membrane staining (short arrow) is evident in lower villus cells, no staining is detected in the microvilli (open arrow). PKC δ is detected in the brush border microvilli of mature cells of the mid-villus (closed arrow). Lateral membrane staining (long arrow) is more intense in mid- and upper villus cells than in those of the lower villus regions. (C) Mid- and upper villus epithelium of the same crypt-villus unit in A and B showing intense immunofluorescence staining for PKC δ in the microvilli (arrowhead; also see inset) and along the lateral membranes. (D) Diagrammatic representation of the distribution of PKC δ in the crypt-villus unit of rat duodenum. Arrows indicate the cell positions at which the subcellular distribution of this isozyme changes. Lateral membrane staining first appears with cell growth arrest (lower arrow). Levels increase in the neck of the crypt (middle arrow). Recruitment of PKC δ to the brush border occurs in cells of the functional zone (F, upper arrow). Bars, 10 μm.
corporation of $^3$H-thymidine was also inhibited following treatment (data not shown). Exposure to 100 nM PMA resulted in 60.7 (±8) % inhibition of cell growth; therefore, this concentration was used for subsequent biochemical and morphological analyses. PKC activity studies revealed that treatment with phorbol ester results in rapid translocation of PKC-specific activity from the cytosol to the particulate subcellular fraction of IEC-18 cells (Table II). Immunofluorescence localization studies demonstrated that untreated cells exhibit a cytoplasmic and nuclear staining pattern for PKC α (Fig. 6 B, 1). The nature of the nuclear staining remains to be established. PMA-induced cell growth inhibition is accompanied by recruitment of PKC α to the plasma membrane of IEC-18 cells (Fig. 6 B, 2, arrow). Western blot analysis revealed that PKC α partitions almost entirely with the cytosolic subcellular fraction in untreated IEC-18 cells (Fig. 5 A, α). Subcellular partitioning and membrane association of intestinal PKC isozymes in isolated crypt, low-to-mid villus, and upper villus cells. Soluble (S) and particulate (P) fractions were subjected to Western blot analysis as described in Materials and Methods. The table below each blot shows the results of densitometric analysis expressed as follows: % Total, fraction of total cellular PKC isozymes found in the soluble and particulate compartments for each cell subpopulation; % relative to crypt, total PKC isozyme detected in each subpopulation expressed relative to that found in crypt cells; % CP, levels of PKC isozyme associated with the particulate fraction of each cell subpopulation expressed relative to that detected in crypt cells. Levels of each PKC isozyme in isolated membranes, determined by densitometric analysis, are expressed relative to those in the crypt fraction (% relative to crypt). Results shown are representative of three or more experiments.

(A) Subcellular partitioning and membrane association of PKC α, β, and γ in rat IEC subpopulations. The levels of each of these isozymes are higher in the particulate subcellular fraction and in isolated membranes of low-to-mid villus (MV) and upper villus (UV) cells relative to crypt (C) cells. Levels of PKC β are significantly higher in mature cells of the upper villus than in cells of the low-to-mid villus. (B) Subcellular partitioning and membrane association of PKC βII in isolated IEC subpopulations. Levels in the particulate fraction decrease as cells migrate towards the villus tips (compare C lanes with MV and UV lanes). Note that the highest levels were found in crypt and low-to-mid villus membranes (representing differentiating cells of the upper crypts and lower villus). (C) Subcellular partitioning and membrane association of PKC ε in IEC subpopulations. Note that this isozyme was undetectable in isolated membranes.
Table II. Analysis of PKC Activity in Subcellular Fractions of IEC-18 Cells

|                  | PKC activity |          |          |
|------------------|--------------|----------|----------|
|                  | pmol/min/mg protein |          |          |
| Cytosolic fraction |              |          |          |
| Control          | 604 ± 80     |          |          |
| PMA-treated      | 52 ± 9       |          |          |
| Particulate fraction | 335 ± 10 |          | 733 ± 89 |

Cells were treated with 100 nM PMA for 15 min. Cytosolic and particulate fractions were prepared as described in Materials and Methods. PKC activity was measured using the GIBCO BRL Protein Kinase C Assay System. Results are expressed as the mean ± SD of two experiments.

6 C). Translocation of an 80-kD PKC α immunoreactive protein from the soluble to the particulate subcellular fraction reflects the membrane association of PKC α observed morphologically following PMA treatment (Fig. 6 C, compare -PMA, lanes S and P with +15 min, lanes S and P). These results parallel those obtained in situ and provide direct support for a model in which PKC α plays a role in IEC growth cessation.

Cytoskeletal Association of PKC Isozymes in Rat Intestinal Epithelial Cells

There is increasing evidence that PKC isozymes can associate with the cytoskeleton, a component of the particulate fraction of cells (e.g., Jaken et al., 1989; Mochly-Rosen et al., 1990; Gregorio et al., 1992, 1994; Omari et al., 1992). Since PKC activity assays demonstrated the presence of the enzyme in the cytoskeletal fraction of IEC (see Table I) and immunofluorescence analysis revealed that enterocyte PKC isozymes can be found in subcellular compartments enriched in cytoskeletal elements (brush border microvilli, terminal web, and junctional complexes), studies were undertaken to examine cytoskeletal associations of PKC isoforms in intact villus cells and in isolated brush borders. The partitioning of PKC isoforms into soluble and particulate material under several cell solubilization and fractionation conditions was compared. The conditions used were (a) 1% Triton X-100 which solubilizes cytosolic and membrane proteins without affecting most cytoskeletal interactions, and (b) RIPA buffer which solubilizes membranes and disrupts many cytoskeletal associations, thus releasing all but a few cytoskeleton-associated proteins. These studies revealed that all intestinal cultures were treated with 0.01, 0.1, and 1 μM PMA, or ethanol for 15 min. Cells (in triplicate wells) were trypsinized and counted 24 h after treatment in three separate determinations. Results are expressed as percent of control cell growth. (B) Cells growing on coverslips were treated with 0.1 mM PMA or solvent for 15 min. Control cells exhibit cytoplasmic and nuclear staining for PKC α (f). Treatment with PMA results in recruitment of PKC α to the periphery of IEC-18 cells. Nuclear staining persists (2). (C) Subconfluent IEC-18 cells were treated with 0.1 mM PMA or solvent for 15 min. The cells were washed and cytosolic and particulate subcellular fractions were prepared as described in Materials and Methods. Western blot analysis of subcellular fractions revealed that PKC α is almost entirely in the soluble fraction (S) of untreated (−) IEC-18 cells. Translocation of PKC α from the soluble (S) to the particulate (P) fraction of the cells was detected after 15 min exposure to PMA (+15 min).

Figure 6. Activation of PKC in IEC-18 immature intestinal crypt cells results in cell growth inhibition accompanied by striking changes in the subcellular distribution of PKC α. (A) Subconfluent

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PKC isozymes exhibit some association with cytoskeletal elements in mature enterocytes. Results obtained for individual PKC isoforms are described below.

PKC \( \alpha \) and \( \delta \) are accumulated at the lateral membranes and in the brush border microvilli of mature enterocytes (Fig. 7, IA, IC2, IA2, and IIC). The majority of both PKC \( \alpha \) and \( \delta \) in IEC is Triton soluble (Fig. 8 A), reflecting their membrane and cytosolic localization; however, a small amount remains associated with the Triton- and RIPA-insoluble fractions (lanes TP and RP). PKC \( \alpha \) and \( \delta \) were also detected in the detergent-insoluble fractions of isolated brush borders, indicating a tight association of these isoforms with the brush border cytoskeleton (Fig. 8 B, lanes TP and RP). PKC \( \beta II \) is localized in the terminal web region of all villus cells (Fig. 7, IB and IIB). A proportion of enterocyte PKC \( \beta II \) is likely associated with cytoskeletal elements, since Triton- and RIPA-insoluble PKC \( \beta II \) could be detected in intestinal scrapings (Fig. 8 A), and brush border PKC \( \beta II \) is almost entirely insoluble in Triton and RIPA buffers (Fig. 8 B). Associations with nonerythroid spectrin (Gregorio et al., 1992, 1994) and/or intermediate filaments (Hocevar and Fields, 1991; Spudich et al., 1991) in the terminal web region and along the lateral membranes are possible.

PKC \( \epsilon \) and \( \xi \) are localized in the apical junctional complex region and along the lateral membranes of villus IEC (Fig. 7, ID, IE, and IID). The majority of PKC \( \epsilon \) is likely associated with cytoskeletal elements, since it is insoluble in both Triton buffer and RIPA buffer (Fig. 8 A) and was not detected in isolated membranes (see Fig. 5). This isozyme was also almost entirely in the insoluble fraction of isolated brush borders under all solubilization conditions (Fig. 8 B), suggesting an association with intermediate filaments (cytokeratins) of the belt desmosome. It is noteworthy in this regard that PKC \( \epsilon \) has been shown to phosphorylate and coimmunoprecipitate with cytokeratins in HT-29 colon adenocarcinoma cells (Omary et al., 1992). In contrast to PKC \( \epsilon \), the majority of PKC \( \xi \) is Triton-soluble in IEC scrapings (Fig. 8 A, lane TS); however, significant levels remain with the Triton-insoluble fraction (Fig. 8 A, lane TP). Most of this cytoskeletal association is displaced in RIPA buffer (Fig. 8 A, lane RS and RP). In isolated brush borders, a large proportion of PKC \( \xi \) was detected in both Triton and RIPA-insoluble fractions (Fig. 8 B), indicating significant association with the brush border cytoskeleton. Collectively, these data suggest that insoluble PKC \( \xi \) is associated with membrane and cytoskeletal elements, possibly with structural elements of the apical junctional complexes.

**Discussion**

This study demonstrates that multiple PKC isozymes (\( \alpha \), \( \beta III \), \( \delta \), \( \epsilon \), and \( \xi \)) are present in the intestinal epithelium and that they exhibit distinct patterns of expression and subcellular localization along the crypt-villus unit. The data clearly demonstrate naturally occurring changes in PKC isozyme subcellular distribution that correlate with specific cellular events. Marked changes in membrane association/solubility properties of intestinal PKC isozymes were detected at specific transition points along the crypt-villus unit associated with cell growth suppression and acquisition of mature function, indicating a role for this enzyme family in these cellular processes (see Fig. 9). Furthermore, PKC isozymes were found to be tightly associated with the brush border cytoskeleton, suggesting an additional role in regulation of the structural organization of the enterocyte. Observed differences in the localization of individual PKC isozymes, together with differential regulation of their expression and redistribution along the crypt-to-villus axis, indicate that each isozyme likely plays a distinct and specialized role in cell signaling in this tissue. Compartmentalization of PKC isozymes within specific regions of the crypt-villus unit may permit positional control of enzyme-substrate interactions, enabling the unique participation of each PKC sub-species in IEC-growth or maturation-inducing signals (see Olson et al., 1993).

**The Role of PKC Isozymes in Growth and Differentiation of Intestinal Epithelial Cells In Situ**

Little is known regarding the mechanisms involved in regulation of cell growth and differentiation in the intestinal epithelium. Data presented in this report suggest that PKC isozymes play a role in control of post-mitotic events in this tissue. In proliferating cells of the intestinal crypts, PKC isozymes exhibit either a diffuse cytoplasmic distribution (PKC \( \alpha \), \( \beta II \), \( \delta \), and \( \xi \)) or a localized distribution pattern which is maintained in all cells along the crypt-to-villus axis (\( \beta III \), \( \epsilon \), and \( \xi \)). Thus, no PKC isozyme analyzed in this study was found to be uniquely compartmentalized in proliferating crypt cells in a pattern characteristic of activation, suggesting that PKC isozymes are not involved in maintenance of the proliferative state in IEC. Instead, several findings support a role for this enzyme family in events related to cell growth cessation and/or establishment and maintenance of mature function (see Fig. 9). These findings include:

1. **Figure 7.** Comparison of the subcellular distribution of intestinal PKC isozymes in villus enterocytes. (I) Immunofluorescence staining of rat duodenal enterocytes for PKC \( \alpha \) (A), \( \beta II \), lower villus (B 1), \( \beta II \), upper villus (B 2), \( \delta \), lower villus (C 1), \( \delta \), upper villus (C 2), \( \epsilon \) (D), and \( \xi \) (E) (first and fourth rows) with corresponding phase contrast micrographs (second and fifth rows) and summary diagrams (third and sixth rows). (A) PKC \( \alpha \) is localized along the lateral membranes (small arrow) and in the brush border microvilli (large arrow) of villus enterocytes. No staining was observed in the junctional complex region or in the basal membrane domains. (B) PKC \( \beta II \) staining is detected in the terminal web region (arrowheads) of all villus enterocytes. Intense lateral membrane staining is apparent in lower villus enterocytes. Note the absence of staining in the brush border microvilli (open arrow). (C) PKC \( \delta \) is detected along the lateral membranes (small arrow) of lower villus enterocytes. No staining is seen in the brush border microvilli (open arrow) of these cells (D). PKC \( \delta \) is detected in the microvilli (large arrow) of upper villus enterocytes (2). Intense lateral membrane staining is apparent in these mature cells. (D) PKC \( \epsilon \) is localized along the lateral membranes and in the junctional complex region (arrow). (E) PKC \( \xi \) staining is also apparent in the apical junctional complex region (arrow) and along the lateral membranes of villus enterocytes. MV, microvilli; TW, terminal web; TJ, tight junction; ZA, zonula adherens; D, desmosome; IF, intermediate filaments. (II) En face views of villus epithelial cells stained for PKC \( \alpha \) (A), \( \beta II \) (B), \( \delta \) (C) and \( \xi \) (D). Arrows indicate staining of the microvilli (A), terminal web (B), and junctional complex (C and D). Bars: (I) 10 \( \mu m \); (II) 5 \( \mu m \).
cell migration into the differentiation zone is accompanied by a 1.7-fold increase in total PKC activity and a 1.4-fold increase in total levels of expression of PKC α, δ, and ε; (b) in general, levels of PKC activity and of PKC isozyme expression in the particulate subcellular fraction (i.e., membrane and cytoskeletal elements) are higher in differentiating and functional cells relative to immature crypt cells; (c) PKC α, βII, δ, and ε are activated in cells of the mid-crypt, precisely in the region associated with cell growth cessation and commitment to differentiation (cell position 14–18 from the crypt base); (d) direct activation of PKC in IEC-18 immature intestinal crypt cells results in inhibition of cell growth accompanied by translocation of PKC α from the soluble to the particulate fraction; (e) PKC βII is activated specifically in cells of the differentiation zone; and (f) acquisition of mature function is accompanied by activation of PKC δ. Taken together, these data indicate an involvement of PKC isozymes in cellular processes related to growth cessation, differentiation, and mature function of IEC in situ. In keeping with this conclusion, PKC ε, which has been shown in a recent report to be expressed at high levels in the small intestine, is localized only in differentiating cells of the villus (Osada et al., 1993).

The in situ data demonstrate a temporal and spatial relationship between PKC activation and IEC growth inhibition/differentiation. A direct role for PKC in negative growth regulation of intestinal epithelial cells is supported by studies presented in this report using the IEC-18 immature crypt cell line. Additional support for such a role is provided by several other in vitro studies using established normal and malignant intestinal epithelial cell lines. Direct activation of PKC by PMA in another small intestinal crypt cell line, IEC-6 (Quaroni and May, 1980), was shown to result in inhibition of cell growth accompanied by translocation of PKC activity from the cytosolic to the particulate subcellular fraction (Baliga and Borowitz, 1988). In addition, rapidly proliferating premalignant and malignant colonic tumor cells exhibit low levels of PKC activity relative to cells of normal colonic mucosa (Guillem et al., 1987; Kopp et al., 1991; Craven and DeRubertis, 1992), and PKC activation in some colon carcinoma cell lines results in growth inhibition and/or induction of markers of differentiation (McBain et al., 1988a, b; Rochette-Egly et al., 1988; Baron et al., 1990). Strong supportive evidence for the involvement of PKC isozymes in IEC growth control also comes from studies in HT-29 colon adenocarcinoma cells where overexpression of PKC βII was found to correlate with growth inhibition and decreased tumorigenicity in nude mice (Cho et al., 1990). In summary, data presented in this report obtained by analysis of rat intestinal tissue and by direct activation of PKC in an established immature crypt cell line, together with evidence from other in vitro and in situ studies, strongly implicate PKC in the control of growth inhibition and differentiation of cells of the intestinal epithelium. Evidence from studies in other epithelial systems including keratinocytes (Isseroff et al., 1989; Dlugosz and Yuspa, 1993), kidney epithelial cells (Godson et al., 1990), and pancreatic islet cells (Ganesan et al., 1990, 1992) indicates that PKC-dependent pathways may be important in the control of differentiation and mature function in epithelial tissues in general.

The mechanisms underlying PKC-mediated regulation of cell growth and differentiation in the intestinal epithelium remain to be established. One possibility involves modulation of the expression/activity of growth factors or growth factor receptors. Activation of PKC in cultured small intestinal crypt cells has been demonstrated to result in downregulation of the EGF receptor and decreased binding affinity for EGF (Baliga and Borowitz, 1988; Baliga et al., 1990). Thus, it is tempting to speculate that one or more PKC isozymes participate in growth control in the intestinal epithelium by downregulating the EGF receptor in cells of the mid-crypt region. It is noteworthy that purified PKC α has been shown...
to phosphorylate the EGF receptor in epidermoid carcinoma cells (Ido et al., 1987).

TGF-β has been shown to function in negative growth regulation in intestinal epithelial cells (Kurokowa et al., 1987; Barnard et al., 1989). Several studies have implicated PKC activation in induction of TGF-β expression (Akhurst et al., 1988; Kim et al., 1990). In addition, studies in a colon carcinoma cell line suggest that at least some effects of TGF-β require the activation of PKC (Chakrabarty, 1992). Thus, another mechanism for PKC-mediated control of enterocytic growth and differentiation might involve upregulation of the expression and/or activity of growth inhibitory factors such as TGF-β.

Association of PKC Isozymes with the Brush Border Cytoskeleton

Assembly of the brush border cytoskeleton is an important aspect of enterocytic differentiation, although the underlying mechanisms are poorly understood (Heintzelman and Mooseker, 1992). This report demonstrates that a proportion of each of the PKC isozymes identified in the intestinal epithelium appears to be concentrated in compartments of the enterocyte which are enriched in cytoskeletal proteins (see Fig. 7), and exhibits tight association with cytoskeletal elements (see Fig. 8). The localization and solubility data presented here, together with evidence from previous reports, suggest that PKC α and δ may be associated with the IEC actin-based cytoskeleton (Jaken et al., 1989), possibly with actin filaments of the microvillus cores, while PKC βII may be linked either directly or indirectly to spectrin (Gregorio et al., 1992, 1994) or intermediate filaments (Spudich et al., 1992, 1983), and PKC ε and θ may interact with structural proteins (e.g., intermediate filaments) of the apical junctional complexes (Omary et al., 1992). While marked alterations in membrane association of PKC isozymes which correlate with post-mitotic events were observed along the crypt-to-villus axis, cytoskeletal associations appeared comparatively static; PKC isozymes are present in cytoskeletal compartments of proliferating, differentiating, and mature cells. Although the brush border is disorganized in immature IEC, the major cytoskeletal proteins are already expressed in their full concentration in the apical cytoplasm, and both junctional complexes and intermediate filament networks are present (Fath et al., 1990). The localization of PKC isozymes in these subcellular compartments, together with evidence that phosphoinositide turnover and elevated PKC activity are associated with control of cytoskeletal assembly and membrane-cytoskeletal linkages (Burn et al., 1988; Forscher, 1989), suggest that brush border assembly may involve the reorganization of a pool of cytoskeletal proteins mediated by PKC isozyme activity. It is interesting in this regard that studies in cultured kidney epithelial cells (MDCK) have indicated that PKC is an important regulator of epithelial...
cell polarization and may play a role in assembly of the tight junction (Baliga et al., 1993). PKC isozymes may also be involved in maintenance of brush border morphology in mature enterocytes (Stidwell et al., 1984; Heintzelman and Mooserke, 1992). A more extensive analysis of these possible PKC isozyme-cytoskeleton associations and their function(s) in the brush border will be addressed in future investigations.

Intestinal epithelial cell migration from the crypts to the villus tips involves programming for proliferation- and differentiation-related events. This report demonstrates that PKC isozyme activation correlates with growth inhibition and differentiation of intestinal epithelial cells in situ. Although modulation of their activities in this tissue appears to be complex, specific PKC isozymes could play different roles integrated in both structural and functional differentiation of these cells. These studies provide a framework for ongoing investigations directed towards identifying isozyme-specific substrates in renewing epithelium to further clarify the participation of individual members of the PKC family in these processes.

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