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The network organization and the phosphorylation of cytokeratins are concomitantly modified by forskolin in the enterocyte-like differentiated Caco-2 cell line

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SUMMARY

Confluent Caco-2 cells, originating from a human colon carcinoma, display morphological and functional characteristics of differentiated enterocytes such as the presence of a polarized monolayer covered by an apical brush border that express several hydrolases. The adaptation of these cells to grow in the continuous presence of forskolin, a drug known to stimulate adenylyl cyclase permanently, has been previously shown to result in a decreased apical expression of hydrolases and in morphological alterations including the disappearance of intercellular spaces and shortening of microvilli. In the present work we have analyzed the possibility that cytoskeletal proteins may be the target of forskolin in living Caco-2 cells. We show that forskolin initiates dramatic changes in the spatial organization of the cytokeratin network that correlate with an increased phosphorylation of cytokeratin molecules, whereas microtubules, microfilaments and vimentin remain mainly unaffected. Indirect immunofluorescence studies show that the cytokeratin network is redistributed from the cell periphery to the cytoplasm. Biochemical experiments indicate that forskolin doesn't interfere with the cytokeratin profile, since the three cytokeratins normally found in intestine (CK 8, CK 18, CK 19) are similarly expressed in both control and forskolin-Caco-2 cells. Analysis of ^{32}P-labeled cytokeratin extracted from the two cell populations demonstrates that forskolin quantitatively increases the phosphorylation of type I cytokeratin (CK 18 and CK 19), whereas the phosphorylation of type II cytokeratin (CK 8) is altered both quantitatively and qualitatively with the emergence of a new phosphorylation site. These results provide a new cell system in which it is possible to control the subcelluar distribution of cytokeratin by changing their phosphorylation status and therefore to study their potential cellular functions.

Key words: cytokeratin, phosphorylation, Caco-2 cell, forskolin

INTRODUCTION

The enterocytic differentiation of human intestinal epithelial cells is characterized by the polarization of the cell monolayer and the emergence of a regular brush border membrane that harbors several brush border hydrolases (Madara, 1991). It has been shown that human colon cancer cell lines, especially HT-29 and Caco-2, may mimic this differentiation process when cultured under specific conditions (Zweibaum et al., 1991). Previous studies have pointed out the crucial role of glucose metabolism in the control of this phenomenon (Pinto et al., 1983; Rousset et al., 1979, 1985; Trugnan et al., 1987; Wice et al., 1985; Zweibaum et al., 1985). Recent experiments have further documented the role of this metabolite in Caco-2 cell differentiation by the use of forskolin (FK) a drug known to stimulate adenylyl cyclase permanently and strongly in several cell types (Seamon and Daly, 1986). Caco-2 cells were adapted to grow in the continuous presence of FK ('FK-Caco-2 cells') and this resulted not only in a 10- to 20-fold permanent increase in the intracellular cyclic AMP content, but also in a 5-fold increase in glucose consumption and in a decreased glycogen content (Rousset et al., 1985). Under these metabolic conditions, no reversal of the differentiated phenotype was observed but some characteristic features of differentiated Caco-2 cells were impaired: the apical expression of brush border hydrolases was reduced, the microvilli were shorter and thicker, and intercellular spaces disappeared (Baricault et al., 1993; Rousset et al., 1989, 1985). These modifications suggested that the cytoskeleton of Caco-2 cells may be a target of the drug. In the present study we have tested this hypothesis by analyzing the network organization of microtubules, microfilaments and intermediate filaments in FK-Caco-2 cells. We found that FK initiates dramatic changes in the subcellular distribution of cytokeratins (CKs), an epithelial-specific class of intermediate filaments (IFs) (Moll et al., 1982), without significant morphological alteration of other cytoskeletal proteins.

Intestinal epithelial cells mainly express cytokeratins as pairs such that each pair is composed of acidic type I (CK 18 and 19) and neutral-basic type II (CK 8) (Moll et al., 1982). The mechanisms by which the CK network organization is controled are far from being clear (Stewart, 1993). However, it has been suggested that phosphorylation may play a key role in monitoring association-dissociation of CKs as well as interactions with other cytoskeletal structures (Coulombe, 1993). We have therefore studied the expression and the phosphorylation of CKs in control and FK Caco-2 cells by using a biochemical approach. We show that the FK-induced perturbation of the CK network organization is associated with quantitative and qualitative changes in the phosphorylation of CK 8, CK 18 and CK 19, but not of vimentin, another IF expressed in Caco-2 cells, suggesting that the drug may activate CK-specific kinase(s). These results provide a new highly polarized epithelial cell system in which it will be possible to study the dynamic properties and the functions of CK.

MATERIALS AND METHODS

Cell culture and drug treatments

Caco-2 cells were obtained from J. Fogh (Memorial Sloan Kettering Cancer Center, Rye, NY; Fogh et al., 1977) and were grown at 37°C in a 10% CO_2/ 90% air atmosphere in Dulbecco's modified Eagle's minimum essential medium (Gibco BRL, Eragny, France) supplemented with 20% heat-inactivated (56°C, 30 minutes) fetal calf serum (Boehringer Mannheim, Germany), 1% non-essential amino acids (Gibco), and 1% penicillin-streptomycin (Gibco), as previously described (Pinto et al., 1983; Rousset et al., 1985). This medium was changed 48 hours after seeding and then daily. For cell maintenance and for biochemical studies, cells were seeded at 1×10^4 cells per cm^2, in 25 cm^2 plastic flasks (Corning Glassworks, Corning, NY). For immunofluorescence studies, cells were seeded at the same density on glass slides in 39 mm Petri dishes (Corning). Caco-2 cells (passages 45-85) were routinely passaged every 6 days and used after 15-20 days of culture. FK was obtained from Calbiochem (La Jolla, San Diego, CA) and used in the range of 2.5×10^{-5} to 5×10^{-5} M from a stock solution made by a 1/2000 dilution in ethanol. Previous work has shown that these concentrations of FK and the presence of diluted ethanol are without effect on cell growth and viability of Caco-2 cells (Rousset et al., 1985). Cells were treated with FK from day 2 on and then throughout the culture period. Under these conditions, the cyclic AMP (cAMP) concentration was shown to be permanently and greatly increased (Darmoul et al., 1991; Rousset et al., 1985).

Antibodies and immunofluorescence studies

Two mouse monoclonal anti-CK 8 antibodies (clone 4.1.18 and clone Ks pan 1-8) were obtained from Boehringer. CK18 was detected using a mouse monoclonal anti-CK18 antibody (clone CK 2) obtained from Boehringer. Mouse monoclonal antibody directed against cytokeratin 19 (clone 170.2.14) was purchased from Boehringer. The mouse monoclonal antibody to actin (clone C4) was purchased from Boehringer. The anti-α-tubulin was obtained from Amersham (Buckinghamshire, England). A mouse monoclonal anti-vimentin antibody (aV2) was kindly provided by S. Georgatos (EMBL, Heidelberg). The fluorescein-conjugated anti-mouse IgG antibody was obtained from Jackson (Interchim, Montluçon, France). The Texas Red-conjugated phalloidin was obtained from Sigma (St Louis, MO).

Indirect immunofluorescence was performed on cells grown on glass coverslips, according to previously published techniques (Darmoul et al., 1992; Zweibaum et al., 1984). After medium removal, cells were rinsed three times with ice-cold PBS, permeabilized and fixed with cold methanol (at −20°C) for 10 minutes, followed by a 10 minute incubation in acetone at 4°C. Alternatively, cells were fixed for 10 minutes at room temperature with 1% paraformaldehyde and permeabilized with a 10 minute incubation in cold methanol or in the presence of 0.075% Saponin diluted in PBS. After immunostaining, samples were mounted in Glycergel (Dakopatts, Copenhagen, Denmark) and were observed using a conventional microscope equiped for fluorescence analysis (Axioplan, Zeiss, Oberkochen, Germany).

[^{32}P]phosphate labeling of cytokeratins in cultured Caco-2 cells

Caco-2 cells grown in 25 cm^2 flask, were preincubated 2× 30 minutes in phosphate-free Dulbecco's modified Eagle's minimum essential medium. Cells were then labeled for 4 hours at 37°C with 200 μCi/ml of carrier-free [^{32}P]orthophosphate (439 mCi/ml, ICN Radiochemicals, Costa Mesa, CA). Cells were washed 3 times with PBS^- (PBS without Ca^{2+} and Mg^{2+}) at 4°C and then scraped with a rubber policeman. CKs were extracted as described below and were analyzed by one- or two-dimensional electrophoresis followed by silver nitrate staining and autoradiography.

Preparation of CK extracts

CK extraction was performed as described by Gilmartin et al. (1984). Briefly, Caco-2 cells were washed two times with PBS^-. Cells were harvested by scraping the cell monolayer with a rubber policeman and washed once in PBS^-. All the subsequent steps were performed at 4°C. Cells were lysed in 20 vols of Tris-Triton buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100) and the buffer-insoluble pellet was collected at 12,000 ***g*** for 15 minutes. The pellet was washed three times in 50 vols of washing buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF) and incubated at 37°C for 30 minutes with occasional shaking. The insoluble material was collected by centrifugation and washed three times. The pellet was extracted at 37°C for 30 minutes in extraction buffer (9 M urea, 100 mM β-mercaptoethanol, 25 mM Tris-HCl, pH 7.4, 1 mM PMSF). Residual insoluble material was pelleted and the supernatant was analyzed as described below. Alternatively, for the labeling experiments we used a simplified CK extraction procedure ('NaF procedure') derived from a previously published technique (Gilmartin et al., 1984). At the end of the labeling period, the medium was removed, the culture flasks were rapidly rinsed with PBS^- and the cells were lysed in 0.1 M Na_2HPO_4, pH 7.5, 1% Triton X-100, 0.1 M NaF, 1 mM EDTA, 1 mM PMSF. The buffer-insoluble proteins were collected by centrifugation at 15,000 ***g***, resuspended according to the procedure of O'Farrel (1975) and analyzed by one-and two-dimensional SDS-PAGE. In order to obtain internal standards, CK were also extracted from 4 month-old Balb/c mouse liver according to the method described by Eichner et al. (1984). One gram of liver was homogenized in 2 ml of homogenization buffer (25 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM PMSF, 1 mM leupeptin, 1 mM aprotinin, 1 mM EDTA, 1 mM EGTA) using a Teflon Potter homogenizer. Samples were centrifuged 10 minutes at 10,000 ***g***. The upper phase was discarded and the lower phase was mixed with an equal volume of homogenization buffer and centrifuged 10 minutes at 10,000 ***g***. The pellet was resuspended in 9.5 M urea, 25 mM Tris, pH 7.4, and 10 mM β-mercaptoethanol. The sample was sonicated and centrifuged for 10 minutes at 10,000 ***g*** and the resulting pellet was resuspended in lysis buffer as described by O'Farrell (1975).

Gel electrophoresis and quantification

CK extracted according to the above described protocols were analysed by one-dimensional gel electrophoresis according to Laemmli (1970) and/or by two-dimensional gel electrophoresis as described by O'Farrell (1975). Gels were either stained with Coomassie Blue or with silver nitrate (Morissey, 1981). For labeling experiments, the gels were dried and radioactivity was detected using

autoradiography on Trimax 3M films. Gels and autoradiograms were scanned and quantified using an Apple OneScanner driven by Ofoto® and Image 1.45® software. An attempt was made to determine the specific activity of phosphate in CK molecules as follows: equal amounts of CK extracted from labeled control or FK Caco-2 cells were separated on two-dimensional gels. Silver nitrate staining was performed for the same duration and using the same batch of reagents, in order to obtain comparable stainings. Autoradiography was also performed in parallel and films were developed in identical conditions. Both silver nitrate-stained gels and autoradiograms were quantified by scanning. The ratio of the surface area of the label of a given CK to the surface area of the corresponding unlabeled CK was calculated and used to represent the specific activity of phosphate in CK molecules.

Western blot analysis

Immunoblot analysis of IFs was performed after two-dimensional gel electrophoresis. Briefly, 100 µg of proteins derived from the indicated cell populations were separated as described above and transferred to nitrocellulose sheets (Schleicher and Schuell, Darrel, Germany) by the method of Burnette (1981). CKs were identified using the above described antibodies. Antigen-antibody complexes were visualized using goat anti-mouse immunoglobulins labeled with alkaline phophatase (Promega Corp., Madison, WI).

Partial digestion of cytokeratins

Identification of cytokeratins was performed by partial digestion of individual cytokeratin proteins. CK were resolved on two-dimensional gel and detected using Coomassie Blue staining. Spots corresponding to putative CK were excised from the gel and subjected to limited proteolysis according to the procedure described by Cleveland et al. (1977). Briefly, CK-containing gel pieces were placed in the sample wells in the presence of 10 ng of V8 protease extracted from *Staphylococcus aureus* (Sigma Biochemicals). Digestion proceeded directly during electrophoresis and the resulting fragments were separated on a 15% acrylamide gel. After migration, the gel was stained with silver nitrate and subjected to autoradiography, if needed.

RESULTS

FK induces change in the CK network organization

To test the hypothesis that FK may alter the cytoskeletal organization of differentiated Caco-2 cells, we used immunocytochemical screening to look for subcellular distribution of microtubules, microfilaments and intermediate filaments in both control and FK-Caco-2 cells. The most obvious and reproducible change was observed in the distribution of CKs (Fig. 1), whereas other cytoskeletal structures were only slightly perturbed. Therefore we decided to focus the present study on the CK network. Confluent Caco-2 cells were stained using three distinct anti-cytokeratin monoclonal antibodies directed against CK 8, CK 18 and CK 19. The choice of these compounds was based on experiments described in the next paragraph and on data from previous studies that have shown that normal and cancerous intestinal epithelial cells expressed this particular subset of intermediate filaments (Franke et al., 1981a; Moll et al., 1982; Rafiee et al., 1992). In control cells, anti-CK antibodies mainly decorated pericellular structures that form regular cylinders all around the basolateral membrane (Fig. 1A,C). CK filaments were also detectable in the supranuclear region of the cytoplasm below the apical surface. The three CKs display a similar subcellular distribution, a result expected from the fact that type I and type II CKs form common filaments in which they are assembled as a pair (Cooper et al., 1985; Franke et al., 1981b; Moll et al., 1982). These results are in good agreement with previous studies that have shown, using immunofluorescence and electron microscopy, that CKs form tonofilaments that extend from lateral desmosomes to the apical surface of intestinal villus cells (Chandler et al., 1991; Franke et al., 1979a,b, 1981a,b; Hirokawa et al., 1982). In addition, CK filaments have been identified in a cytoskeletal formation, the terminal web (Hirokawa et al., 1982; Hull and Staehelin, 1979), localized below the apical cell surface, which may play a role in the formation and/or the maintenance of microvilli (Hirokawa et al., 1982). Therefore Caco-2 cells, which originate from a human colon carcinoma, appear to display a cytokeratin network similar to the one observed in normal differentiated epithelial intestinal cells (Franke et al., 1979a, 1981a), a result that confirms that these cultured cells are capable of mimicking several aspects of the enterocytic phenotype (Pinto et al., 1983; Zweibaum et al., 1991). In FK-Caco-2 cells the distribution of CK was dramatically perturbed (Fig. 1D,F). In the vast majority of the cells anti-cytokeratin antibodies mostly stained the cell cytoplasm. Interestingly, FK doesn't similarly alter the subcellular distribution of individual CK. CK 8 and CK 18 labels fill the cell cytoplasm in a diffuse manner. Filamentous structures were still detectable but observations at different focus have shown that they were essentially perpendicular to the basal-apical cell axis. This would suggest that the network formed by CK 8 and CK 18 is reorganized rather than destroyed. CK 19 staining reveals a more heterogeneous pattern. In some cells, CK 19 is concentrated around the cell nucleus, suggesting a retraction of CK-19-containing IFs. In other parts of the cell monolayer, CK 19 was found in the cytoplasm as previously seen with CK 8 and CK 18, suggesting that CK 19 still contributed to IF formation with these CKs. Finally, CK 19 was also detected in vesicular structures whose nature remains to be determined. It is known that CK 19 is the only CK described so far that presents a very short (13 residues) tail domain (Eckert, 1988). Such a structure may explain the particular CK 19 subcellular distribution in FK Caco-2 cells, since it has been suggested that this part of the molecule may be involved in phosphorylation-dependent IF assembly and/or in interactions with other cytoskeletal structures (Coulombe, 1993). This may also refer to the fact that CK 19 does not play the same essential role in filament formation as the paired keratins do (Stasiak et al., 1989). Finally, it should also be noted that some FK-Caco-2 cells display a CK network similar to the one observed in control cells. This differential response to FK treatment probably reflect the fact that the Caco-2 cell line is heterogeneous (Beaulieu and Quaroni, 1991; Howell et al., 1993; Jalal et al., 1992). FK-induced changes in CK distribution were similarly detected after fixation and permeabilization with methanol alone, methanol plus acetone, paraformaldehyde plus methanol or paraformaldehyde/ saponin (not shown), indicating that the observed effects were not a fixation artifact.

Changes in the CK network organization are not associated with changes in the CK profile in FK Caco-2 cells

The above described alterations in the CK network in FK Caco-2 cells may be due either to a change in the CK profile

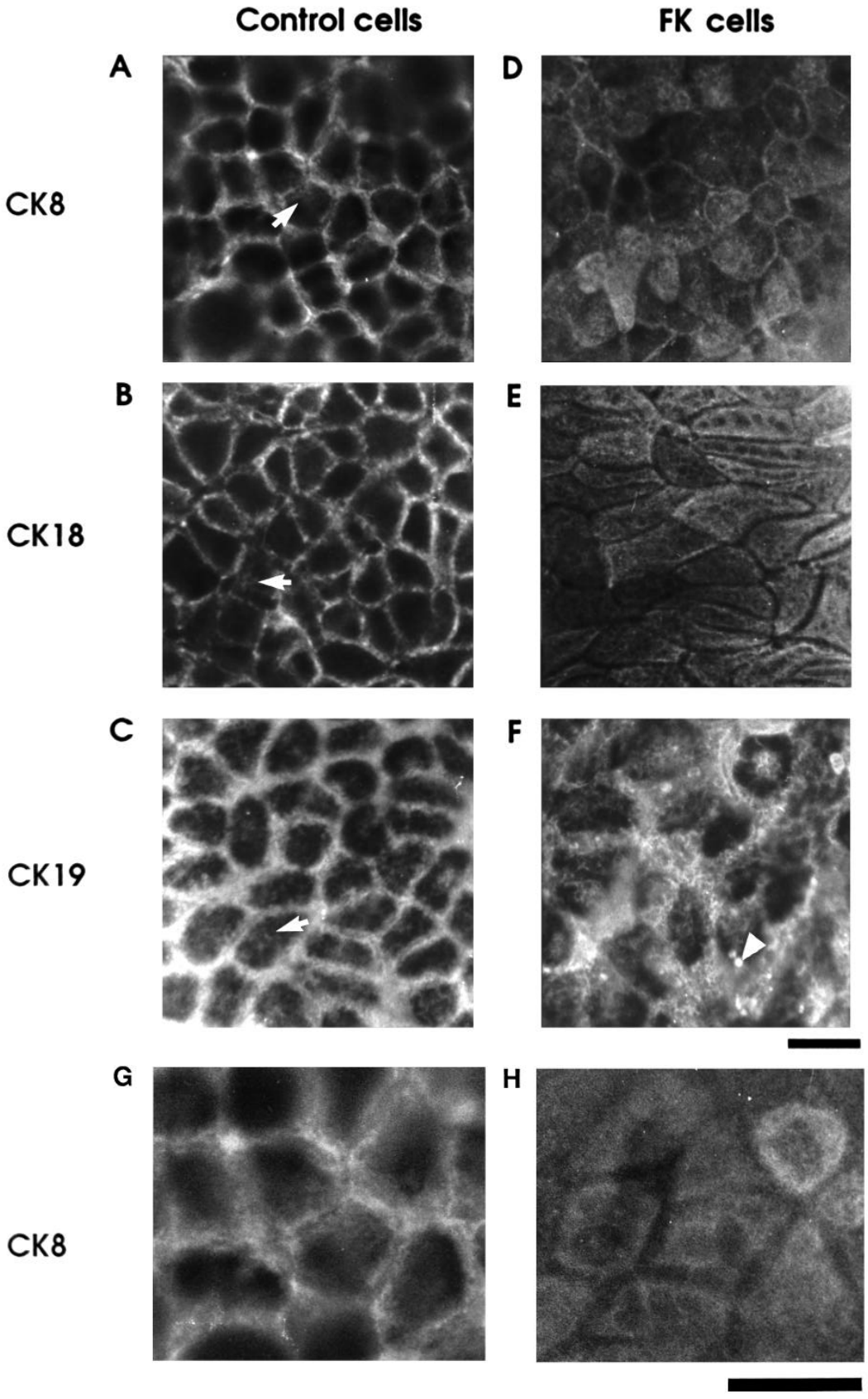

Fig. 1. Indirect immunofluorescence of cytokeratins in control (A-C) and FK-Caco-2 cells (D-F). Control and FK-Caco-2 cells were fixed and permeabilized using cold methanol and acetone and then stained with: anti-CK8, clone 4.1.18, used at 1/50 (A and D); anti-CK 18, clone CK 2, used at 1/50 (B and E); and anti-CK19, clone 170.2.14, used at 1/50 (C and F), monoclonal antibodies and a fluorescein-labeled anti-mouse IgG as secondary antibody. In control cells cytokeratins mainly localized at the cell periphery and some subapical structures were also stained (arrows). FK induces a reorganization of the CK network as shown by the almost complete disappearance of the CK staining at the cell periphery and the presence of a diffuse cytoplasmic labeling. Note in FK-Caco-2 cells the presence of positive vesicles when using anti-CK 19 antibody (F, arrowhead). Similar results were obtained using different fixation procedures (not shown). Bar, 25 μm. (G and H) Higher magnification of the CK 8 distribution in control and FK Caco-2 cells, respectively.

or to post-translational modification of CK molecules. It has been previously shown, for example, that during the differentiation process of intestinal cells the subcellular distribution of CKs was modified, together with their expression level

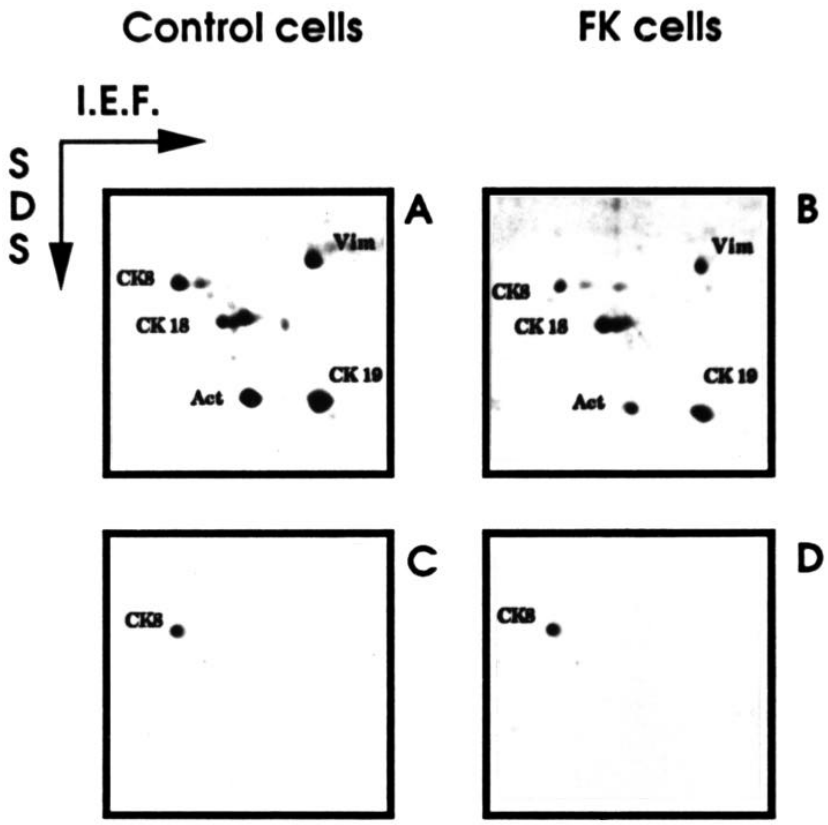

Fig. 2. Two-dimensional gel electrophoresis and western blot of CKs extracted from control and FK-Caco-2 cells. CKs were extracted using a high-salt precipitation protocol and analyzed using IEF (first dimension) followed by SDS-PAGE (second dimension). (A and B) Silver nitrate staining. Note that CK patterns in control (A) and FK cells (B) are comparable except that a more acidic form of CK 8 is detectable in FK cells. Duplicate gels were transferred onto nitrocellulose sheets and CK 8 was revealed by western blotting with clone 4.1.18 monoclonal antibody (C and D). CK 8 was visualized using a phosphatase-conjugated anti-mouse antibody. Development time was adjusted so that no cross-reaction appeared, thus explaining that in both control and FK Caco-2 cells only one spot, corresponding to the unphosphorylated CK 8, is detectable. Vim, vimentin; Act, actin.

(Chandler et al., 1991), and included a new CK that is now known to be keratin 20, which is specifically expressed in differentiated rat intestinal cells (Calnek and Quaroni, 1992; Quaroni et al., 1991). We subsequently tested the possibility that FK Caco-2 cells may display a different CK profile as compared to control cells. It is well known that intestinal epithelial cells mainly express CK 8, CK 18, CK 19 (Moll et al., 1982). Several human colon cancer cell lines were also shown to express the same set of CKs (Moll et al., 1982; Rafiee et al., 1992). Whether Caco-2 cells also express this set of CK and whether FK may modify this profile was explored by biochemical analysis. CKs from the two Caco-2 cell populations were extracted and were subjected to two-dimensional gel electrophoresis. Silver nitrate staining of the gels revealed five major groups of spots in the CK region in control as well as in FK cells (Fig. 2A,B). Each group corresponds to a particular apparent molecular mass, of 40 kDa, 41 kDa, 48 kDa, 55 kDa and 57 kDa, respectively. Proteins separated on two-dimensional gels were transferred onto nitrocellulose sheets and analyzed by western blotting. Staining of the nitrocellulose sheets with monoclonal antibodies allowed us to identify the 55 kDa protein as CK8 (Fig. 2C,D). Spots corresponding to 55 kDa (CK 8) and 48 kDa (putative CK 18) were cut off two-dimensional gels and were subjected to V8 protease digestion together with authentic CK 8 and CK 18 from mouse liver, according to the method described by Cleveland et al. (1977). Comparison of the peptides obtained from Caco-2 cells CKs with those of mouse liver CKs clearly establishes that control and FK-Caco-2 cells express CK 8 and CK 18 (Fig. 3, see also Fig. 6A). The three remaining spots were identified as vimentin (57 kDa), actin (41 kDa) and CK 19 (40 kDa), using specific monoclonal antibodies (not shown). It can therefore be concluded that control as well as FK Caco-2 cells express the same set of cytokeratins as other normal or cancerous intestinal epithelial cells (Moll et al., 1982), except for the presence of vimentin, which can probably be ascribed to the fact that experiments were done on cultured cells (Ben-ze'ev, 1984). The possibility that some CKs may be in a soluble form in FK cells was also explored. Western blot analysis using a 'pan' anti-CK antibody and silver nitrate staining were used to study the CK content of all the fractions obtained during the CK purification procedure. No detectable amount of CK was observed in these fractions in either control or FK cells (not shown). These observations rule out the possibility that the adaptation of Caco-2 cells to grow in the continuous presence of FK may interfere with CK content or solubility in these cells. Therefore, we turn to the hypothesis that the observed CK network perturbations may be related to post-translational modification of CK molecules.

The CK network reorganization is associated with changes in CK phosphorylation in FK Caco-2 cells

Control and FK-treated cells were metabolically labeled for 4 hours with [^{32}P]orthophosphate, and CKs were extracted and analyzed on SDS-PAGE, using both silver nitrate staining (Fig. 4A) and autoradiography (Fig. 4B). Silver nitrate staining of the gels indicated that the protein profile was similar and that comparable amounts of cytoskeletal proteins were present in the two cell populations. As expected, IFs were labeled with [^{32}P]phosphate in control as well as in FK Caco-2 cells. Three major radioactive spots were detected that correspond to CK 8, CK 18 and CK 19 and it clearly appears that the labeling of

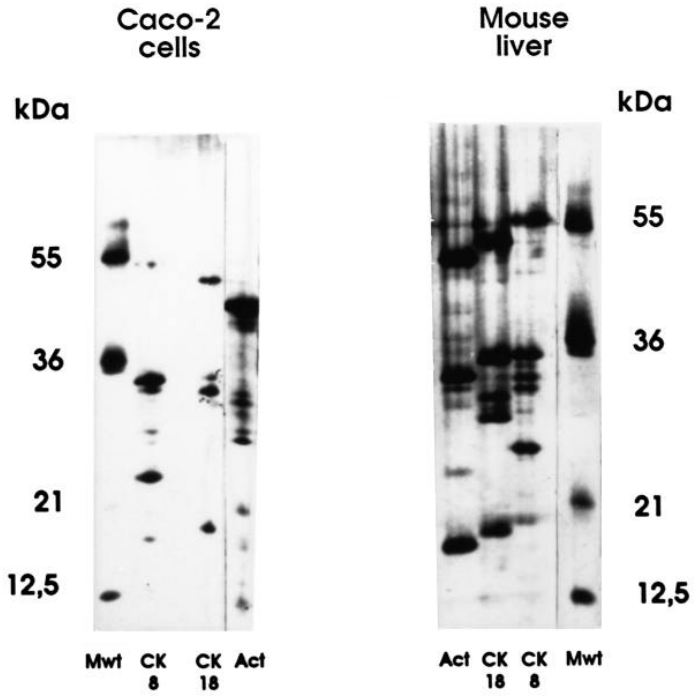

Fig. 3. V8 protease digestion assay on CK 8 and CK 18 extracted from Caco-2 cells and mouse liver. CK were extracted and separated on two-dimensional gels as in Fig. 2. Proteins were analyzed using Coomassie Blue staining. Spots corresponding to the putative CK 18 and CK 8 were scraped off and subjected to V8 protease digestion as described by Cleveland et al. (1977). CK 8 and CK 18 digestion produces a band pattern identical to the one observed with authentic mouse liver CK 8 and CK 18. Note that actin (Act) extracted from mouse liver and from Caco-2 cells displays a similar pattern. Mwt, molecular mass standards.

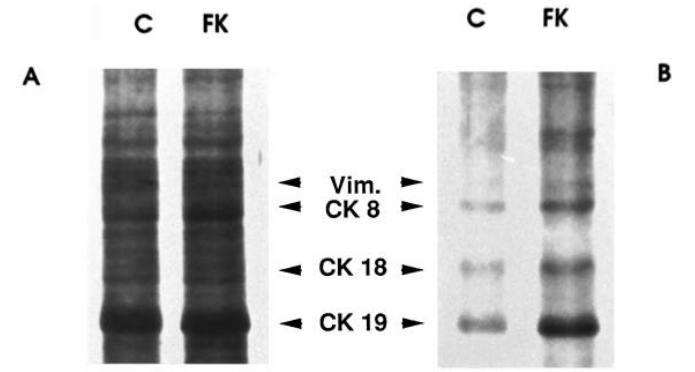

Fig. 4. One-dimensional analysis of ^{32}P-labeled Caco-2 cells. Control and FK-Caco-2 cells were labeled for 4 hours using [^{32}P]orthophosphate. CKs were extracted using the NaF procedure as described by Gilmartin et al. (1984) and analyzed on SDS-PAGE. An identical amount of protein was loaded on each slot. Proteins were stained using silver nitrate (A) and gels were then subjected to autoradiography (B). Silver nitrate staining reveals similar profile and comparable amount of proteins in control and FK-treated cells. In contrast, the incorporation of ^{32}P into the three CKs (CK 8, CK 18 and CK 19) is dramatically increased in FK-Caco-2 cells as compared to control cells, whereas vimentin appears to be only faintly labeled in the two cell populations.

CKs is much higher in FK Caco-2 cells as compared to control cells. Interestingly, it should be pointed out that the amount of radioactivity detectable in the 57 kDa region was low, suggesting that vimentin is only poorly phosphorylated under the particular labeling conditions used. However, since it is known that vimentin becomes more soluble when it is hyperphosphorylated (Inagaki et al., 1987), our results do not allow us to conclude definitively that the overall vimentin phosphorylation is low.

CK phosphorylation was further analyzed using two-dimensional gel electrophoresis (Fig. 5). The amount of radioactivity incorporated into CKs was determined using two distinct methods. Scanning measurements of the radioactive CK spots were performed on three independent experiments. Alternatively, radioactive CK spots were excised from the gels and counted (not shown). Both methods gave similar results and indicated that the amount of radioactive phosphate incorporated into the three CKs increased by 3- to 15-fold in FK-Caco-2 cells (Fig. 5E). Comparison of the silver nitrate staining (Fig. 5A and B) with the corresponding autoradiogram (Fig. 5C and D) shows that each CK behaves differently. CK 19 appears as a single spot in both silver nitrate staining and autoradiography. However, a careful superimposition of the two images indicates that the radioactive spot is shifted to the acidic pH, as compared to the silver nitrate-stained spot. CK 18 is detectable as two distinct spots, with the more acidic being the only one labeled with [^{32}P]phosphate. For these two CKs no additional labeled spot could be detected in FK cells. This conclusion is strengthened by the data presented in Fig. 6. Labeled CKs were extracted from control and FK-Caco-2 cells and separated on two-dimensional gels. Spots corresponding to CK 18 and CK 19 were cut off and were subjected to V8 protease digestion and SDS-PAGE electrophoresis. Analysis of the silver nitrate staining and the corresponding autoradiogram of these gels indicated that CK 18 and CK 19 display identical band patterns in control and FK-Caco-2 cells, and that no additional band could be visualized in FK cells. From the data displayed in Fig. 5 and from duplicate experiments, it was possible to determine the specific activity of phosphate in CK molecules (Fig. 7), which was significantly increased in FK cells, suggesting a stimulation of phosphate turnover in these cells. Therefore, it should be concluded that FK increases the phosphorylation of CK 18 and CK 19 essentially in a quantitative manner. CK 8 displays a totally different pattern, since an additional labeled acidic spot could be detected in FK Caco-2 cells (compare Fig. 5A,B with C,D). The V8 protease digestion of CK 8 (Fig. 6) confirms this conclusion, since it resulted in the appearance of additional labeled peptides in FK Caco-2 cells, as compared to control cells. However, as for CK 18 and CK 19, the specific activity of phosphate in CK8 increases significantly. These results indicate that, as well as a quantitative increase, CK 8 phosphorylation is also modified in a qualitative manner, with the emergence of a new phosphorylation site in FK Caco-2 cells, thus indicating that FK does not induces an overall hyperphosphorylation but acts in a more specific way on the cytokeratin family.

DISCUSSION

The correlation between modifications of the CK network

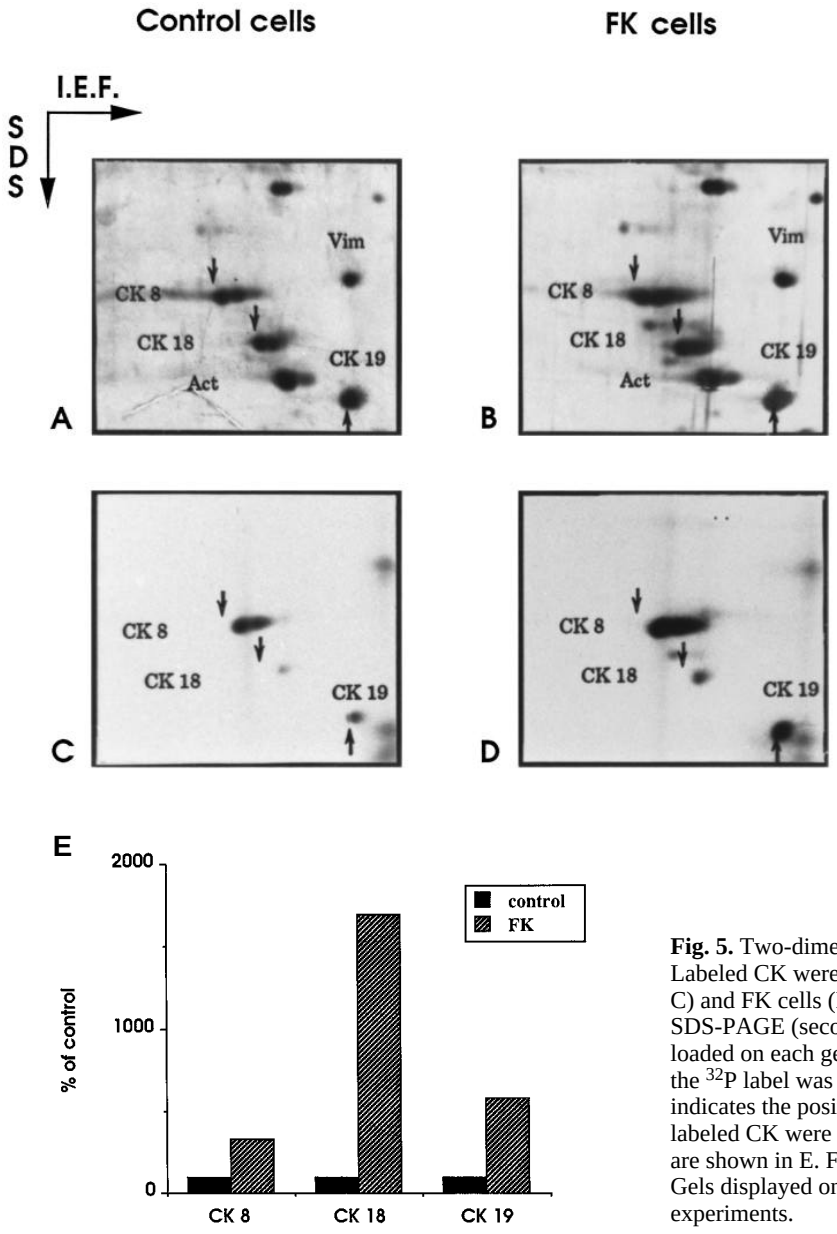

Fig. 5. Two-dimensional gel electrophoresis of ^{32}P-labeled Caco-2 cells. Labeled CK were extracted using the NaF procedure from control (A and C) and FK cells (B and D), and analyzed using IEF (first dimension) and SDS-PAGE (second dimension). An identical amount of protein was loaded on each gel. Protein were stained using silver nitrate (A and B) and the ^{32}P label was visualized using autoradiography (C and D). Arrows indicates the position of the unphosphorylated form of each CK. ^{32}P-labeled CK were quantified by scanning the gel and the resulting values are shown in E. FK induces an hyperphosphorylation of the three CKs. Gels displayed on this figure are representative of three independent experiments.

organization and the phosphorylation status of these molecules has been previously reported in some other living cell systems (for review see Skalli et al., 1992). It is now well established that, during mitosis, IFs, including CK, undergo a dissociation/association cycle in which the phosphorylation of IF molecules plays a key role (Chou et al., 1989, 1990; Jones et al., 1985). A similar phosphorylation-related reorganization of the CK network has also been described during the interphase of some cell systems. In EGF-stimulated newborn rat hepatocytes, a specific phosphorylation of the hepatic type II CK, CK 8, is associated with a reorganization of CK8/CK18 filaments from the cell periphery to the cytoplasm (Baribault et al., 1989). During the meiotic maturation of *Xenopus* oocytes it has also been established that hyperphosphorylation of the type II CK expressed in oocytes causes the disappearance of the cortical filament system (Klymkowsky et al., 1991). The involvement of type II CK phosphorylation in remodelling the keratin network has been further documented by the analysis of point mutations of the head domain of type II CK observed in human hereditary skin diseases (Chan et al., 1993; Steinert and Parry, 1993). These results were obtained mainly on undifferentiated or unpolarized cell systems, whereas we present here results on a highly polarized and differentiated cell line. However, previous results fit very well with our data, since we show here that, when Caco-2 cells are grown in the presence of FK, the type II CKs (namely, CK 8) also undergo a particular hyperphosphorylation, that correlates with the disappearance of the CK network from the cell periphery and its redistribution into the cytoplasm. Whether this phenomenon is due to the disassembly of paired cytokeratin filaments or to a dissociation of these filaments from other cellular structures, as suggested previously (Knapp et al., 1983), remains to be explored.

The mechanism by which forskolin interferes with the phos-

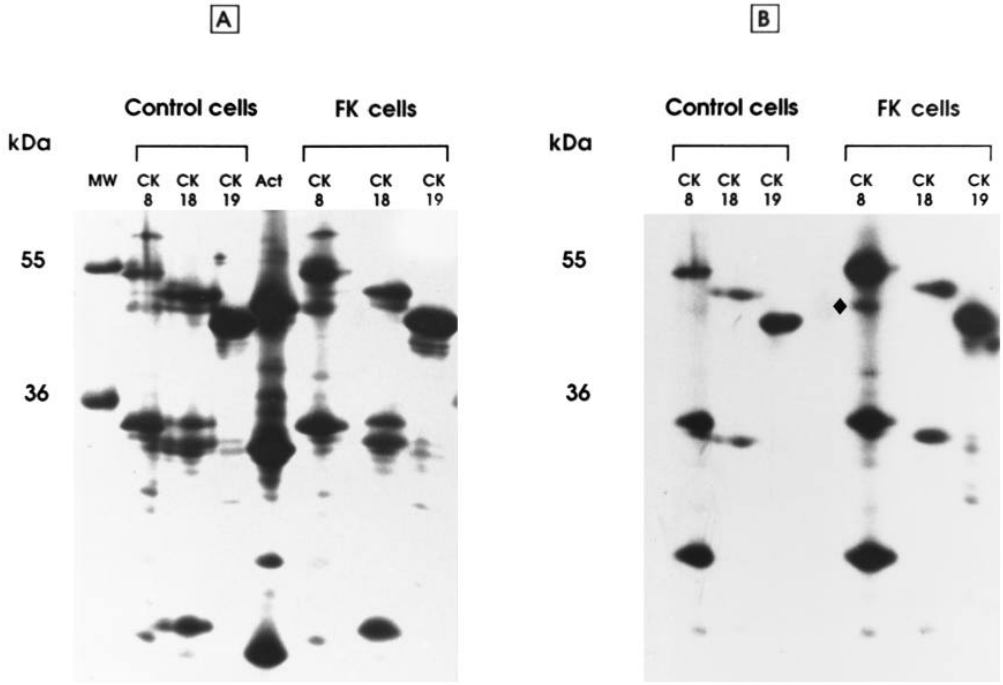

Fig. 6. V8 protease digestion of ^{32}P-labeled CK 8, CK 18 and CK 19 from control and FK-Caco-2 cells. CKs were prepared as for Fig. 5 and separated on two-dimensional gels. After Coomassie Blue staining, spots corresponding to CK 8, CK 18 and CK 19 were cut from the gels and subjected to partial digestion using V8 protease. (A) Silver nitrate staining of the gels. (B) Corresponding autoradiograms. The CKs pattern was similar in control and FK cells except that additional labeled peptides were detectable (◆) in CK 8 extracted from FK cells.

phorylation of CK remains to be further studied. The most pertinent hypothesis is that FK will act directly through its well known stimulating effect on adenylyl cyclase (Seamon and Daly, 1986). Such an increase in cyclic AMP has been shown to stimulate the phosphorylation of IFs molecules in Swiss 3T3 cells (Escribano and Rozengurt, 1988). In vivo as well as in vitro experiments have also provided evidence indicating that cyclic AMP-dependent protein kinase(s) A can readily phosphorylate serine sites in the amino-terminal domain of IFs

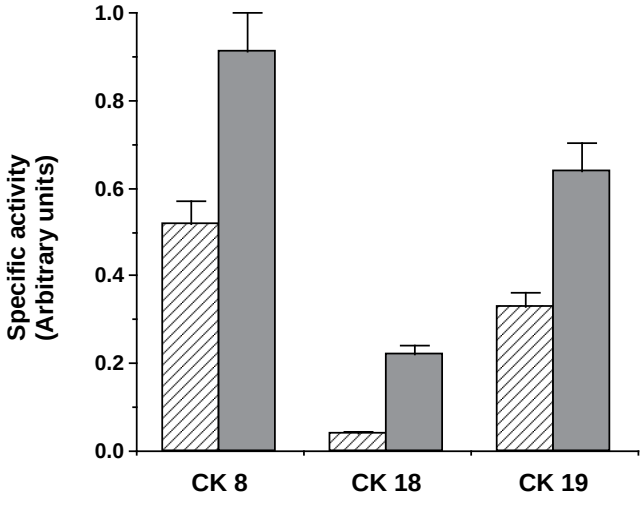

Fig. 7. Specific activities of labeled phosphate in CK 8, 18 and 19 from control and FK Caco-2 cells. Silver nitrate-stained gels and autoradiograms from Fig. 5, together with data from two other similar experiments, were scanned and the ratio of the labeled to the unlabeled surface area (arbitrary units) of each CK was determined in control (hatched bars) and FK Caco-2 cells (grey bars). Note the increase of specific activity of phosphate in the three CKs extracted from FK cells.

(Geisler and Weber, 1988; Inagaki et al., 1987; O'Connor et al., 1981). To support this hypothesis it should be mentioned that preliminary data from our laboratory have shown that dideoxy-forskolin, an analog of FK that doesn't stimulate adenylyl cyclase (Laurenza et al., 1989; Seamon and Daly, 1986), is without effect on both the phosphorylation of CKs and their network organization. However, FK may interferes with Caco-2 CKs through a more indirect pathway, since this compound is also known to display several adenylyl cyclase-independent effects. In this regard, it is worth noting that the ε catalytic subunit of protein kinase C has been recently shown to associate with and phosphorylate CK 8 and CK18 in HT-29 cells, another human colon cancer cell line (Omary et al., 1992). It would therefore be of interest to determine if this or other protein kinases may be involved in the FK-dependent stimulation of CK phosphorylation.

In the present work we have demonstrated that the adaptation of Caco-2 cells to grow in the continuous presence of FK leads to a complete reorganization of the CK network, which is associated with changes in the phosphorylation status of the three CKs expressed in this differentiated human colon cancer cell line. Despite the severity of this reorganization, morphological alterations were modest. Although the cell shape appears to be modified (see Fig. 1), no reversal of the differentiated phenotype could be observed (Rousset et al., 1985). The cells remain fully polarized and still form a tight monolayer, capable of transporting solute, as assessed by the presence of domes in the plastic culture flasks (Grasset et al., 1984). This would suggest that the archetypal function of CKs, i.e. their contribution to the maintenance of cell architecture, may be not so pivotal and it should be borne in mind that actin, tubulin and vimentin networks are mostly unchanged in FK Caco-2 cells. Whether CKs may exert other cellular functions remains one of the key questions and it may be interesting to note, in this regard, that we recently described a blocking of the targeting of brush border hydrolases from the *trans*-Golgi

network to the apical membrane in the same FK Caco-2 cells as those used in this study (Baricault et al., 1993; Rousset et al., 1989). Further study will be designed to determine if CK network disorganization and the blocking of a trafficking step may be related events.

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