Rescue of atypical protein kinase C in epithelia by the cytoskeleton and Hsp70 family chaperones

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Summary

Atypical PKC (PKCι) is a key organizer of cellular asymmetry. Sequential extractions of intestinal cells showed a pool of enzymatically active PKCι and the chaperone Hsp70.1 attached to the apical cytoskeleton. Pull-down experiments using purified and recombinant proteins showed a complex of Hsp70 and atypical PKC on filamentous keratins. Transgenic animals overexpressing keratin 8 displayed delocalization of Hsp70 and atypical PKC. Two different keratin-null mouse models, as well as keratin-8 knockout cells in tissue culture, also showed redistribution of Hsp70 and a sharp decrease in the active form of atypical PKC, which was also reduced by Hsp70 knockdown. An in-vitro turn motif rephosphorylation assay indicated that PKCι is dephosphorylated by prolonged activity. The Triton-soluble fraction could rephosphorylate PKCι only when supplemented with the cytoskeletal pellet or filamentous highly purified keratins, a function abolished by immunodepletion of Hsp70 but rescued by recombinant Hsp70. We conclude that both filamentous keratins and Hsp70 are required for the rescue rephosphorylation of mature atypical PKC, regulating the subcellular distribution and steady-state levels of active PKCι.

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Introduction

The protein kinase C (PKC) family in humans comprises three subclasses: conventional, novel and atypical. They control essential cellular functions ranging from cell proliferation to differentiation, share a highly conserved kinase domain, and differ in their regulatory domains. PKCι (ιota, ι/ι; one of the two atypical isoforms, which are collectively referred to as aPKC) is an oncogene product causative of lung non-small cell carcinoma and is a predisposing factor for colon cancer when overexpressed (Fields et al., 2007). It is also a key regulator of asymmetry in various tissues, and is conserved in evolution. It was originally identified as such in C. elegans along with its partners (partition deficient, PAR) PAR3 and PAR6 (Rose and Kemphues, 1998). In neurons, PKCι controls axonal polarity under extrinsic Wnt signaling (Zhang et al., 2007). In epithelial cells, the PKCι-PAR3-PAR6 complex is associated with tight junctions and generates a signaling gradient that specifies the apical domain and the localization of the junctions. The tight-junctional scaffold for this complex includes Crumbs, Pals1 and PatJ (Suzuki and Ohno, 2006). The functionality of the PKCι kinase domain depends on its conformation, controlled by phosphorylations in the activation loop (T403 in PKCι) by PDK-1 and in the turn motif (T555 in PKCι) (Newton, 2003).

Unlike other kinases, PKC isozymes lose their activation phosphorylation and conformation in a substrate-dependent manner (Dutil et al., 1994), i.e. they become inactivated as a consequence of their own function. This characteristic has been used to inactivate conventional and novel PKC by prolonged treatment with phorbol esters (Savart et al., 1992; Hansra et al., 1999; Lang et al., 2004). The loss of PKC conformation is controlled by phosphatases and followed by attachment to still-unidentified cytoskeletal elements in the Triton-insoluble fraction (Gao et al., 2008). Once dephosphorylated, the PKC molecules are ubiquitinylated and degraded (Chen et al., 2007). Hsp70 chaperone proteins (Hsp70.1 hereafter referred to as Hsp70, and the constitutively expressed Hsc70) (Daugaard et al., 2007) bind dephosphorylated PKC (including α, β and the atypical ι isoforms) (Gao and Newton, 2002) and rescue them from degradation to regain function (Gao et al., 2006). The activation loop and turn motif are rephosphorylated, although it is unclear whether PDK-1 or other kinases are involved in the process. This mechanism effectively extends the active life of PKC (Newton, 2003) and, conceivably, can regulate the overall steady-state cellular levels of the kinase.

A role of Hsp70 in rescuing dephosphorylated atypical PKCι isoform has not yet been reported. However, based on the structural conservation of the PKC catalytic domain and the presence of the invariant Leu554 motif (NFDSQFTNEPQL*TPDDDDI) necessary for Hsp70 binding (Gao and Newton, 2006), it is reasonable to hypothesize that Hsp70 interaction with atypical PKCι is also involved in maintaining the functional pool of this kinase. Phosphorylation in the turn motif (T555) abrogates Hsp70 interaction (Gao and Newton, 2002), thus making this phosphorylation an ideal marker for the active-conformation PKCι pool that cannot interact with Hsp70. To our knowledge, no attempts have been made to identify the specific cytoskeletal proteins that bind PKCι in the...
inactive conformation. However, various chaperones, including Hsp70 involved in PKC rescue, are bound to keratin intermediate filaments (IFs) (Liao et al., 1995; van den Ijssel et al., 1999; Planko et al., 2007). IFs are formed by family of cytoskeletal proteins, encoded by approximately 60 different genes in mammals. Mutations identified in IF proteins responsible for human disease outnumber the pathogenic mutations in both actins and tubulins. Mutations in IFs do not abrogate filament polymerization or structural integrity (Omary et al., 2004), and, therefore it is unlikely that the phenotype results from mechanical weakness of the cells. An emerging body of evidence suggests that IFs have non-mechanical functions (Oriolo et al., 2007a), including antipapoptotic activity (Marceau et al., 2007) related to intracellular signaling, but mechanistic explanations for those functions are still missing (Magin et al., 2007; Toivola et al., 2005).

In addition, we reported a broad loss of the apical domain phenotype in the villus enterocytes of keratin 8 (K8)-null mice. Yet, a molecular model for that phenotype has been elusive (Ameen et al., 2001). Together, these facts led us to hypothesize that a pool of PKC ι might be associated with a keratin scaffold in epithelial cells. The results not only corroborated the existence of a pool of keratin-bound PKC ι, but also showed a novel function of the cytoskeleton in the post-translational regulation of PKC ι expression and localization.

**Results**

A functional pool of PKC ι co-distributes and co-purifies with the keratin cytoskeleton

The CACO-2 (human colon carcinoma) cell line is an extensively used model of intestinal epithelium, slowly polarizing after 5 days in culture (Pinto et al., 1983). Like in other epithelial cells (Suzuki and Ohno, 2006), XZ confocal sections showed PKC ι distributed only in the apical domain of CACO-2 cells, colocalizing with PAR3 only in the apical-most part of the cell-cell contacts (Fig. 1A,B). In the rest of the apical region, away from tight junctions, it colocalized with PAR6 (not shown), along with keratin IFs (Fig. 1B,C), but not with PAR3. Triton-100-insoluble PKC ι (Fig. 1C, green) colocalized with the apical keratin signal (Fig. 1C, red). PAR3 was not detected in the Triton X-100 extracted monolayers (not shown). To characterize the cytoskeletal association of PKC ι and its partners, we used an extraction method modified from a well-established method to isolate keratins that dissects actin. The Triton X-100 soluble supernatant (S1) contains cytosolic and membrane proteins. The Triton-insoluble (cytoskeletal) pellet was further extracted in 1.5 M KCl, and separated into supernatant (S2) and pellet (P). Together, the three fractions contained the entire set of cellular proteins. Actin was present in S1 and S2, but not in P (Fig. 1D). Keratins were found almost exclusively in P (Fig. 1D). PKC ι and pT555 PKC ι were present in all three fractions. It was surprising to find pT555 PKC ι in the P fraction because previous publications indicated that only the non-phosphorylated inactive forms of PKC bind to the cytoskeleton (Gao et al., 2008). Well-known PKC ι partners and components of the conventional tight-junction scaffold that binds PKC ι, including PAR3, PAR6, 14-3-3 (PAR5 homolog), ZO-1 and PALS1 (Suzuki and Ohno, 2006), were not present in the keratin pellet P, but were found in S1 or S2. Hsp70 proteins, however, were present in all three fractions (Fig. 1D). Protein partitioning in lipid rafts represents an exception to the Triton insolubility criterion of cytoskeletal association. We ruled out raft association of the Triton-insoluble PKC ι by lack of co-purification with the ganglioside GM1, found only in...
PKCι in the intermediate filament fraction is capable of substrate phosphorylation. CACO-2 cells were grown to confluency in 75-mm plates for 10 days, serum starved for 24 hours, and subjected to the three-fraction (S1, S2 and P) preparation described in Fig. 1D. PKC activity was measured using a PKC kinase assay kit. Samples of 20 μg protein were diluted into the kinase assay dilution buffer and loaded on 96-well plates coated with a PKC substrate peptide.

(A) Inhibitors were added to the appropriate wells in the following concentrations: 1 μM PKA inhibitor H-89, 1 μM GF10923X (at this concentration inhibiting only conventional and novel PKCs), 1 μM RO-31-8220 (inhibits more effectively atypical PKCs). Relative kinase activity was normalized per μg of protein. The data are means ± s.d. from three independent experiments (Student’s t-test significance, *P<0.001). (B) A similar assay was performed from the three fractions obtained from cells expressing a specific anti-PKCι shRNA or infected with empty vector lentiviral particles (ctrl). After 10 days in culture and puromycin selection, the cells were fractionated in S1, S2 and P as described in Fig. 1D, and relative kinase activity was measured in each fraction as described above. The data are means ± s.d. from three independent experiments (t-test significance, **P<0.001). (C) In parallel experiments, S1, S2 and P fractions from cells expressing anti-PKCι shRNA and controls were analyzed by immunoblot. Ponceau-S staining of the same membrane is shown as loading control.

Table 1. Distribution (%) of PKCι protein and enzymatic activity in CACO-2 cell fractions

|                | S1     | S2     | P       |
|----------------|--------|--------|---------|
| Cellular protein* | 75±4.3 | 18±3.3 | 7±1.5   |
| PKCι/μg of total protein¹ | 79±7.3 | 15±4.4 | 5±1.2   |
| pT555-PKCι/μg of total proteinφ | 83±6.5 | 13±2.0 | 3±0.8   |
| PKC activity¹ | 83±5.2 | 13±2.1 | 4±1.4   |
| PKC activity abrogated by anti-PKCι shRNAφ | 16±2.0 | 33±2.3 | 93±3.4  |

Data are taken from the experiments shown in Figs 1 and 2.

*Percentage of total cellular protein was obtained by Lowry measurements used to normalize protein load in each experiment.

¹Percentage of total PKC and pT555-PKCι were measured by densitometry on immunoblots using the anti-PKCι (total protein) or anti-pT555-PKCι antibodies, respectively. For all measurements, non-saturated images were used.

φPercentage of total PKC activity in the cell was calculated from the data in Fig. 2A.

§Percentage of total PKC activity in cells expressing shRNA was calculated from the data in Fig. 2B. The data are means ± s.d. from at least three independent experiments.
to the results from overlay assays, we did not detect any pT555 signal in the PKCζ bound directly to intermediate filaments or in the additional PKCζ bound to the filaments in the presence of Hsp70 (Fig. 3D, pull-down, pT555), albeit the input did contain pT555 PKCζ. This result indicates that only the pT555-dephosphorylated form of PKCζ interacts with Hsp70 in a ternary complex with IF. This is consistent with the binding of another atypical isoform, PKCζ, to Hsp70 that occurs only when the turn motif is dephosphorylated (Gao and Newton, 2002). Keratins and Hsp70 post-translationally regulate steady state levels of PKCζ Hsp70 and Hsc70 are products of two different genes that display 86% identity at the amino acid level. Hsc70 is constitutively expressed whereas Hsp70 is expressed only under stress in normal tissues (Daugaard et al., 2007) such as intestinal epithelium (Baumler et al., 2007). However, in some tissue culture cells, including CACO-2 cells, both Hsp70 and Hsc70 are constitutively expressed (Hirasaka et al., 2008; Broquet et al., 2003), albeit Hsp70 expression can be further upregulated by stress (Pierzchalski et al., 2008). Importantly, whereas it is unclear to what extent the functions of both proteins are redundant, Hsp70 and Hsc70 share several clients and a substantial level of functional overlap has been demonstrated (Singh et al., 2008; Freeman and Morimoto, 1996; Haag Breese et al., 2006).

To understand the possible biological implications of a PKCζ-Hsp70 interaction based on filamentous keratins, we started by knocking down IFs using lentiviral-mediated expression of anti-K8 shRNA. This is possible because differentiated CACO-2 cells express only one type-II keratin (K8; see two-dimensional gel analysis in supplementary material Fig. S2A), which is necessary to form the obligate heterodimers of a type-II and a type-I keratin. As in previous similar experiments (Wald et al., 2008), the knockdown was efficient, although some cells still expressed filaments (Fig. 4A, dark blue). Cells in which IF knockdown was very effective showed no PKCζ, except for some remnant signal in the tight-junction region (Fig. 4A, green). Both Hsp70 and Hsc70 were modestly decreased and delocalized (Fig. 4A, red). Immunoblot analysis of total extracts confirmed the K8 knockdown, and showed a modest reduction in Hsp and/or Hsc70, an almost 70% reduction in total PKCζ, and extensive loss (>90%) of the pT555 PKCζ signal (Fig. 4B,C). After testing several sequences, we have found only one shRNA sequence that effectively knocks down K8 in CACO-2 cells. The lack of a second independent shRNA sequence carried a small risk of observing ‘off-target’ effects. However, having observed a similar decrease of PKCζ in K8-null mouse enterocytes (see Fig. 7L), we can reasonably rule out that possibility for the PKCζ readout. The reduction in total PKCζ was clearly much larger than the size of the PKCζ pool that co-purifies with IFs (Table 1), suggesting that the interaction of PKCζ with IF-Hsp70 is necessary to maintain a population of PKCζ molecules that is normally not attached to IFs.

To determine whether changes in PKCζ steady state levels were transcriptionally regulated, we measured PKCζ mRNA levels by qPCR. Triplicate determinations (normalized to GAPDH) yielded 1.25±0.01 (control) and 1.28±0.02 (K8 knockdown), showing that K8 knockdown did not significantly affect steady-state levels of PKCζ mRNA. By contrast, incubation of CACO-2 cells with the proteasome inhibitor MG-132 for only 6 hours resulted in a threefold increase in the steady-state amount of PKCζ (along with a tenfold increase in total protein ubiquitinylation) (supplementary material Fig. S3), indicating that, like in other cells (Gao and Newton, 2006), PKC turnover is very high. Along with the lack of transcriptional effects shown before, this result highlighted the
Rescue of aPKC and the cytoskeleton

Because of the protective role of Hsp70 in PKC regulation, it was important to test the prediction that Hsp70-mediated rescue is involved in the maintenance of normal steady-state levels of PKCι. In CACO-2 cells, two members of the Hsp70 family, Hsp70 and Hsc70, are expressed in similar amounts because Hsp70 expression is leaky (Broquet et al., 2003; Liu et al., 2003). Accordingly, two different shRNA sequences were designed to knockdown Hsc70 (shRNA1) or Hsp70 (shRNA2) and were delivered by lentiviral transduction followed by puromycin selection. Both shRNAs knocked down Hsp70 and Hsc70 (Fig. 5A, possibly because of the very high level of sequence homology between the two mRNAs (>90%). Both shRNAs had no effect on other proteins, such as actin or K8, which were used as load controls (Fig. 5A). However, there was a 60-70% decrease in total PKCι levels. More importantly, knockdown of Hsp and/or Hsc70 proteins with both shRNAs abrogated pT555 PKCι signal (Fig. 5A,B). Because knockdown of Hsp70 proteins results in apoptosis (Aghdassi et al., 2007), these experiments were performed within 48 hours of the lentiviral infection. Within this time window there was no increase in annexin V binding, although longer times of Hsp70 knockdown resulted in massive cell death (not shown). Additional internal controls for lack of caspase-3 activation and cleavage were also performed (Fig. 5C). Similar Hsc and/or Hsp70 knockdown experiments were analyzed by immunofluorescence and confocal microscopy. They confirmed that the knockdown of Hsp and/or Hsc70 proteins was extensive in the majority of the cells (Fig. 5D,E), and indicated that Hsp70 knockdown did not affect the expression or localization of IFs (Fig. 5F,G, also see Fig. 5A), but abrogated the pT555 PKCι signal (Fig. 5H,I).

Hsp70 is involved in facilitating the correct conformation of many proteins, so it is likely that several client kinases might be affected. Likewise, the effect of K8 knockdown could be a general phenomenon affecting several kinases. To test this idea we run a screen of 12 different kinases in a commercial facility that uses a panel of validated, normalized antibodies for immunoblot. The

Fig. 4. Intermediate filaments are necessary for the apical expression of PKCι and Hsp70 in CACO-2 cells. (A) CACO-2 cells were cultured on Transwell filters. At 4 days after seeding, the cells were transduced with lentiviral particles carrying shRNA against K8 (K8 shRNA) or empty vector particles (control). After 10 days in culture and puromycin selection, the cells were fixed and processed with anti-K8 antibody (blue channel), anti-PKC antibody (green channel), anti-Hsp70 (total, Hsp70 and/or Hsc70) antibody (red channel) and DAPI (light blue), and analyzed by confocal microscopy. XZ sections are shown with the apical side up. DAPI staining is shown in light blue. Scale bars: 10 μm. (B) CACO-2 cells were transduced as described above, but total SDS extracts were analyzed by immunoblot. Mr of standards is ×10^3. (C) The reduction in band intensity was obtained from the ratios of band intensity in knockdown and control cells, normalized by the intensity of the actin band re-probed in the same lane. For all measurements, non-saturated images were used. The means ± s.d. from three independent experiments are shown.

Fig. 5. Hsp70 and/or Hsc70 are necessary to maintain a pool of pT555 PKCι in steady state in CACO-2 cells. (A) Downregulation of Hsp70 by shRNA leads to the decrease in the amount of pT555 and total PKC protein in CACO-2 cells. At 7 days after seeding, cells were infected with two sets of lentiviral particles carrying two different shRNA constructs against Hsp70 (shRNA1, shRNA2). Two days after infection, cells were extracted and analyzed by immunoblot. Actin is shown as a loading control. (B) The reduction of each band was calculated as in Fig. 4C. (C) Apoptosis in Hsp70 shRNA infected cells was controlled using caspase-3 cleavage. A positive control of apoptosis was performed by incubating CACO-2 cells in 30 mM H2O2 for 2 hours (arrowhead, cleaved caspase 3). Mr of standards is ×10^3. (D-I) CACO-2 cells were grown on Transwells and infected with lentiviral particles expressing shRNA1 as described above (E,G,I) or empty vector particles (D,F,H). The cells were probed by immunofluorescence using anti-Hsc70 antibody (green channel, D,E), anti-pT555 PKC antibody (red channel, H,I), and anti-K8 antibody (K8, purple channel, F,G). DAPI, light blue. Scale bar: 10 μm.
Table 2. Effect of K8 knockdown on steady-state levels of twelve different kinases in CACO-2 cells

| Kinase Probes | Control | K8 shRNA | Decrease (%) | Confirmed Hsp70 client† |
|--------------|---------|-----------|--------------|-------------------------|
| PKCα (atypical) | Total | 1029 | 295 | 69 | This work |
| Chk-1 | Total | 372 | 240 | 36 | Yes (Arlander et al., 2006) |
| Raf-1 | pS259 | 173 | 175 | 0 | Ind (Nollen et al., 2002) |
| PKCα | pS657 | 212 | 213 | 0 | |
| PKCδ | pT641 | 50 | ND | 100 | Yes (Gao and Newton, 2002) |
| PKCε | pS645 | 117 | 82 | 30 | |
| PKCζ | pT629 | 36 | ND | 100 | |
| PKCζ (atypical) | pT655 | 81 | ND | 100 | |
| PKCζ (atypical) | pT410 | 131 | ND | 100 | Yes (Gao and Newton, 2002) |
| PKA, catalytic β | pS338 | 1604 | 1240 | –17* | Yes (Gao and Newton, 2002) |
| PKA, catalytic α | pT197 | 223 | 404 | –81* | |
| PKBα (Akt) | pT308 | 0.0 | ND | 100 | Yes (Gao and Newton, 2002; Doong et al., 2003) |
| PDK-1 | Total | 152 | 139 | 8 | |

Identical amounts of protein from total SDS extracts from 1.5×10^5 CACO-2 cells infected with empty lentiviral particles (Control) or particles expressing anti-K8 shRNA and selected in puromycin were analyzed in a commercial facility (Kinexus Kinetworks KCPS 1.0, Vancouver, Canada) by immunoblot using a panel of validated standardized antibodies. The antibody mixtures were validated by the provider to prevent cross-reactivity among target proteins. Normalization was based on the intensity of fluorescence detection of immunoreactive standard proteins (Pelech, 2004). All the normalization and statistics were done by Kinexus. The values of normalized units are, therefore, comparable throughout the assay. Probe indicates the antibody used, either against total protein or a phospho-epitope in a consensus regulatory domain. Chk-1, Checkpoint kinase 1; ND, not detected.

*– signifies an increase.
†Yes, confirmed direct clients; Ind, kinases known to indirectly depend on Hsp70.

pattern of relative kinase expression and activation in control cells (Table 2) was identical to those published before from various pools of cell extracts (see table 1, 8 days, in Wald et al. (Wald et al., 2008)), showing the consistency of the method from one set of experiments to another analyzed at the same facility. Comparing extracts from CACO-2 cell cultures transduced with either anti-K8 shRNA and selected in puromycin were analyzed in a commercial facility (Kinexus Kinetworks KCPS 1.0, Vancouver, Canada) by immunoblot using a panel of validated standardized antibodies. The antibody mixtures were validated by the provider to prevent cross-reactivity among target proteins. Normalization was based on the intensity of fluorescence detection of immunoreactive standard proteins (Pelech, 2004). All the normalization and statistics were done by Kinexus. The values of normalized units are, therefore, comparable throughout the assay. Probe indicates the antibody used, either against total protein or a phospho-epitope in a consensus regulatory domain. Chk-1, Checkpoint kinase 1; ND, not detected.

*– signifies an increase.
†Yes, confirmed direct clients; Ind, kinases known to indirectly depend on Hsp70.

Table 3. Effect of K8 knockdown on steady-state levels of PKCζ and Akt in isolated villous enterocytes (FVB/n mice)

| Kinase Probes | Control | K8-null | Decrease (%) | Significance |
|--------------|---------|---------|--------------|--------------|
| PKCζ (atypical) | pT410 | 0.4±0.1 | 0.06±0.12 | 85 | P<0.01 |
| PKBα (Akt) | pT308 | 2.4±1.6 | 0.27±0.33 | 89 | P<0.05 |

Cells were isolated from 15-cm loops including the duodenum and part of jejunum from three control and four knockout animals as described in Materials and Methods and subjected to SDS-PAGE and immunoblot. The band intensity was measured and normalized to the corresponding actin band reprobed in the same lane as described in Fig. 4C (relative units). Control animals were were K8+/– littermates.

differences shown by the kinase screen in CACO-2 cells were reproducible in the animal model and statistically significant for the other atypical PKC isoform PKCζ (pT1410) and for pT308 Aktα (Table 3), two known Hsp70 clients (Gao and Newton, 2002).

To confirm our results in vivo, we used two mouse knockout models. The K19-null, K18+/– and hK18 R89C (knock-in) mice (K18R89C animals) have been shown to lack IFs in the intestinal crypts, where keratins form small, non-filamentous aggregates (Hesse et al., 2007). In normal littermates of K18R89C mice, the small intestinal crypts showed apical layers of Hsc70 and pT555 PKCζ (Fig. 6A,G). By contrast, in K18R89C transgenic mice the apical localization of Hsc70 and pT555 PKCζ was abrogated along with the expression of IFs (Fig. 6C,D,I,J), with the signals close to those in antibody negative controls (Fig. 6E,F,K,L). In the same animals, the expression and distribution of Hsc70 and pT555 PKCζ were normal in the villi (not shown). The second knockout model we used, K8-null mice, lack intermediate filaments in the villus, but display normal filaments in the crypts, because of the expression of redundant K7 (Baribault et al., 1994; Ameen et al., 2001). Accordingly, in K8-null mice the apical layer of Hsc70 was not observable in villous enterocytes (Fig. 7C). The pT555 PKCζ signal in villus enterocytes from control littermates was present only in the tight-junctional compartment (Fig. 7G, arrows). In K8-null mice, the IFs disappear after enterocytes move over the crypt-villus boundary (Ameen et al., 2001; Wald et al., 2008). Unlike in control...
animals, K8-null mice displayed the pT555 PKCι signal associated with tight junctions only until the mid-villus region (Fig. 7I, arrows), not reaching the tip of the villus (Fig. 7I, arrows) as in controls. This suggests that even the tight-junction pool of PKCι needs IFs to subsist.

To validate these observations at the biochemical level, villus enterocytes were isolated from the mucosa of control and K8-null mice and analyzed by immunoblot. The intensity of Hsc70 signal was very variable among all the animals, but it was not significantly decreased in K8-null mice. The average actually showed a modest, non-significant increase (negative values, Fig. 7L). This effect was different from CACO-2 cells under K8 knockdown (Fig. 4) and might indicate some degree of cellular stress in the epithelium in some animals. Conversely, pT555 signal was greatly and significantly decreased in all knockout animals as expected (Fig. 7K,L, where n=3 and n=4, respectively). Because

the best-known function of atypical PKC is to maintain the structure and localization of tight junctions (Hurd et al., 2003) and paracellular permeability (Hirose et al., 2002), we hypothesized that a decrease in pT555 PKCι might affect tight junctions in the upper part of villi. We did not observe any morphological changes by electron microscopy (not shown). However, transepithelial uptake of fluorescent Dextran 3000, a bona fide probe for paracellular permeability, was significantly increased in small intestine loops with their normal blood supply (Fig. 7M). No evidence of inflammation was observed microscopically, or by myeloperoxidase assay of the intestinal mucosa in the same animals (control, 0.124±0.05 OD units/50 μg protein; K8-null, 0.05±0.08 OD units/50 μg protein). Therefore, the decrease in PKCι correlates with a modest but significant increase in permeability of the tight junction in the small intestine, as predicted by previous studies on PKCι functions.

Hsp70 and filamentous keratins are essential for the rephosphorylation of the turn motif in mature PKCι. The results described above indicate that Hsp70 and the keratin cytoskeleton are necessary to maintain the steady-state levels of PKCι but do not distinguish between direct participation of those molecules in the rescue mechanism and indirect effects mediated by other molecules, such as kinases. To independently corroborate the role of Hsp70 and the cytoskeleton in the maintenance of PKCι levels we designed an in-vitro rescue of PKCι turn motif phosphorylation assay, using different cellular fractions. Because PKCι lacks the phorbol-ester binding domain, it was impossible to dephosphorylate PKCι by phorbol-ester overstimulation, as with conventional PKCs. Therefore, we incubated S1, S2 and P fractions of CACO-2 cells (described in Figs 1 and 2) in the presence of ATP and an excess of the consensus PKCι substrate peptide. The experiments were performed in the presence of proteases and proteasome inhibitor cocktails, but in the absence of phosphatase inhibitors.

After 5-hour incubations with the PKC substrate peptide, we succeeded in substantially decreasing the pT555 PKCι signal in all three fractions, without noticeable changes in the PKCι protein levels (Fig. 8A for S1, not shown for S2 and P; the fractions containing dephosphorylated PKCι are hereafter referred to as S1* and P*). Then, the peptide was removed by ultrafiltration, and ATP was replenished. Separately, none of the fractions were able to rephosphorylate PKCι (S1*+ATP, Fig. 8A; P*+ATP, Fig. 8B). Mixing S2 with either S1 or P in the presence of ATP also failed to achieve any PKCι rephosphorylation (not shown). However, when fractions S1* and P* were mixed together in the presence of ATP, pT555 signal was restored, in most cases to levels similar to those at the beginning of the experiment (S1*+P*+ATP, Fig. 8C).

Because the P fraction is enriched in keratins but still contains keratin-associated proteins, we also used a highly purified preparation of keratins to supplement S1*. When analyzed by two-dimensional gels and silver stain, these keratins were found to be >99.9% pure [no contaminants were detected within the sensitivity of silver stain; supplementary material Fig. S2A (Oakley et al., 1980)]. Such a preparation was found to be as equally active as the P fraction in supplementing S1* for PKCι rephosphorylation (S1*+IF+ATP, Fig. 8D). By contrast, single isolated non-filamentous K8, K18, or K19 failed as supplements for S1* to induce PKCι rephosphorylation (Fig. 8E). The specificity of Hsp70 and/or Hsc70 for the PKCι rephosphorylation was tested by immunodepleting S1* with three anti-Hsp70 and anti-Hsc70 antibodies. As expected,

![Fig. 6. Hsc70 and PKCι distribution depend on the integrity of intermediate filaments in small intestine crypts of K18R89C mice. Small intestines from K18<sup>−/−</sup>, K19<sup>−/−</sup>, hK18 R89C knockout/knock-in mice (C,D,LJ) (ko) or control littermates (A,B,E,H,K,L) (wt and control) were fixed in 10% TCA and frozen. Frozen sections were processed with anti-Hsc70 antibody (A-D, green) or anti-pT555 PKCι antibody (G-J, green), and anti-K8 antibody (keratin, red), and counter-stained with DAPI (light blue). Notice that intestinal epithelium does not express Hsp70 when not stressed. Control sections were stained with non-immune IgG at the same dilutions (E,F,K,L). The images are single confocal optical sections. Scale bars: 10 μm.](image-url)
after immunodepletion of Hsp70 and/or Hsc70, S1* supplemented with purified filamentous keratins also failed to rephosphorylate PKCι (Hsp70-depleted, S1*+IF+ATP, Fig. 8F). However, if the same immunodepleted S1* fraction was supplemented with recombinant purified Hsp70 (single-band by silver stain, supplementary material Fig. S2B), it regained its ability to rephosphorylate pT555 PKCι (Fig. 8G).

Finally, we wanted to check whether Hsp70 that is bound to IF in the pellet fraction (P) of CACO-2 cells is functionally competent to enable PKCι rephosphorylation. To that end, we added the intermediate filament pellet P* fraction to the PKCι-dephosphorylated Hsp70-immunodepleted S1* fraction (Fig. 8H). Indeed, we found that addition of the P* fraction and ATP (+P+ATP, Fig. 8H) resulted in PKCι rephosphorylation, whereas purified IFs, lacking Hsp70, did not rescue pT555 signal (+IF+ATP, Fig. 8H) in the presence of Hsp70-immunodepleted S1* (Fig. 8H). We conclude that Hsp70 and/or Hsc70 and filamentous keratins, present together in an insoluble cytoskeletal pellet compatible with the complexes shown in vitro in Fig. 3, are equally necessary to rescue dephosphorylated PKCι in the inactive conformation and enable rephosphorylation of the turn motif.

Overexpression of keratin filaments affects the subcellular distribution of PKCι

Bearing in mind that there is an excess of soluble Hsp70 in intestinal cells (Fig. 1), and that filamentous keratins are essential for the rescue of PKCι phosphorylation (Fig. 8), we hypothesized that overexpression of keratin filaments might affect the distribution of PKCι within an epithelial cell, because overexpressed keratins result in redistributed and mislocalized excessive IFs (Wald et al., 2005). The rationale was that the assembly of the ternary complex (PKCι, Hsp70, filamentous keratins) might be the rate-limiting step for PKCι rescue, considering that the keratin-Hsp70 compartment is much smaller than the cytosolic compartment of soluble Hsp70 (Table 1). The prediction was that additional IFs would increase the ability of the cell to rescue more PKCι. Furthermore, the additional newly refolded and rephosphorylated PKCι would be expected to interact with nearby membranes through its C-terminal domain and become mislocalized. To test these predictions of the model, mild K8 overexpressors (approximately twofold increase in K8 protein) were used first. Unlike ‘severe’ HK8-4 animals with an intestinal phenotype (approximately fourfold increase in K8) (Casanova et al., 1999; Wald et al., 2005), these animals display only a chronic pancreatic phenotype (Toivola et al., 2008). The typical distributions of pT555 PKCι (Fig. 9B, arrows) and Hsc70 (Fig. 9J) were not
changed by K8 overexpression, but, for both of them, a signal above background was visualized in the cytoplasm, especially in regions where abundant supernumerary IFs were observed (arrows, Fig. 9E-F; Fig. 9M-N). Second, we also analyzed the distribution of Hsc70 and pT555 antibodies in the presence of the substrate peptide was unaffected by either Hsp70 immunodepletion or incubation with the normal rabbit serum (S1*). However, reduction of Hsp70 protein in S1 fraction resulted in the abrogation of mature PKC rephosphorylation in the presence of the substrate peptide was unaffected by either Hsp70 immunodepletion or incubation with the normal rabbit serum (S1*). However, reduction of Hsp70 protein in S1 fraction resulted in the abrogation of mature PKC rephosphorylation in the presence of the substrate peptide.

(F-H) PKC rephosphorylation in the presence of purified IFs is Hsp70-dependent. (F) Immunodepletion by incubation with anti-Hsc70 and/or anti-Hsp70 antibodies resulted in substantial decrease in the amount of Hsp70 protein in S1 fraction (Hsp70-depleted). Incubation of S1 fraction with normal rabbit serum served as a control (control). The activity-dependent phosphorylation of PKC in the presence of the substrate peptide was unaffected by either Hsp70 immunodepletion or incubation with the normal rabbit serum (S1*).

50 μg of protein in the equally dephosphorylated pellet (P*) as in D, or with the recombinant K8 (S1*+K8+ATP), K18 (S1*+K18+ATP) or K19 (S1*+K19+ATP) in the presence of 1 mM ATP for 4 hours. (F-H) PKC rephosphorylation in the presence of purified IFs is Hsp70-dependent. (F) Immunodepletion by incubation with anti-Hsc70 and/or anti-Hsp70 antibodies resulted in substantial decrease in the amount of Hsp70 protein in S1 fraction (Hsp70-depleted). Incubation of S1 fraction with normal rabbit serum served as a control (control). The activity-dependent phosphorylation of PKC in the presence of the substrate peptide was unaffected by either Hsp70 immunodepletion or incubation with the normal rabbit serum (S1*).

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Discussion

This work highlights the importance of chaperone-mediated rescue in maintaining functional levels of atypical PKC and also indicates that filamentous keratins are essential for that function. Because overexpression of active PKC is causative of neoplasia, understanding PKC rescue might be important for interventions in abnormal cell growth. From a functional perspective, the apical polarity phenotype of keratin knockdown (Salas et al., 1997) or knockout (Ameen et al., 2001; Toivola et al., 2004; Satoh et al., 1999) has been documented, and includes loss or mistargeting of apical membrane proteins, depolarization of γ-tubulin, and disorganization of microtubules. It is conceivable that some of these anomalies might be due to the steep decrease in active PKC activity.

In agreement with this notion, here we report an increase in paracellular permeability in the intestine (Fig. 7M), as expected from the functions of aPKC on tight junction assembly and integrity reported by others (Hirose et al., 2002).

The data in this work, along with previous publications (Satohisa et al., 2005), support a model of two different apical PKC pools, one associated with PAR3 at the tight-junction, and the other located in the apical membrane, away from junctions (Fig. 10A). In vivo, the latter is observed in mouse intestinal crypts, which CACO-2 cells mimic well, whereas the former is observed in the more differentiated villus enterocytes. Upon continuous activity, PKC from both pools becomes dephosphorylated and acquires an inactive conformation (Fig. 10B). This continuous and rapid turnover was
PKC is conserved in PKC

The reconstitution experiments enabled us to independently conclude that there is a direct role for Hsp70 and filamentous keratin in PKC1 conformational rescue behind the phenotype of keratin knockdown and knockouts. However, there remains the possibility that the keratin knockdown effect might be mediated by the observed decrease in steady-state Hsp70 levels. This possibility seems to be dispelled by the high levels of Hsc70 in K8-null enterocytes. Conversely, the Hsp70 knockdown did not affect the steady-state amount of keratins. Together, these data allow us to conclude that filamentous keratins and Hsp70 are independent but concurrent requirements for the first step of the PKC rescue mechanism, acting in a complex attached to the cytoskeleton.

The apical distribution of keratin IF in intestinal cells is conserved from worms to vertebrates (Oriolo et al., 2007a). From the results in Fig. 8, it seems safe to conclude that the rescue of PKC1 in the IF pellet (Figs 1, 2) suggests that the release step may produce after rephosphorylation. In the absence of a positive identification of the kinase responsible for rephosphorylating the turn motif, we are currently unable to analyze the steps that follow the interaction of PKC1 with Hsp70 and/or Hsc70. However, it was surprising that PKC1 in the IF pellet can be functional in the absence of all its known PAR partners and membranes, just associated with the keratin cytoskeleton. Whereas the size of PKC1 cytoskeletal compartment is modest (Table 1), the presence of an active PKC1 pool on the cytoskeleton, away from the plasma membrane, might have important implications for other functions (e.g. membrane traffic) involving PKC1-dependent phosphorylations within 1 μm or less from the plasma membrane.
Materials and Methods

Vectors and reagents

The antibodies used were as follows: mouse monoclonal anti-PKCι (BD Biosciences); rabbit anti-PKCι (Santa Cruz Biotechnology); rabbit anti-phospho-T555 (pT555) PKCι (Biosource Invitrogen); rabbit anti-phospho turn motif aPKC (Epitomics); PKCζ (atypical) pT410 and PKB (Akt) pT308 (Cell Signaling); mouse anti-V5 (Invitrogen); mouse anti-K8 (Biomeda); anti-pan-cytokeratin (Sigma); anti-K8 TROMA 1 (Hybridoma Bank); rabbit anti-Hsc70 (constitutive form of Hsp70; Stressgen Bioreagents); rabbit anti-Hsp70 (inducible form of Hsp70; Stressgen Bioreagents); rabbit anti-Hsp70 (against constitutive and inducible forms of Hsp70; Cell Signaling), rabbit polyclonal anti-caspase 3 (detects full length caspase-3 and its large cleavage fragment; Cell Signaling); mouse anti-actin (CAMP; Biomedicals); rabbit anti-ratERK (Abcam); rabbit anti-PAR3 (Upstate); mouse anti-par-14.3.3 (clone CG15; Lab Vision); rabbit anti-Pals1 (Upstate) and mouse anti-ZO1 (Zymed Laboratories). Affinity-purified secondary antibodies with no cross-reactivity were obtained from Jackson ImmunoResearch Laboratories. Recombinant purified K8, K18 and K19 were obtained from US Biological. Recombinant active Hsp70 was obtained from Stressgen. Recombinant PKCι was from Invitrogen. Protease inhibitor cocktail (Sigma, cat. no. P-8340) two phosphatase inhibitor cocktails (Calbiochem, cat. no. 524624 and 52625), and proteasome inhibitor MG-132 (Calbiochem, cat. no. 474790) were used.

Cell cultures, lentivirus production and infection

CACO-2 cells were obtained from American Type Culture Collection. The cells were cultured as described (Salas, 1999). HEK 293TN cells were obtained from System Biosciences as a part of the lentivirus packaging system. PKCι shRNA (5'-CCGGGCTGGAATACTGTTATGGGTTGAATGTCAATCCAGGCTTG-3') was obtained from Open Biosystems (cat. no. TRCN0000060375) in the pLKO.1 lentivirus vector. Anti-Hsc70 shRNA (5'-AGGGCTTCCAAAGGGTGTGTT-3') was obtained from Open Biosystems. Mission shRNA lentiviral particles carrying shRNA against human K8 (NM_002273) were from Sigma (5'-CCGGGCAAGATGCTCGAGTG-3') and from Open Biosystems. Recombinant PKCι and K19 were obtained from US Biological. Recombinant active Hsp70 was obtained from Stressgen. Recombinant purified K8, K18 were obtained from Jackson ImmunoResearch Laboratories. Affinity-purified secondary antibodies with no cross-reactivity were obtained from Stressgen. Recombinant active Hsp70 was obtained from Stressgen. Recombinant PKCι was from Invitrogen. Protease inhibitor cocktail (Sigma, cat. no. P-8340) two phosphatase inhibitor cocktails (Calbiochem, cat. no. 524624 and 52625), and proteasome inhibitor MG-132 (Calbiochem, cat. no. 474790) were used.

Caco-2 cell extraction and fractionation

The procedure was modified from Steinert et al. (Steinert et al., 1982). At 10 days after seeding, cells were washed in PBS and then extracted in PBS containing 1% Triton X-100, 2 mM EDTA (extraction buffer, EB) supplemented with cocktails of protease and phosphatase inhibitors (Calbiochem) at room temperature. After 15 seconds (three 5-second intervals) of sonication the cell extract was spun for 10 minutes at 16,000 g. This first supernatant was referred to as the S1 fraction. The pellet was resuspended in 1.5 M KCl, sonicated for 15 seconds (three intervals), incubated for 10 minutes on ice, and spun for 10 minutes at 16,000 g. The resulting supernatant was referred to as the S2 fraction, and the pellet was referred to as the P fraction. For functional assays, S1 was used directly, S2 desalted by ultrafiltration, and P resuspended as a coarse suspension by a 3-second sonication. For immunoblots, S1 and S2 were aceton-precipitated, washed in double-distilled H2O, and resuspended in sample buffer. In all cases protein was determined by Lowry assay.

PKC activity assay

PKC activity was measured using a PKC Kinase Non-Radioactive Assay Kit (Assay Designs, Stressgen, Ann Arbor, MI) as follows: CACO-2 cells were grown to confluency for 10 days in 75-mm plates, serum starved for 24 hours, washed twice with PBS and subjected to three fractions preparation (S1, S2 and P) as described above. About 18-20 μg of protein was diluted into the kinase assay dilution buffer (Stressgen) and loaded per well in 96-well plates coated with a PKC substrate peptide. Inhibitors were added to appropriate wells in the following concentrations: 1 μM PKA inhibitor H-89, 1 μM GF10923X (at this concentration inhibiting only conventional and novel PKCs), 1 μM Ro-31-8220 (inhibits atypical PKCs more effectively). The PKC assay was performed according to manufacturer’s specifications.

Non-denaturing PKCι purification, blot overlay, pull-down assays and immunoprecipitation

PKCι was transferred into HEK293TN cells, harvested, purified on Ni2+ columns under non-denaturing conditions and used in blot overlays as described (Wald et al., 2005). Pull-down experiments were performed as described elsewhere (Oriol et al., 2007b). Briefly, highly purified intermediate filaments from CACO-2 cells were covalently bound to CNBr-activated Sepharose beads (Amersham) according to manufacturer’s protocol (25 μg of keratin/10 mg beads per reaction). The beads were blocked with 1% casein, incubated with 2 μg/ml recombinant PKCι (Invitrogen) in the presence (2 μg/ml) or absence of recombinant Hsp70 (Stressgen) in PBS containing 1% Triton X-100 overnight at 4°C with gentle shaking and then extensively washed. Sepharose beads coupled to normal rabbit serum (Jackson ImmunoResearch Laboratories) served as a negative control. Immunoprecipitation of PKCι was performed as described before (Wald et al., 2005), except that the protein-A-coupled beads (Santa Cruz Biotechnology) were used instead of Sepharose beads. A rabbit...
polycional anti-PKCζ antibody (Santa Cruz Biotechnology) was used for immunoprecipitation.

Transgenic mice, analyses of intestinal epithelial, and immunofluorescence

The K8-null transgenic mice in the FVB/n background were originally obtained by Baribault and coworkers (Baribault et al., 1994), and the intestinal phenotype is described elsewhere (Ameen et al., 2001). Mice deficient in intermediate filaments in intestinal crypts [K18−/−, K19−/−, K18R89C (K18R89C animals) (in a mixed FVB/n x C57BL/6 x 129P2 genetic background)] and K8 overexpressors have been described (Hesse et al., 2007; Toivola et al., 2008; Casanova et al., 1999). Isolation of villus enterocytes was described by McNicholas et al. (McNicholas, 1994). For paracellular permeability assays, animals were deeply anesthetized, and a 1-cm loop comprising duodenum and the first part of the jejunum was cut, leaving the normal blood supply. A solution of 10 mM Texas-red-coupled Dextran 3000 in 1:1 H2O:PBS supplemented with 5 mg/ml glucose was instilled into the lumen and then the open ends of the loop were clamped. Blood was collected at time 0 from the tail, and by cardiac puncture at 50 minutes at the time of euthanizing the animal. Texas-red fluorescence was analyzed in 70-μm slices of samples in a spectrophotometer. Myeloperoxidase assay was performed on mucosa scraped from 2 cm of jejunum just distal to the Dextran-perfused loop, as described elsewhere (Bradley et al., 1982).

Procedures for immunofluorescence, frozen sectioning, and confocal microscopy have been described before (Ameen et al., 2001). Triton X-100 extraction before fixation is described elsewhere (Salas et al., 1988) and was performed with the following modification in the extraction buffer: PBS was supplemented with 0.5% Triton X-100, 1 mM MgCl2, 1 mM EGTA and the Sigma cocktail of antiproteases described above. Trichloroacetic acid (TCA) fixations were performed in 10% TCA in water for 10 minutes. Immunofluorescent labelling was performed in a Leica TCS A1 confocal microscope. Confocal stacks were normally collected at 0.4-μm intervals

Analysis of intermediate filament-dependent repolarization of PKCζ in the soluble fraction of Caco-2 cells

Caco-2 cells were grown and fractionated (S1, S2 and P) as described above, with the exception that the extraction buffer was not supplemented with phosphatase inhibitors. To induce the activity-dependent dephosphorylation of PKCζ, the S1, S2 and P fractions were incubated in the presence of 150 μM PKC-substrate peptide (Upstate, cat. no. 12-536) and 1 mM ATP at 30°C with gentle shaking for 5 hours. After treatment, the peptide was removed by ultrafiltration in Centricon YM-3 (Upstate, cat. no. 12-536) and 1 mM ATP at 30°C with gentle shaking for 5 hours. The disordered amino-terminus of SIMPL interacts with members of the 70-kDa heat-shock protein family. DNA Cell Biol. 25, 704-714.

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