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Functional analysis of keratin-associated proteins in intestinal epithelia: heat-shock protein chaperoning and kinase rescue

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Abstract

A growing body of evidence from several laboratories points at non-mechanical functions of keratin intermediate filaments (IF), such as control of apoptosis, modulation of signaling, or regulation of innate immunity, among others. While these functions are generally assigned to the ability of IF to scaffold other proteins, direct mechanistic causal relationships between filamentous keratins and the observed effects of keratin knockout or mutations are still missing. We have proposed that the scaffolding of chaperones such as Hsp70/40 may be key to understand some IF non-mechanical functions if unique features or specificity of the chaperoning activity in the IF scaffold can be demonstrated. The same criteria of uniqueness could be applied to other biochemical functions of the IF scaffold. Here we describe a subcellular fractionation technique, based on established methods of keratin purification. The resulting keratin-enriched fraction contains several proteins tightly associated with the IF scaffold, including Hsp70/40 chaperones. Being non-denaturing, this fractionation method enables direct testing of chaperoning and other enzymatic activities associated with IF, as well as supplementation experiments to determine the need for soluble (cytosolic) proteins. This method also permits to analyze inhibitory activity of cytosolic proteins at independently characterized physiological concentrations. When used as complementary approaches to knockout, knockdown, or site-directed mutagenesis, these techniques are expected to shed light on molecular mechanisms involved in the effects of IF loss of function.

1. INTRODUCTION

Chaperones are associated with the intermediate filaments

Intermediate filaments (IF) are components of the cytoskeleton present in most but not all metazoans with remarkable differences with respect to the better-known actin and tubulin cytoskeleton (Koster, Weitz, Goldman, Aebi, & Herrmann, 2015). There are 6 types of intermediate filament proteins. Except for lamins (type V), the rest is expressed in the cytoplasm in a tissue-specific manner (Eriksson et al., 2009). Here, we will focus on types I and II IF, also known as keratins (Krt), which are characteristic of epithelial tissues. Keratins have long been recognized for conferring mechanical properties to epithelia, which are critically important in the skin. However, for several years, many groups recognized that a

number of proteins unrelated to IF actually bind to keratin filaments. These observations were common and a consensus was reached accepting that the mechanically strong IF are scaffolds decorated by other proteins.

Chaperones were among the first proteins to be found attached to the IF scaffold. Omary and coworkers (Liao, Lowthert, Ghorri, & Omary, 1995) noted that Hsp70 forms a stable complex with keratins early during filament biogenesis. Quinlan (Quinlan, Carte, Sandilands, & Prescott, 1996) discovered the association of the small chaperone alpha-crystallin with filensin IF. Mrj, a member of the Hsp40 family, an essential co-chaperone of Hsp70, was found to bind the C-terminal region of Krt18 (Izawa et al., 2000).

Bearing in mind that the bulk of the chaperone protein is soluble, i.e. not associated with IF, it is natural to ask what is the function of chaperones attached to the IF scaffold. One possibility is that Hsp70/40 and other chaperones are necessary to maintain the structure and function of the IF themselves. Supporting this view, several investigators showed chaperone functions in keratin turnover (Watson, Geary-Joo, Hughes, & Cross, 2007), folding (Janig, Stumptner, Fuchsbichler, Denk, & Zatloukal, 2005), as well as associated with keratin aggregates in disease (Gu & Coulombe, 2005). Conversely, a growing body of evidence in keratin knockout mice and knockdown cell lines suggests that the other, non-excluding, alternative is also possible. Namely, that IF may be necessary for chaperoning non-IF proteins. In this regard, it is important to mention that the Krt8 KO mouse hepatocytes are approximately 100-fold more sensitive to TNF α -induced apoptosis than controls (Caulin, Ware, Magin, & Oshima, 2000). A similar observation was made in Krt18 KO mouse liver (Leifeld et al., 2009). Furthermore, Ser30 O-linked glycosylation of Krt18 protects against apoptosis and is necessary to maintain Akt levels (Ku, Toivola, Strnad, & Omary, 2010) in liver and pancreas. Intriguingly, Coulombe and coworkers (Lessard et al., 2013) uncovered a regulatory function of Krt16 on innate immunity and the role of Krt17 regulating protein synthesis (Kim, Wong, & Coulombe, 2006). Non-mechanical functions of keratin IF have been comprehensively reviewed (Homberg & Magin, 2014; Oriolo, Wald, Canessa, & Salas, 2007; Snider & Omary, 2014). A long list of interactions between keratins and several signaling molecules has been reviewed elsewhere (Pan, Hobbs, & Coulombe, 2013). Overall, however, the mechanistic explanations of how the IF scaffold affects unrelated molecules often physically distant from the IF, remain elusive.

There are different families of chaperones, which not only aid the correct folding or refolding of other proteins ("clients") but also facilitate multiprotein arrangements (Quinlan & Ellis, 2013). This review will focus on Hsp70 and Hsp40 chaperones attached to the IF scaffold. Because Hsp or Hsc70 and Hsp40 chaperones work together, control many cellular functions (Clerico, Tilitsky, Meng, & Gierasch, 2015; Saibil, 2013), and show anti-apoptotic properties (Wang, Chen, Zhou, & Zhang, 2014), just like keratins, it is natural to hypothesize that the Hsp70/40 scaffolding on IF may be responsible for some of the non-mechanical functions of keratin IF. Three mechanistic questions come to mind to test this hypothesis:

- Is Hsp70/40 associated with keratin IF active as a chaperone?

- If so, bearing in mind that the bulk of the Hsp70 and 40 chaperones is soluble, and not associated with IF, what fraction of chaperoning occurs on IF as compared with total Hsp70 chaperoning activity?
- If IF-associated Hsp70/40 is active, is there any substrate (“client”) specificity for the IF-bound chaperones? In other words, is it possible that a subset of Hsp70/40 clients can be folded only on IF, and not in the cytosol?

In the following sections, we will briefly summarize evidence supporting positive answers for all three questions in the case of PKC ϵ in intestinal epithelia. This work will focus on reductionist methods to separate the IF-Hsp70 scaffold from cytosolic Hsp70 aimed to compare the relative chaperoning activity and substrate selectivity in both compartments. Because IF and cytosol are in intimate contact in the cell, it is a given that all the proteins in the filaments, including keratins, must be in equilibrium with the cytosol counterparts. Also, because there is no known barrier between filaments and cytosol, one must assume that all the cytosolic components are freely available on the filaments. Therefore, any functional difference, such as substrate selectivity, must be due to the interaction with the keratins or other molecules in the keratin filament. Along with the methods, we will summarize proof of concept evidence supporting the notion of a chaperoning-active, substrate-specific IF scaffold.

2. ISOLATION OF KERATIN INTERMEDIATE FILAMENTS FOR FUNCTIONAL ASSAYS

The original procedure to isolate an IF fraction was developed by Goldman and coworkers (Steinert, Zackroff, Aynardi-Whitman, & Goldman, 1982). It consists of two parts. In the first one, the cells are extracted in non-ionic detergent, and the insoluble fraction is further extracted in 1.5M KCl. The second part involves successive steps of solubilization in urea, followed by dialysis and filament reassembly. When completed, it yields highly purified keratin filaments, devoid of detectable contaminants. We adapted the first part, which yields >90% enriched keratins, with a number of proteins that co-purify with keratins as minor components. These proteins can be easily detected in 2D gels stained with silver (e.g. see Fig. 1 in (Salas, 1999)). The method to obtain keratins along with IF-associated proteins from confluent, differentiated, cultured epithelial cells is as follows (Mashukova, Forteza, Wald, & Salas, 2012; Mashukova et al., 2009):

- a. After extensively washing the cells with saline buffer (e.g. PBS), they are extracted in PBS supplemented with 1% Triton X-100, 2 mM EDTA, 1 mM ATP, and separate protease and phosphatase inhibitor cocktails at the dilutions recommended by the manufacturer (antiproteases, Sigma P8340, antiphosphatases, Calbiochem 524624 and 524627). Typically, 2×10^6 cells are extracted in 0.5 ml of the extraction buffer. This extraction is performed at room temperature to facilitate solubilization of lipid rafts. Addition of ATP in the extraction buffer is important to dissociate actin-myosin complexes. If ATP is omitted, actin frequently contaminates the final IF fraction (kP). In some functional assays involving dephosphorylation (Section 4), the anti-phosphatase cocktail must be omitted.

- b.** Immediately sonicate the mix for 15 seconds on ice (3 x 5 second intervals with 5 second lapses).
- c.** Spin down at 16,000 g for 10 minutes at room temperature. The supernatant of this centrifugation is the soluble fraction (S), and contains cytosol, nucleoplasm, and membrane-associated proteins, including those from organelles.
- d.** The resulting pellet is resuspended in 1.5 M KCl, sonicated as described in b, and kept on ice for 10 minutes. The mix is then spun as described in c.
- e.** The pellet from the centrifugation in d is the desired IF fraction (kP), and the supernatant is enriched in actin and actin-binding proteins.

To process these 3 fractions for SDS-electrophoresis and ensuing immunoblot, the S fraction and the KCl supernatant from (d) are acetone-precipitated. The latter, originally from the pellet in (c) is, therefore, named aP (actin pellet, after the acetone step). Then, the resulting pellets, as well as kP are washed three times in distilled water. For the 2×10^6 cells, the protein pellet from S is solubilized in 0.5 ml, and aP or kP in 0.25 ml 1% SDS in loading buffer without bromophenol blue. Samples of these solutions are used to determine protein by Lowry assay. Then, the extracts are diluted in complete SDS sample buffer up to 1 mg/ml and run in SDS-electrophoresis (usually 40 μ g/lane, depending on gel size). A typical blot from these fractions is shown in Fig. 1, using Ponceau S staining for total protein (Fig. 1A) and immunoblot for chaperones, actin and Krt8 (Fig. 1B). It is important to note that, although the same amount of protein was loaded in the 3 lanes, to our knowledge there is no valid loading control possible. Accordingly, we use keratins, actin, and fully soluble proteins (e.g. GAPDH) to validate the extraction conditions, and to compare loading among the same fractions from different preparations or from different experimental conditions.

The presence of various chaperones, atypical protein kinase C (aPKC), actin-binding proteins and cytoskeletal proteins in these 3 fractions, as determined by immunoblot in previous publications, is shown in Table 1. It is important to highlight that all cellular proteins are included in one or more fractions, as the procedure does not discard any fraction. Also, it is important to consider that the S fraction represents the bulk of the cellular protein (75%) while the kP fraction represents less than 10% of the total cellular protein. Therefore, functions associated with IF, such as chaperoning, must display some different or unique feature as compared to the cytosol to be quantitatively relevant at the cellular level.

The results in Table 1 apply specifically for Caco-2 (human intestinal epithelial) cells. In other cell types or for different IF proteins the results may vary. For example, Hsp27 preferentially binds to Glial Fibrillary Acidic Protein (GFAP) and not keratin (Perng et al., 1999). Accordingly, we have found it in the S fraction in intestinal cells (Fig. 1).

For functional assays in the following sections, processing of the fractions should be different. To maintain non-denaturing conditions and change the buffers to those used in specific biochemical reactions, S and aP, the extract from the KCl extraction, need to be desalted. We use centrifugal ultrafiltration devices with 3,000 kD cutoff (e.g. Centricon Ultracel YM-3). We replace the extraction buffer by the buffer desired for a functional assay

by 2-volume washes (2 consecutive protein concentrations followed by resuspension in the buffer for a functional assay) of the retenates from S and aP. This step is used also for protein concentration or dilution to adjust identical protein concentrations in all the fractions. In addition, this step enables to remove ATP from S fraction which is necessary for negative controls. The kP fraction which is a pellet, is washed twice in the desired buffer and finally resuspended in the same by a 2-second sonication step.

There are two caveats that need to be considered. First, the extraction procedures yielding the kP are stringent, and involve at least 1 hour of manipulation of the keratins. Therefore, it is conceivable that keratin associated proteins with a weak interaction, or a relatively fast dissociation may be solubilized in the process and appear either in S or aP. Longer manipulations of the keratins must be avoided as the keratin monomers slowly dissociate from the pellets, resulting in a slow loss of filamentous material if several changes of solution are performed over a period of hours. Second, conversely, this is a co-purification of highly insoluble protein aggregates. We have not determined the presence of lamins or insoluble chromatin scaffold proteins in kP, but it is conceivable that contaminants from other insoluble subcellular pools of protein may be present in kP as well. Accordingly, co-purification must be validated independently by fluorescence or EM co-localization, to ensure that co-purifying proteins are actually from the keratin IF. In the case of Hsp70, colocalization with keratin IF has been reported before (Liao et al., 1995). Likewise, Hsp40 / keratin colocalization was reported as well (Yamazaki, Uchiumi, & Katagata, 2012).

3. DETECTION OF CHAPERONING ACTIVITY ON KERATIN INTERMEDIATE FILAMENTS, RELATIVE TO CYTOSOLIC ACTIVITY

For a direct measurement of Hsp70 chaperoning activity, the luciferase refolding assay is commonly used (Lu & Cyr, 1998). The general principle is to measure luminescence from luciferase before and after chemical denaturation in guanidinium. Then, upon removal of the denaturing agent, luciferase refolds in the presence of a complete Hsp70 chaperoning complex and ATP. In doing so, it regains most (typically 60 – 70%) of the original luminescence within 4–5 hours.

- a. The fractions are resuspended by ultrafiltration (S, aP) or sonication (kP) in refolding buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 1 mM ATP), as described in the previous section.
- b. Luciferase is diluted to 0.3 mg/ml in denaturation buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 6 M guanidinium-HCl, and 5 mM DTT). Denaturation is carried out at 25°C for 1 hour. Also, 1 µl samples in triplicate of luciferase are equally diluted in 125 µl PBS in parallel, to be used as a measure of 100% luciferase activity. These samples are further diluted as described in the next paragraph.
- c. Chemically denatured luciferase (1 µl) is diluted in 125 µl refolding buffer to dilute guanidinium. Then, 15 µg protein from each fraction (S, aP and kP) are supplemented with 1 µl of diluted luciferase solution and incubated at 25°C. Another set of samples without ATP is prepared as negative controls. Additional

samples are supplemented with an identical amount of PBS or albumin to measure residual luminescence after guanidinium denaturation. Ideally, all samples should be prepared in triplicates.

- d. Aliquots of 1 μ l are removed from the folding reactions at various times (e.g. 1, 2 and 4 hours refolding) and luminescence is measured with a GloTM Luciferase Assay System (Promega) in a luminometer.

S and kP display similar levels of ATP-dependent luciferase refolding chaperone activity for similar protein concentrations. This is not surprising because of the high concentration of soluble Hsp70/40 chaperones in S, but considering the enrichment of IF in kP, it is remarkable that chaperoning activity actually enriches in kP as much as keratins, which represent the bulk of the protein in that fraction. On the other hand, there is no specificity for luciferase refolding in any of the fractions. Soluble or keratin-associated chaperones seem to be equally effective (Mashukova et al., 2014).

4. REFOLDING OF aPKC BY Hsp70/40 IN KERATIN INTERMEDIATE FILAMENTS

PKCs are AGC kinases which comprise, in humans, 10 isoforms divided in 3 categories: conventional (PKC α , β I, β II and γ), novel (PKC δ , ϵ , θ , and η), and atypical (PKC ι / λ and ζ). We have focused our study on atypical PKCs (aPKC), but some of the concepts that follow can be also extended to conventional or novel PKCs. All isoforms share a highly conserved catalytic domain. They are activated by PDK1-mediated phosphorylation of the activation loop and autophosphorylation of the turn motif (T555 in human PKC ι) (Newton, 2003). Importantly, kinase activity leads to a faster dephosphorylation of these phosphosites (Gould & Newton, 2008), by exposing them to phosphatases (Gould et al., 2011). Dephosphorylated PKC molecules are subjected to rapid ubiquitination and degradation (Lu et al., 1998). An Hsp70-mediated rescue mechanism enables refolding of PKC (Gao & Newton, 2006), immediately followed by the same sequence of PDK1-dependent phosphorylation (Mashukova et al., 2012) and autophosphorylation.

Because there is no direct readout of aPKC folding, the autophosphorylation of the turn motif may be used as an indirect surrogate. The basis of the assay is to enable endogenous PKC activity in various cell fractions (S, aP and kP) with ATP and a substrate peptide until PKC becomes fully dephosphorylated in about 5 hours. This process is dependent on the endogenous phosphatases. We have not determined which phosphatases are present, but it is known that PP2A is in a complex with aPKC in epithelial cells (Nunbhakdi-Craig et al., 2002). Ubiquitin is partially lost in the ultrafiltration (~80% retention expected in each cycle for a 8.5-kDa protein, http://kirschner.med.harvard.edu/files/protocols/Millipore_Centricons.pdf) and then diluted. For this reason, we speculate that aPKC protein is not degraded during the assay (Mashukova et al., 2009). Upon removal of the substrate peptide by ultrafiltration, aPKC is allowed to undergo Hsp70-dependent refolding and PDK1 phosphorylation. The readouts are immunoblots for PKC ι protein and pT555-PKC ι and ζ (the phospho-epitope antibody recognizes the phosphorylated turn domain of both kinases).

- a. Dephosphorylation step: Cell fractions (S, aP and kP) are supplemented with 150 μ M PKC substrate peptide (ERM₁PRKRQGSVRRRV) and 1 mM ATP. The peptide phosphorylation is allowed to proceed at 30°C for 5 hours.
- b. Peptide is removed by ultrafiltration and replaced by the rephosphorylation buffer as described in section 2.
- c. Refolding / re-phosphorylation: 50 μ g of protein from S, or 20 μ g of kP protein are incubated separately or together with 1 mM ATP (or negative controls without ATP) at 30°C for 4 hours. Samples from the fractions before and after step a (original phosphorylation level and fully dephosphorylated level respectively), and after the incubation in step c, are analyzed by immunoblot using antibodies against pT555 turn domain and total PKC ϵ protein.

We have shown that, separately, each fraction is unable to rephosphorylate PKC ϵ or ζ . However, combining S and kP enables rephosphorylation, while combinations of either one with aP do not (Mashukova et al., 2009). Bearing in mind that all three fractions contain Hsp70 and 40, the result suggested the specific need of IF for the rephosphorylation. It was later shown that supplementing kP with recombinant purified PDK1 alone was sufficient to enable rephosphorylation of aPKC. Also, immunodepletion of endogenous PDK1 from S prevented the S + kP mixture from supporting aPKC rephosphorylation (Mashukova et al., 2012). The IF fraction, therefore, is sufficient to refold endogenous aPKC. The only soluble component necessary to complete the readout of the reaction is PDK1, which is present in S.

Our laboratory has started to use these techniques to test the loss of function of keratin mutants when and if such mutants are able to form filaments. For this purpose, keratin-deficient cells such as SW-13/cl 2 (Schweitzer et al., 2001) or keratin-deficient keratinocytes (Seltmann, Cheng, Wiche, Eriksson, & Magin, 2015) represent the ideal background to express mutant keratin along with the corresponding normal keratin partner.

We have also used the same fractionation assay to measure PKC kinase activity using a PKC Kinase Non-Radioactive Assay kit (Assay Designs, Stressgen, Ann Arbor, MI). Because there is very little isoform specificity in this phosphorylation assay, the 3 cellular fractions can also be obtained from cells depleted in specific isoforms with RNAi treatments to assess isoform-specificity of the activity. Alternatively, individual fractions can be treated with PKC inhibitors, including pseudosubstrate peptides. Using these techniques we showed that atypical PKC is responsible for most of PKC activity in the kP fraction (Mashukova et al., 2009). It is conceivable that other enzymes may be enriched in the IF scaffold as well.

5. SUPPLEMENTATION ASSAYS USING CYTOSOL (S) AND ISOLATED KERATIN INTERMEDIATE FILAMENTS (kP)

Using separate fractions not only allows specific immuno-depletion, but also to supplement the fractions with purified proteins. For example, supplementing the S fraction with 15 μ g (for every 50 μ g of S protein) fully purified filamentous keratins without detectable contaminants (either recombinant or after cycles of urea denaturation followed by dialysis), is sufficient to enable aPKC rephosphorylation in the assay described above. Conversely,

single recombinant keratins (5 µg Krt8 or 18 / 50 µg of S protein), which do not form filaments, do not allow rephosphorylation in the S fraction (Mashukova et al., 2009). Finally, the S fraction immuno-depleted in Hsp70 fails to supplement highly purified (with no Hsp70) IF to enable aPKC rephosphorylation. Accordingly, this rephosphorylation can be rescued with recombinant Hsp70. Those results show specificity of the Hsp70/40 attached to IF to refold aPKC.

It has been extensively shown that individual keratin molecules assemble into IF directly from the cytosol, and likewise are shed back to the cytosol as non-filamentous subunits (Kolsch, Windoffer, Wurflinger, Aach, & Leube, 2010). Although there are areas of the cytoplasm where incorporation or disassembly is favored, there is consensus that filamentous and soluble non-filamentous subunits are in continuous exchange. Likewise, supplementation of highly purified filamentous keratins with Hsp70 described above supports the notion of equilibrium between soluble chaperones and chaperones attached to IF. While we and others have measured physiological changes in total cellular Hsp/Hsc70 concentrations (Mashukova et al., 2009), those changes are quantitatively small (e.g. up to 3 fold) to allow meaningful changes in the keratin-bound chaperones. One of the small chaperones, however, shows greater variability.

The small chaperone Bag-1 binds Hsp70 via a conserved N-terminal Bag domain. There are 4 isoforms of Bag-1, products of alternative start codons on the same transcript: Bag-1L (large) which contains a nuclear localization signal, Bag-1M (medium), Bag-1, and Bag-1S (small) (Alberti, Esser, & Hohfeld, 2003). The Bag-1 cellular concentrations are variable over a 20-fold range (Maki et al., 2007). We found that Bag-1M total cellular content varies 6–7 fold in mouse small intestine villus enterocytes under pro-inflammatory stimulation, and 3–4 fold in Caco-2 human intestinal epithelial cells under TNFα stimulation. Importantly, only Bag-1M and not Bag-1S was found attached to the IF fraction (Fig. 1B). Furthermore, the levels of IF-bound Bag-1M were dependent on the total cellular Bag-1M (Mashukova et al., 2014). These data suggest that attached Bag-1M is in equilibrium with soluble Bag-1M. To confirm the functional implications of Bag-1M - IF attachment supplementation assays can be used. However, to supplement kP fractions it is critically important to know the cytosolic concentration of the ligand (Bag-1M in this case). This can be achieved by determining the total cellular concentration and then measuring the fraction of the total cellular volume represented by the cytosol.

- a. Total cellular Bag-1M can be determined by semiquantitative immunoblot at different protein loads per lane to determine Bag-1M / total cellular protein. Then, the same are compared with known amounts of recombinant Bag-1M in the same blots.
- b. To determine a concentration, it is necessary to estimate cell volumes. Total protein per cell volume is estimated at 200 mg/ml (Milo, 2013). Using this constant, we estimated the basal levels of Bag-1M in non-stimulated cells at 0.8–1 µM. Because Bag-1M is excluded from the nucleus or organelles and mostly cytosolic, an estimation of the volume of the cytosol will provide a better estimation of the Bag-1M concentration around IF. These measurements were performed for

different cell lines using fluorophores (Tan et al., 2012). For Caco-2 cells, in particular, the cytosol represents 50% of the cell volume.

Using this technique, we supplemented the fractions for luciferase refolding assays (as in Section 3) with recombinant Bag-1M at the predetermined concentrations. We found that Bag-1M is necessary for Hsp70 activity at basal concentrations (around 1 μ M) but inhibits chaperoning activity in kP at concentrations usually found in epithelial cells under inflammatory stimulation (around 6 μ M). Importantly, the inhibitory concentration is higher for soluble, cytosolic Hsp70 which showed another aspect of specificity for chaperones attached to the keratin scaffold (Mashukova et al., 2014). Moreover, these results suggested that the Hsp70 keratin scaffold may not be functional in cells expressing high levels of Bag-1M, such as some carcinomas (Maki et al., 2007).

6. SUMMARY

The reductionist methods described here need to be complementary of cell- or animal-based evidence indicating that IF are necessary for a certain function. These approaches are designed to provide mechanistic insight on whether or not a given biochemical activity, in the cases described here protein chaperoning, can be carried out specifically by keratin filaments. For example, we demonstrated that Krt8 KO mice which lack IF in the small intestine villus enterocytes, also display decreased steady-state levels of aPKC. Conversely, mice overexpressing keratins display increased amounts of aPKC in the same cells. Finally, while we have only tested the role of Hsp70 in aPKC refolding, we also found steep decreases in the steady-state levels of protein or turn-motif phosphorylation in PKC β II, ϵ , γ , and PKB α (Akt) in keratin deficient cells. On the other hand, other known Hsp70 clients such as Chk-1 were unaffected by keratin knockdown (Mashukova et al., 2009). These results suggest that only a subset of Hsp70 clients is dependent on IF. Finding that IF knockout or knockdown negatively impacts on the steady-state levels of a protein, without affecting transcription/translation, seems to be a prerequisite to hypothesize that IF-based chaperoning is necessary for that protein. “Scaffolding” of a certain protein on IF has been shown several times. Considering that in many cases the same protein is present in the cytosol or other compartments in larger amounts than in IF, it is difficult to understand how passive binding of a small population of the molecules may have a biologically significant impact at all. Conversely, if the isolated IF scaffold performs a unique function different from the cytosol or other cellular compartments then it seems valid to conclude that association to IF is essential.

Although progress has been made to unravel the molecular structure of filamentous keratins (Lee, Kim, Chung, Leahy, & Coulombe, 2012), the organization of the protein machinery around IF is still poorly understood. The molecular reasons why Hsp70/40 refold dephosphorylated aPKC specifically in the IF scaffold are unknown. Likewise, the binding mechanism of Bag-1M to IF is also unknown. Binding affinity may explain why Bag-1M is more efficient inhibiting Hsp70 on the filaments, but direct mechanistic evidence supporting that is still missing. Finally, to our knowledge, the approach described here has not been used in other types of IF. The techniques shown here may be helpful to understand the

functional need of IF for other “downstream” mechanisms and functions identified by keratin loss-of-function mutations or keratin knockouts.

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Figure 1.

(A) Blot of S, aP and kP fractions obtained from a confluent differentiated culture of Caco-2 cells (40 $\mu\text{g}/\text{lane}$) stained with Ponceau S. First lane on the left hand side are MW standards, values represent $M_r \times 10^3$. (B) Immunoblots from the same (or parallel) membrane for the antibodies indicated on the left were developed by chemiluminescence.

Table 1

Proteins co-purifying with keratin (kP), actin (aP) and soluble (S) fractions in Caco-2 intestinal epithelial cells.

Protein	S	aP	kP	References
Keratins 8 / 18	–	–	+++	(Mashukova et al., 2009)
Actin	++	++	–	Fig. 1
tubulin	+++	+	–	(Mashukova et al., 2012)
PKC ζ	+	+	+	(Mashukova et al., 2009)
pT555-PKC ζ	+++	++	+	(Mashukova et al., 2009)
Hsc/Hsp70	+++	++	++	(Mashukova, Wald, & Salas, 2011)
Hsp 40	+++	++	+	Fig. 1
Hsp 27	++	–	–	Fig. 1
14-3-3	+	–	–	(Mashukova et al., 2009)
Bag-1M	+++	+	+	(Mashukova et al., 2014)
Bag-1S	+++	+	–	(Mashukova et al., 2014)
ZO-1	++	+	–	(Mashukova et al., 2009)
Par6	+++	+	–	(Mashukova et al., 2009)
Par3	+	–	–	(Mashukova et al., 2009)
Pals1	++	+	–	(Mashukova et al., 2009)
PDK1	+	+	–	(Mashukova et al., 2012)
FRACTION OF CELLULAR PROTEIN (%)	75	18	7	(Mashukova et al., 2009)

The + signs represent relative magnitudes of the bands as compared among the 3 fractions. For example, +++ means the band is stronger than ++. The – sign indicates that no band could be detected, even in over-exposed immunoblots. Note that 14-3-3 has been shown to bind to IF (Ku, Michie, Resurreccion, Broome, & Omary, 2002). Therefore, it is an example of IF-associated proteins that do not co-purify in the kP fraction.