Identification of the Junctional Plaque Protein Plakophilin 3 in Cytoplasmic Particles Containing RNA-binding Proteins and the Recruitment of Plakophilins 1 and 3 to Stress Granules[□]

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Recent studies on the subcellular distribution of cytoplasmic plaque proteins of intercellular junctions have revealed that a number of such proteins can also occur in the cyto- and the nucleoplasm. This occurrence in different, and distant locations suggest that some plaque proteins play roles in cytoplasmic and nuclear processes in addition to their involvement in cell–cell adhesive interactions. Plakophilin (PKP) 3, a member of the *arm*-repeat family of proteins, occurs, in a diversity of cell types, both as an architectural component in plaques of desmosomes and dispersed in cytoplasmic particles. In immuno-selection experiments using PKP3-specific antibodies, we have identified by mass spectrometric analysis the following RNA-binding proteins: Poly (A) binding protein (PABPC1), fragile-X-related protein (FXR1), and ras-GAP-SH3-binding protein (G3BP). Moreover, the RNA-binding proteins codistributed after sucrose gradient centrifugation in PKP3-containing fractions corresponding to 25–35 S and 45–55 S. When cells are exposed to environmental stress (e.g., heat shock or oxidative stress) proteins FXR1, G3BP, and PABPC1 are found, together with PKP3 or PKP1, in “stress granules” known to accumulate stalled translation initiation complexes. Moreover, the protein eIF-4E and the ribosomal protein S6 are also detected in PKP3 particles. Our results show that cytoplasmic PKP3 is constitutively associated with RNA-binding proteins and indicate an involvement in processes of translation and RNA metabolism.

INTRODUCTION

Over the past decade, several studies on the subcellular distribution of the plaque proteins of adhering junctions have revealed that a number of such proteins are not only constituents of cell–cell contact structures but also found dispersed in the cytoplasm and nucleus. This dual location suggests that in addition to establishing and maintaining cell adhesive functions these proteins may also play roles in nuclear and ribonucleoprotein processing mechanisms. Such proteins include members of the *arm*-repeat family, which are characterized by variable numbers of an ~42-amino acid motif (Peifer *et al.*, 1994). Among these nonjunctional functions the signaling roles of β -catenin and protein p120 have been rather well characterized (for reviews, see Anastasiadis and Reynolds, 2000; Huelsken and Birchmeier, 2001; Nelson and Nusse, 2004). For example, β -catenin is a key player in the Wnt pathway, directly mediating downstream events

through transactivation of transcription factors of the Lef1/TCF family to coordinate the activation of gene targets (Clevers and van de Wetering, 1997). Additional more recent evidence indicates that protein p120 also regulates cadherin turnover at the cell surface, thereby controlling the amount of cadherin available for cell adhesion (Kowalczyk and Reynolds, 2004; Reynolds and Roczniak-Ferguson, 2004).

Less is known on the functions of the plakophilins (PKPs), which are characteristic plaque proteins of desmosomes and also occur nearly ubiquitously and constitutively in the cytoplasm or nucleus of a wide range of cells (Heid *et al.*, 1994; Mertens *et al.*, 1996; Schmidt *et al.*, 1997, 1999; Bonn *et al.*, 1999). These basic proteins (isoelectric points ranging from 9.3 to 10.1) represent different isoforms of the products of three genes, and the corresponding proteins (PKP1–3) are deeply integrated in desmosomal plaques in a cell differentiation-dependent manner. PKP1 has been found primarily in desmosomes of stratified and complex epithelia (Franke *et al.*, 1983; Kapprell *et al.*, 1988; Schfer *et al.*, 1993; Hatzfeld *et al.*, 1994; Heid *et al.*, 1994). In contrast, PKP2 is characteristic of desmosomes of simple and certain nonepithelial tissues possessing desmosome-like junctions such as the myocardium and the dendritic reticulum cells of lymph node follicles, but it has also been localized to certain complex and stratified epithelia colocalizing with PKP1 and/or PKP3 (Mertens *et al.*, 1996, 1999). PKP3 has been found primarily in desmosomes of both simple and stratified epithelia but not in myocardium and hepatocytes (for reviews, see Hatzfeld, 1999; Schmidt and Jger, 2005); in addition, PKP3

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Abbreviations used: FXR1, fragile-X-related protein; G3BP, Ras-GAP-SH3-binding protein; PABPC1, cytoplasmic poly (A) binding protein; PKP, plakophilin.

has been noted to occur in an easily extractable fraction (Schmidt *et al.*, 1999).

To elucidate the function of PKP3 in its nonjunction-bound state, we decided to perform immunoselection experiments, followed by SDS-PAGE separation and mass spectrometric analysis. Using such an experimental approach, we have already been successful in identifying novel interaction partners of other junctional proteins: Thus, we have found the tight junction plaque protein symplekin to occur in both the karyo- and the cytoplasm of *Xenopus laevis* oocytes, in which it is associated with RNA cleavage and polyadenylation specificity factor, suggesting that symplekin is involved in both, 3'-end processing of pre-mRNA in the nucleus and the regulated polyadenylation in the cytoplasm (Hofmann *et al.*, 2002). Moreover, nuclear particles containing PKP2 were associated with the largest subunit of RNA polymerase III, in combination with the other polymerase III subunits as well as transcription factor TFIIB (Mertens *et al.*, 2001).

Here, we report on the identification of PKP3-containing complexes that are associated with the RNA-binding proteins poly (A) binding protein (PABPC1), fragile-X-related protein (FXR1), and ras-GAP-SH3-binding protein (G3BP), as identified by immunoselection and particle fractionation. Moreover, we have found that PKP3 is accumulated in so-called "stress granules" known to accumulate factors of stalled translation initiation complexes. We hypothesize that cytoplasmic PKP3 is involved in certain fundamental cell processes such as translation and/or RNA metabolism.

MATERIALS AND METHODS

Cell Lines, Culture Conditions and Transfections

The following human cell lines were used: HaCaT keratinocytes (Boukamp *et al.*, 1988), mammary gland carcinoma MCF-7 and colon carcinoma line CaCo2 (American Type Culture Collection, Manassas, VA). For stress treatments, cells were maintained at 43°C for 30 min ("heat shock") or were incubated with 0.5 or 1 mM arsenite for 30 min (oxidative stress).

Antibodies and Reagents

As PKP3-specific antibodies, the murine monoclonal antibodies (mAbs) PKP3-270.6.2 and PKP3-310.9.1 (available from Progen Biotechnik, Heidelberg, Germany), the guinea pig antisera PP3-1 (Schmidt *et al.*, 1999), and the broadly reacting PKP3-X-H, generated by immunization with the peptides MHENHFLMSALQPH and RTIRAPAMRTLQRF derived from the *X. laevis* PKP3 amino acid sequence were used. Further monoclonal antibodies applied in this study were against plakoglobin (clone 11E4; kindly provided by M. J. Wheelock, University of Toledo, Toledo, OH), PKP2 (clone CM86; Mertens *et al.*, 1996; available from Progen Biotechnik) desmoglein 1-2 (clone DG3.10; Schmelz *et al.*, 1986; available from Progen Biotechnik), and E-cadherin, p120, G3BP (all from BD Biosciences Pharmingen, Heidelberg, Germany) as well as protein FXR1 (clone 3FX; kindly provided by E. W. Khandjian, Centre de Recherche Hôpital Saint François d'Assise, Quebec, Canada; Khandjian *et al.*, 1998) and PABPC1 (clone 10E10; Immuquest, Cleveland, United Kingdom; see Görlach *et al.*, 1994). In addition, the following specific rabbit antibodies against the following proteins were used: FXR1 (FXR1 ML13; kindly provided by E. W. Khandjian; Khandjian *et al.*, 1998), PABPC1 (kindly provided by R. E. Lloyd, Baylor College of Medicine, Houston, TX) and S6 ribosomal protein (Cell Signaling Technology, New England Biolabs, Frankfurt am Main, Germany). Finally, a guinea pig antiserum against PKP1 (Schmidt *et al.*, 1997) and a goat serum against TIA-1 (Santa Cruz Biotechnology, Santa Cruz, CA) were used.

Secondary antibodies used for immunofluorescence microscopy were Alexa 488, Alexa 568, or Cy3-conjugated antibodies to immunoglobulins of mouse, guinea pig, rabbit, or goat, respectively (Dianova, Hamburg, Germany). For immunoblot analysis, horseradish peroxidase-conjugated secondary antibodies were applied in combination with the enhanced chemiluminescence system (New England Nuclear, Cologne, Germany).

Sucrose Gradient Centrifugation

For sucrose gradient centrifugation, cells grown on 10-cm plates were lysed either with 0.8 ml of ice-cold 5:1 buffer (80 mM KCl, 20 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 0.2% NP-40) or physiological salt buffer (140 mM NaCl, 1 mM MgCl₂, 15 mM HEPES, pH 7.4, and 1% NP-40). Cells

were scraped off with a rubber policeman, resuspended by pipetting, and transferred to 1.5-ml tubes. To analyze the influence of RNase, extracts in 5:1 buffer were incubated with 20 µg/ml RNase A or 100 U of RNase T1 (Roche Diagnostics, Mannheim, Germany) for 15 min at 37°C. After centrifugation at 15,000 × g and 4°C for 10 min, the supernatants were layered on linear 10–40% (in physiological salt buffer without detergent) or 10–60% (in 10 mM Tris-HCl, pH 7.5) sucrose gradients and subjected to centrifugation for 18 h at 23,000 rpm (10–40%) or for 18 h at 35,000 rpm (10–60%) in an SW40 rotor (Beckman Instruments, Munich, Germany). Fractions of 0.4 ml (from 10 to 40% gradients) or 0.8 ml (from 10 to 60%) gradients were collected from top to bottom and proteins were analyzed by SDS-PAGE, followed by Western blotting. In parallel gradients, bovine serum albumin (BSA) (Sigma, Deisenhofen, Germany), catalase, thyroglobulin (Amersham Pharmacia, Freiburg, Germany), or ribosomal subunits from *X. laevis* ovaries were used as reference proteins or particles.

Immunoselection Experiments

For immunoselection, cultured cells were washed twice with phosphate-buffered saline (PBS), lysed in immunoprecipitation (IP) buffer (140 mM NaCl, 5 mM EDTA, 20 mM HEPES, pH 7.5, and 1% NP-40), scraped off with a rubber policeman, and resuspended by pipetting. Lysates were centrifuged for 10 min at 15,000 × g. Immunoselection was performed with Dynabeads (DynaL, Hamburg, Germany) coated with antibodies specific to mouse IgG or protein A. Samples were cleared by addition of Dynabeads for 2 h on a rotating wheel at 4°C. The beads were then separated, and the supernatants were transferred to a tube containing beads preloaded with the specific antibodies. After incubation overnight at 4°C on a rotating wheel, the Dynabeads were washed four times in ice-cold IP buffer and then boiled in sample buffer, processed by SDS-PAGE, and either stained with Coomassie brilliant blue or blotted to polyvinylidene difluoride (PVDF) membranes. As a control, the Dynabeads used for preclearing were processed in parallel.

Coomassie brilliant blue visible bands were excised and processed for peptide mass fingerprinting. Trypsin digestion and matrix-assisted laser desorption/ionization mass spectrometry was performed as described previously (Hofmann *et al.*, 2002).

"Far Western" and Northwestern Blotting

A sample obtained by immuno-selection using PKP3-specific monoclonal antibodies was separated by SDS-PAGE and transferred to nitrocellulose membrane sheets. [³⁵S]Methionine-labeled PKP3 and β-catenin were generated by coupled in vitro transcription/translation of corresponding cDNA's using the coupled transcription and translation reticulocyte system (Promega, Mannheim, Germany). "Far Western" binding assays were performed as described previously (Mertens *et al.*, 2001).

Northwestern blotting was performed according to Angenstein *et al.* (2002). Total poly (A) mRNA was prepared from HaCaT cells according to the manufacturer's protocol (Oligotex; QIAGEN, Hilden, Germany), labeled using [α-³²P]ATP and poly (A) polymerase (Ambion, Austin, TX), precipitated with ammonium acetate/ethanol, washed, and finally resuspended in 10 mM Tris-HCl, pH 8.0.

For both blot binding assays, bound radioactivity was detected by phosphorimaging (BAS-1800II; Fujifilm, Tokyo, Japan).

Immunofluorescence Microscopy

For immunofluorescence microscopy studies on cultured cells, cells grown on coverslips were fixed in methanol (5 min; −20°C), followed by acetone (30 s; −20°C), washed twice with PBS, and incubated with antibodies for 40 min at room temperature. Before antibody incubation, some specimens were incubated in PBS containing 0.2% Triton X-100 for 5 min (for details, see Mertens *et al.*, 1996). After several PBS washes, cells were incubated for 30 min with the appropriate secondary antibodies, washed in PBS, dehydrated in ethanol, air-dried, and mounted in Fluoromount (Biozol, Eching, Germany). In some experiments, cells were stained with 4',6'-diamidino-2-phenylindole (0.1 µg/ml; Serva, Heidelberg, Germany). Micrographs were taken with an Axiopt microscope (Carl Zeiss, Jena, Germany). Confocal laser scanning immunofluorescence microscopy was done on a Zeiss LSM 510 UV instrument (Carl Zeiss) as described previously (Hofmann *et al.*, 2002).

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) was performed as described by Herold *et al.* (2001) with minor modifications. HaCaT cells with or without heat treatment were washed with PBS, fixed in 2% formaldehyde in PBS for 10 min, and permeabilized for 5 min in 0.1% Triton X-100 in PBS. To detect polyadenylated mRNA, cells were incubated for 15 min at 37°C in prehybridization buffer (2× SSC, 20% formamide, 0.2% BSA, 1 µg/ml total yeast tRNA, and 10% dextran sulfate). For hybridization, the coverslips were transferred to a humidified chamber and covered with 30 µl of hybridization buffer (prehybridization buffer supplemented with 0.1 pmol/µl oligo(dT)₅₀ fluorescently end labeled with Cy3 (Biospring, Frankfurt am Main, Germany). Cells

Figure 1. Intracellular localization of endogenous (A and B) and GFP-tagged (C) PKP3. Cultured HaCaT keratinocytes were fixed in methanol/acetone and incubated in PBS with (A) or without (B and C) Triton X-100 and subsequently immunostained for PKP3 (A and B). (C) HaCaT cells have been transiently transfected with EGFP-N1-PKP3 constructs. Note the intense localization at desmosomes. Without Triton treatment, a strong PKP3 reaction is also evident in the cytoplasm (B and C). Bar, 20 μ m.

were hybridized for 2 h at 37°C and washed successively two times for 5 min in 2 $\times$ SSC/20% formamide (at 42°C), 2 $\times$ SSC (at 42°C), 1 $\times$ SSC, and PBS. Subsequent immunofluorescence microscopy was performed as described above.

Cloning of PKP3-pEGFP and Transfection

To clone the full-length cDNA coding for PKP3 (clone ph PP3 CA 3-1) into vector pEGFP-C1 (BD Biosciences Clontech, Heidelberg, Germany), an *Eco*RI restriction site was generated by PCR at the N terminus and a *Kpn*I restriction site at the C terminus of the PKP3 sequence (forward primer, 5'-TCG AAT TCT CTA TGC AGG ACG GTA AC-3'; reverse primer containing no stop codon, 5'-GGG GTA CCC TAT GGG CCC AGG AGT C-3'). Subsequently, the cDNA was cloned into the *Eco*RI and *Kpn*I sites of the pEGFP-C1 vector. For cloning into vector pEGFP-N1 (BD Biosciences Clontech), a *Kpn*I restriction site at the C terminus of PKP3 was created by PCR with a reverse primer containing no stop codon (reverse primer: 5'-GGG GTA CCG TTG GGC CCA GGA GTC-3'). Cloning into the pEGFP-N1 vector was performed using *Eco*RI and *Kpn*I sites.

Cultured cells were transiently transfected with PKP3-pEGFP-C1 and PKP3-pGFP-N1 constructs and hDcp1-GFP construct (kindly provided by B. Seraphin, Centre de Génétique Moléculaire, CNRS, Gif sur Yvette, France; Cougot *et al.*, 2004) at a cell density of 70% using FuGENE 6 (Roche Diagnostics) according to the manufacturer's instructions. Fluorescence microscopical analyses were performed 2 d after transfection.

Figure 2. Immunoselected proteins of PKP3-containing complexes. Mild detergent extracts from HaCaT cells have been immunoselected with the PKP3-specific antibody PKP3-270.6.2. The precipitates have been solubilized with sample buffer, separated by SDS-PAGE, and stained with Coomassie brilliant blue (lane 1). The prominent protein bands (lane 1) have been identified by peptide mass fingerprinting. In addition to PKP3, the desmosomal plaque protein desmoplakin and cytokeratin 5 and the RNA-binding proteins PABPC1, FXR1, and G3BP were identified. Lane 1, immunoselection; lane 2, negative control. Molecular weight markers are indicated.

RESULTS

Significance of the Cytoplasmic Proportion of PKP3

In HaCaT cells, PKP3 antibody PKP3-270.6.2 reacted with cell-cell junctions, colocalizing with various desmosomal markers such as the transmembrane protein desmoglein and the plaque protein desmoplakin (Figure 1, A and B; see also Schmidt *et al.*, 1999). Fixed cells that had not been treated with Triton-containing buffers before immunofluorescence microscopy showed, in addition, a prominent cytoplasmic staining (Figure 1B). Similar observations were made in other cells and with other PKP3-antibodies (our unpublished data). In cells transfected with PKP3-GFP constructs, localization of the fusion protein at cell-cell contacts as well as in the cytoplasm was seen (Figure 1C), and in the latter localization in a fine granular appearance was obvious.

Figure 3. Reciprocal immunoselection experiment using antibodies specific for PABPC1, FXR1, or G3BP (lane 1, immunoselection; lane 2, negative control). Note that plakophilin PKP3 is enriched in all three samples (lane 1', immunoselection; lane 2', negative control). The smaller PKP3-reactive bands might represent degradation products (compare Figure 2). Molecular weight markers (dots from top to bottom) 158, 116, 97, 66, 55, 43 kDa.

Figure 4. Sucrose gradient centrifugation analysis of proteins present in HaCaT cell extracts in a linear 10–40% sucrose density gradient. Fractions were collected from top to bottom and analyzed by SDS-PAGE and immunoblotting for proteins PKP3, FXR1, PABPC1, and G3BP. L, loading sample. P, pellet fraction. Size references: BSA, 4.3 S; thyroglobulin, 16.3 S; *X. laevis* ribosomal subunits of 40 S and 60 S.

PKP3 Is Associated with RNA-binding Proteins

When living HaCaT cells were lysed with buffers containing mild detergents a portion of PKP3 was easily extractable (Schmidt *et al.*, 1999). To identify proteins associated with PKP3 in its nonjunctional state, such lysates were subjected to immunoselection experiments using mAb PKP3-270.6.2 or the guinea pig antiserum PP3-1. The immunoselected proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue (Figure 2). The major protein bands, ranging from 50 up to 250 kDa, which were not visible in samples obtained after preclearing were excised and subjected to mass spectrometric analysis. The following proteins were identified: desmoplakin (accession no. NP_004406.2), PKP3 (accession no. NP_009114), PABPC1 (accession no. NP_002559.2), FXR1 (accession no. NP_005078.2), G3BP (accession no. NP_005745), cytokeratin 5 (accession no. NP_000415.1), and a degradation product of PKP3 (accession no. NP_009114). The proteins identified could be grouped as follows—desmoplakin, the double band most probably representing the splicing isoforms desmoplakins I and II (Mueller and Franke, 1983; Green *et al.*, 1990; Virata *et al.*, 1992); PKP3 (Schmidt *et al.*, 1999); and cytokeratin 5 (Moll *et al.*, 1982)—and are associated with the desmosomal plaque. The proteins PABPC1 (Görlach *et al.*, 1994; for reviews, see Mangus *et al.*, 2003; Kühn and Wahle, 2004), FXR1 (Siomi *et al.*, 1995), and G3BP (Parker *et al.*, 1996; for review, see Irvine *et al.*, 2004) all had in common RNA-binding motifs and were known as components of RNA metabolism.

To verify the specificity of the interaction of PKP3 with the mRNA-binding proteins, antibodies against PABPC1, FXR1, and G3BP were used for immunoselection (Figure 3). With all three antibodies, the specific protein was enriched (Figure 3, compare lanes 1 and lanes 2). The PVDF membranes were probed in parallel with PKP3-specific antibodies, and PKP3 seemed to be enriched in immunoselected samples using PABPC1-, FXR1-, or G3BP-specific antibodies, compared with negative controls (Figure 3, compare lane 1' and lane 2'), thus indicative of a stable interaction of PKP3 with the RNA-binding proteins.

In a first attempt to analyze whether PKP3 directly binds to any of the three RNA-binding proteins, we performed a “Far Western” blotting experiment (Supplemental Figure

S1). Samples obtained by immunoselection using PKP3-specific antibodies were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with ^{35}S -labeled PKP3. The radioactive labeled protein was bound to itself, whereas the other protein bands were not prominently labeled under those experimental conditions. In parallel, such a membrane was overlaid with ^{32}P -labeled poly (A) mRNA. Such a Northwestern blot experiment indicated strong binding of poly (A) mRNA to a band corresponding to PABPC1 and a much weaker decoration of a band corresponding to PKP3 (Supplemental Figure S1).

To analyze the physical state of PKP3-containing complexes in the detergent extracts, the particles were further subjected to centrifugation in 10–40% sucrose gradients. The resulting fractions were analyzed by SDS-PAGE and immunoblotting using PVDF membranes (Figure 4). PKP3 was recovered in fractions 10–22, with a first maximum in fractions 11 and 12, corresponding to 25 S, and a second maximum in fractions 18 and 19, corresponding to 50 S. In addition, some PKP3 is also detected in the pellet fraction. When the sucrose gradient fractions were analyzed with antibodies against FXR1, PABPC1, and G3BP, a partial co-distribution was observed. FXR1 was recovered in fractions 10–22, with a maximum in fractions 18–20, corresponding to 50–55 S. PABPC1 was recovered in fractions 12–19, and G3BP was mainly found in fraction 10–13 with a maximum in fraction 12, corresponding to 25 S. Remarkably, in these fractionations no codistribution with desmosomal marker proteins was noted such as desmoplakin, which is detected in fractions corresponding from 4 S to 8 S. After prolonged exposure, a small portion of soluble PKP3 is also found in fractions ranging from 4 S to 8 S (Supplemental Figure S2; compare Hofmann *et al.*, 2000). The other plakophilins, PKP1 and PKP2, showed a similar distribution pattern in additional fractions corresponding to larger S values (Supplemental Figure S2; for PKP2; see also Mertens *et al.*, 2001).

PKP3 Is a Component of Cytoplasmic Stress Granules

It has been reported that the RNA-binding proteins PABPC1, FXR1, and G3BP are located in so-called stress granules, which occur when cells are exposed to environmental stress (e.g., heat, hyperosmolarity, or oxidative con-

Figure 5. Laser scanning confocal microscopy showing the results of double-labeling experiments of untreated HaCaT cells (untreated, A–E'') and HaCaT cells after treatment at elevated temperature (heat shock, F–J''). Optical sections are shown. In red, localization of PKP3 with guinea antibodies of serum PP3–1 (A and F) and mAb PKP3-270.6.2 (B and G), Cy3-labeled oligo(dT) [poly (A); C, H], mAb to desmoglein (Dsg; D and I), or mAb to E-cadherin (E-cad; E and J) are presented. In green, localizations of proteins G3BP (A' and F'), FXR1 (B', D', E', G', I', and J') or EGFP-N1-PKP3 (PKP3-GFP; C' and H') are shown. For experimental details, see *Materials and Methods*. Arrowheads allude to the colocalization of PKP3 and markers for stress granules. The corresponding merged pictures are presented in (A''–J''). Bar, 20 μ m.

ditions) and represent sites of accumulation of stalled translation initiation complexes (Mazroui *et al.*, 2002; Kimball *et al.*, 2003; Tourrière *et al.*, 2003; for review, see Anderson and Kedersha, 2002). We wondered whether PKP3 was also present in such granules. Therefore, we analyzed HaCaT cells without treatment (Figure 5, A–E'') and after heat shock (Figure 5, F–J''). In untreated cells, the localization of PKP3 differed from those of G3BP (Figure 5, A–A'') or FXR1 (Figure 5, B–B''). After heat treatment, the distribution of PKP3 had markedly changed (Figure 5, F and G). Correspondingly, the localization of PKP3 in desmosomes was diminished. PKP3 was now found in cytoplasmic granules colocalizing with the RNA-binding proteins G3BP (Figure 5, F'–F''), FXR1 (Figure 5, G'–G''), PABPC1, and TIA-1 (our unpublished data), a marker protein of so-called stress gran-

ules (Gilks *et al.*, 2004). In heat-shocked cells, the PKP3 localization was identical in cells that were treated either with or without Triton-containing buffers (our unpublished data).

We also applied in situ hybridization using Cy3-labeled oligo(dT) probes to localize polyadenylated RNA and compared the distribution of mRNA with that of a PKP3-GFP fusion protein. In heat-treated cells, stress granules were seen that consisted of polyadenylated RNA and PKP3-GFP (compare Figure 5, C–C'' and H–H''), indicating that PKP3 is a component of these granules. No such colocalization was observed with other desmosomal marker proteins such as desmoglein or desmoplakin, neither in untreated nor in heat-treated HaCaT cells (Figure 5, D–D'' and I–I''). We still noticed desmosomal contacts although the cell layer had lost

Figure 6. Distribution of the proteins PKP3, FXR1, and PABPC1 in mild detergent cell extracts (lane 2) and the residual pellet fraction (lane 3) in untreated and heat-shocked cells. In lane 1, the total amount is shown, as detected by immunoblot using specific antibodies. Note PKP3 and FXR1 are enriched in the pellet fraction after heat shock.

its sheet-like appearance. The transmembrane protein, E-cadherin, characteristic for adherens junctions, was also not detected in stress granules (Figure 5, E-E'' and J-J'').

Various reagents have been used to induce the formation of stress granules; therefore, we also applied oxidative stress by incubating cells with medium containing arsenite. Under these conditions the PKP3 distribution was changed showing a colocalization with proteins PABPC1, FXR1, G3BP, and TIA-1 (Supplemental Figure S3).

To compare the proportion of PKP3 in mild detergent extracts of heat-shocked and untreated cells, equal amounts of lysate and pellet fraction were processed for immunoblotting. The portion of easily extractable PKP3 was diminished after heat shock, indicating that PKP3 is now structurally bound (Figure 6). This was also observed for protein FXR1, whereas the distribution of other proteins accumulating in stress granules, such as PABPC1, G3BP, and TIA-1 as well as desmosomal protein desmoglein 2, in lysate and pellet fraction was unchanged (Figure 6; our unpublished data).

We also examined whether other junctional *arm*-repeat proteins were associated with stress granules. The closely related *arm*-repeat protein PKP1 was detected in stress granules (Figure 7, A-A'') in contrast to PKP2, which was never detectable in stress granules (Figure 7, B-B''). Similarly, other *arm*-repeat proteins such as β -catenin (Figure 7, C-C''), plakoglobin (Figure 7, D-D''), or protein p120 (Figure 7, E-E'') were not detected in stress granules after heat shock treatment. At the elevated temperature, none of the proteins under study was degraded after treatment for 30 min, as controlled by Western blotting (our unpublished data).

We also wondered whether PKP3 was enriched in stress granules of other cultured cells. Indeed, in MCF-7 cells, a cell line derived from mammary gland carcinoma, PKP3 colocalized with G3BP in stress granules (compare Figure 8, A-A'' and B-B''). In colon carcinoma-derived CaCo2 cells, PKP3 was detected in small cytoplasmic dots when cells were kept for 30 min at elevated temperature. As indicated by the distribution of G3BP, the stress granules in this cell line were rather small compared with those seen in MCF-7 or HaCaT cells (Figure 8, C-C'').

In the past, several reports (Mazroui *et al.*, 2002; Hua and Zhou, 2004) have shown that upon forced expression of certain RNA-binding proteins by transfection cytoplasmic particles have been formed colocalizing with marker proteins of stress granules. Therefore, we transfected MCF-7 cells with PKP3-GFP constructs and compared the localization of desmosomal proteins and RNA-binding proteins.

Figure 7. Laser scanning confocal microscopy showing the results of double-labeling experiments of HaCaT cells at elevated temperature. Micrographs have been taken with a confocal laser scanning microscope, and single optical sections are shown. In red, immunolocalizations of PKP1 (A) or PKP2 (B), β -catenin (β -Cat; C), plakoglobin (PG; D), or protein p120 (E) are presented. In green, immunolocalizations of proteins FXR1 (A', B', D', and E') or G3BP (D'') are shown. Arrowheads indicate the colocalization of PKP1 and FXR1. The corresponding merged pictures are given in (A''-E''). Bar, 20 μ m.

The PKP3-GFP fusion protein detected at cell-cell contacts colocalized with desmosomal marker proteins such as desmoplakin (Figure 8, D-D''). Cytoplasmic aggregates consisting of PKP3-GFP contained any of the RNA-binding proteins under study nor polyadenylated mRNA (Figures 8, E-E'' and 5, C-C'').

Very recently, specific cytoplasmic foci, called processing bodies, Dcp-bodies, or GW-bodies, have been described as sites of mRNA decay in human cells (cf. Sheth and Parker, 2003; Cougot *et al.*, 2004; for review, see Fillman and Lykke-Andersen, 2005). We compared the localization of protein hDcp1-GFP, which is a marker protein of processing bodies, and PKP3 in untreated MCF-7 cells and detected no localization of PKP3 in processing bodies (Figure 8, F-F''). Processing bodies and stress granules induced by arsenite treat-

Figure 8. Laser scanning confocal microscopy showing the results of double-labeling experiments in different cultured human cells of lines HaCaT (A–A''), MCF-7 (B–B''), and CaCo-2 (C–C'') at elevated temperature and upon forced expression of PKP3-GFP (D–E'') and Dcp1-GFP (F–G'') in MCF-7 cells. Micrographs have been taken with a confocal laser scanning microscope. In red, immunolocalization of PKP3 using the guinea pig antiserum PKP3-X-H (A–C, F, and G), desmoplakin (DP; D), and protein PABPC1 (E) is presented. In green, immunolocalization of protein FXR1 (A', B', and C'), PKP3-GFP (D' and E'), and Dcp1-GFP (F' and G') is shown. In G–G'', MCF-7 cells have been treated with 1 mM arsenite for 30 min. The corresponding merged pictures are given in (A''–G''). Bar, 20 μ m.

ment were reported to be dynamically linked sites of mRNP remodeling (Kedersha *et al.*, 2005). Therefore, we compared the localization of proteins PKP3 and hDcp1-GFP in arsenite-treated cells and no colocalization was observed (Figure 8, G–G'').

To analyze the kinetics of PKP3 assembly in stress granules (cf. Tourrière *et al.*, 2003), HaCaT cells were fixed after various time points at elevated temperature and double-labeled with PKP3 and G3BP antibodies. After 5 min at elevated temperature, PKP3 and G3BP were diffusely distributed throughout the cytoplasm (Figure 9, B–B''). After 10 min, however, the distribution of G3BP started to change into a more punctate appearance. PKP3 was still enriched at cell–cell contacts but was also diffusely distributed over the cytoplasm (Figure 9, C–C''). Then, after 20-min treatment at elevated temperature, the PKP3 distribution changed and seemed more dotted in the cytoplasm, partially colocalizing with G3BP (Figure 9, D–D''), and this was even more evident after 30 min (Figure 9, E–E'').

Similarly, the distribution of PKP3 in stress granules depended on the concentration of arsenite applied for stress treatment. Cells treated with 0.5 mM arsenite showed a stronger diffuse cytoplasmic staining and some granules clearly positive for protein G3BP showed only a weak localization for PKP3; at 1.0 mM arsenite, the diffuse cytoplasmic PKP3 localization was reduced, and stress granules were strongly marked by PKP3-specific antibodies (Figure 9, F–F'' and G–G'').

PKP3 Particles Contain Translation Initiation Factors and RNA

Because the “heat shock” granules represent sites of accumulation of stalled translation initiation complexes, we have examined whether the ribosomal 40 S subunit and initiation factors are associated with PKP3.

Fractions obtained after centrifugation in 10–40% sucrose were probed with antibodies against protein S6, a component of the ribosomal 40 S subunit, and initiation factor eIF-4E, which binds to the cap structure of mRNA (for review, see Preiss and Hentze, 2003). The S6 protein was recovered in fractions 14–22 with a maximum in fractions 17 and 18, corresponding to 40 S as estimated in parallel gradients using ribosomal subunits as reference (Figure 10A). Thus, the large PKP3-containing particles recovered in fractions 17–23 clearly cofractionated with the endogenous small ribosomal subunit. By contrast, the protein eIF-4E was detected in fractions 1–14, with a major portion in fractions 1–3, representing most probably a free soluble form, and a smaller portion with a peak at fraction 11, corresponding to 25 S, thus cofractionating with the smaller PKP3-containing particle that also contained protein G3BP (compare Figures 4 and 10).

The two proteins S6 and eIF-4E were also detected in immunoselected samples as minor bands (Figure 2). Moreover, when mild detergent extracts were treated with RNase A or RNaseT1, PKP3 was no longer recovered in the fractions corresponding to higher S values (Figure 10B), indicating that the PKP3-containing complexes were associated with RNA.

DISCUSSION

After our identification of the desmosomal plaque protein plakophilin 2 (PKP2) as a constitutive component of karyoplasmic particles containing storage forms of RNA polymerase III (Mertens *et al.*, 2001) and of specific nuclear and cytoplasmic ribonucleoprotein particles containing the tight

Figure 9. Laser scanning confocal microscopy showing the results of double-labeling experiments of HaCaT keratinocytes after various times at elevated temperature (A–E'') and various concentrations of arsenite (F–G''). Micrographs taken with a confocal laser scanning microscope and single optical sections are shown. HaCaT keratinocytes have been analyzed after 0 min (A–A''), 5 min (B–B''), 10 min (C–C''), 20 min (D–D''), and 30 min (E–E'') at elevated temperature and after incubation with 0.5 mM arsenite (F–F'') or 1.0 mM arsenite (G–G''). The immunolocalization of PKP3 (red; A–G) is compared with that of protein G3BP (green; A'–G'); the merged

junction plaque protein symplekin (Hofmann *et al.*, 2002; see also Barnard *et al.*, 2004), we can now report the existence of class of cytoplasmic ribonucleoprotein particles containing PKP3, a widespread protein known as an architectural component of desmosomes. In the present study, we have demonstrated, by biochemical and immunological methods, that a proportion of PKP3 is associated with ribonucleoprotein particles and under environmental stress incorporated into "stress granules," accumulation sites of stalled translation initiation complexes. These findings suggest that PKP3 can be involved in processes of translation and/or RNA metabolism.

Previous attempts to identify binding partners of PKP3 have concentrated on desmosomes, where they have shown its binding to the transmembrane glycoproteins desmogleins and desmocollins and the plaque proteins plakoglobin and desmoplakin (Bonné *et al.*, 1999; 2003; Schmidt *et al.*, 1999). Our finding that PKP3 is immunoselected with desmoplakin verifies our previous report on distinct soluble complexes containing PKP1 and desmoplakin (Hofmann *et al.*, 2000) and results for PKP1–3 obtained by yeast two-hybrid screens (Hatzfeld *et al.*, 2000; Chen *et al.*, 2002; Bonné *et al.*, 2003).

Although in general cyto- and nucleoplasmic forms of PKP3 have been reported (Bonné *et al.*, 1999; Schmidt *et al.*, 1999), no distinct entities have been identified. The large ribonucleoprotein particles in which PKP3 is complexed with the RNA-binding proteins PABPC1, FXR1, and G3BP are therefore a novel general cell biological entity occurring in a diversity of cell lines from HaCaT to MCF-7 and CaCo-2 cells. Obviously, the detailed functional role of PKP3 in these particles will have to be elucidated.

A similar association with a ribonucleoprotein particle has not yet been reported for any other junctional *arm*-repeat protein, whereas some other nuclear functions of *arm*-repeat proteins have been identified. For example, the protein β -catenin is a key player in the Wnt signaling pathway, and its cellular levels are tightly regulated by phosphorylation and degradation processes to control transcriptional events (Clevers and van de Wetering, 1997; Huelsken and Birchmeier, 2001; for a recent review, see Nelson and Nusse, 2004) and protein p120 regulates the cadherin turn over by binding directly to cadherin (Ireton *et al.*, 2002; Davis *et al.*, 2003; for review, see Reynolds and Rocznia-Ferguson, 2004) and binds directly to transcription factor Kaiso (Daniel and Reynolds, 1999; for review, see van Roy and McCrea, 2005). PKP1 closely related to PKP3 was also detected in stress granules, suggesting that this protein may have similar functions. In contrast, PKP2, a near-ubiquitous protein, is a component of polymerase III holoenzyme (Mertens *et al.*, 2001). Although all three plakophilins are found in easily extractable particles, they are components of different particles (Mertens *et al.*, 2001). Moreover differences between those three proteins are emphasized by knockdown phenotypes and inherited disorders causally linked to plakophilins, leading to abnormal skin fragility in cases of PKP1 defects and of cardiomyopathies for PKP2 (McGrath *et al.*, 1997; Gerull *et al.*, 2004; Grossmann *et al.*, 2004; for review, see McGrath, 2005).

pictures are presented in (A''–G''). Note that the distribution of G3BP begins to change after 10 min of heat shock, whereas after 20 min cytoplasmic PKP3 seems increasingly in dispersed granules. Arrowheads indicate the colocalization of PKP3 and G3BP in stress granules, brackets allude to the junctional localization of PKP3. Bar, 20 μ m.

Figure 10. Gradient centrifugation analysis of particles extracted with mild detergent solutions from HaCaT keratinocytes in linear sucrose density gradients (10–40% in A; 10–60% in B). Fractions were collected from top to bottom and analyzed by SDS-PAGE and immunoblotting for the plakophilin PKP3, ribosomal protein S6, and protein eIF-4E. L, loading sample. Size references: BSA, 4.3 S; thyroglobulin, 16.3 S; *X. laevis* ribosomal subunits, 40 S and 60 S. (B) Mild detergent HaCaT cell extracts without or with RNase A treatment were subjected to sucrose gradient centrifugation, fractionated, and analyzed by immunoblotting with PKP3-specific antibodies after SDS-PAGE separation. Note after RNase treatment that PKP3 is no longer recovered in fractions corresponding to higher S values. The smaller PKP3 reactive band most probably represents a degradation product (compare Figure 2, lane 1).

Because cytoplasmic PKP3 is found in RNA-containing particles associated with other RNA-binding proteins, one wonders whether PKP3 is capable to bind directly to RNA. On the one hand, PKP3 does not contain any known RNA-binding sequence motif, but as a very basic protein should be able to bind direct to RNA via electrostatic interaction. The outcome of the Northwestern experiment suggests that PKP3 can bind directly to poly (A) mRNA. It would be important to test whether PKP3 is bound to a special category of ribonucleoprotein complexes such as PABPC1, which decorates polyadenylated mRNA or is recruited to specific RNA sequence motifs (for review, see Dean *et al.*, 2004).

Regarding the specific functions of the PKP3-containing particles one can presently only offer hypotheses on the basis of the known functions of the associated ribonucleoproteins. Because PKP3 is detected in two differently sized particles of 25–35 S and 45–55 S and is bound to different portions of the RNA-binding proteins FXR1, PABPC1, and G3BP several functions are possible. An involvement in translation, e.g., is suggested by its cofractionation with ribosomal proteins and translation initiation factors and its recruitment to stress granules would be compatible with its inclusion in translation initiation complexes (Anderson and Kedersha, 2002; Preiss and Hentze, 2003; Kedersha *et al.*, 2005), an interaction with components of RNA biogenesis (e.g., SMN protein; Hua and Zhou, 2004) or roles in RNA-localization (Thomas *et al.*, 2005) and mRNA degradation (Wilczynska *et al.*, 2005). Alternatively, one cannot yet rule out other roles of PKP3 in other RNA based cellular processes. Moreover, the association with PABPC1, which has been reported to be involved in translation initiation and mRNA stability, offers a putative link to the mRNA decay pathway (Mangus *et al.*, 2003; Kühn and Wahle, 2004). In addition, the binding to FXR1 might indicate an involvement in RNA localization (Schaefer *et al.*, 2003), and proteins of this gene family have recently been detected as components of the micro-RNA pathway, a novel RNA-silencing mechanism (Jin *et al.*, 2004; Pham *et al.*, 2004; Sontheimer, 2005). However, the exclusion of PKP3 from processing bodies in untreated and stressed cells suggests PKP3 not being involved in mRNA decay taking place in those cytoplasmic foci (Sheth and Parker, 2003; Cougot *et al.*, 2004; for review, see Fillman and Lykke-Andersen, 2005).

The finding that the smaller PKP3-containing particle contains protein G3BP, which can bind to ras-GTPase activating protein (ras-GAP; Parker *et al.*, 1996), and mRNA 5'-cap

binding protein eIF-4E necessary for cap-dependent translation (Preiss and Hentze, 2003), raises the broader question of PKP3's possible roles in processes of translational control (Gebauer and Hentze, 2004; Mamane *et al.*, 2004; Richter and Sonenberg, 2005) and signal transduction (Irvine *et al.*, 2004).

Future in vitro experiments using immunodepleted extracts or function-oriented studies of PKP3 knockdown phenotypes should give a clearer picture on its possible non-junctional state roles. An especially important question, however, is whether both PKP3 forms, the junctional and cytoplasmic form, are part of an interchangeable pool, which is suggested from the loss of juxtamembranous PKP3 staining after heat shock and its occurrence in stress granules, or whether these two forms are differentially regulated, e.g., by posttranslational modification.

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