Journal of Structural Biology 194 (2016) 415–422

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Branching of keratin intermediate filaments

Soufi Nafeey [a], Ines Martin [b], Tatiana Felder [c], Paul Walther [a,*], Edward Felder [c]

[a] Central Facility for Electron Microscopy, Ulm University, 89081 Ulm, Germany
[b] Institute of Experimental Physics, Ulm University, 89081 Ulm, Germany
[c] Institute of General Physiology, Ulm University, 89081 Ulm, Germany

ARTICLE INFO

Article history:
Received 3 November 2015
Received in revised form 22 March 2016
Accepted 30 March 2016
Available online 31 March 2016

Keywords:
Intermediate filaments
Keratin
Branching
Keratinocyte
Electron tomography
Electron microscopy (EM)

ABSTRACT

Keratin intermediate filaments (IFs) are crucial to maintain mechanical stability in epithelial cells. Since little is known about the network architecture that provides this stiffness and especially about branching properties of filaments, we addressed this question with different electron microscopic (EM) methods. Using EM tomography of high pressure frozen keratinocytes, we investigated the course of several filaments in a branching of a filament bundle. Moreover we found several putative bifurcations in individual filaments. To verify our observation we also visualized the keratin network in detergent extracted keratinocytes with scanning EM. Here bifurcations of individual filaments could unambiguously be identified additionally to bundle branchings. Interestingly, identical filament bifurcations were also found in purified keratin 8/18 filaments expressed in *Escherichia coli* which were reassembled *in vitro*. This excludes that an accessory protein contributes to the branch formation. Measurements of the filament cross sectional areas showed various ratios between the three bifurcation arms. This demonstrates that intermediate filament furcation is very different from actin furcation where an entire new filament is attached to an existing filament. Instead, the architecture of intermediate filament bifurcations is less predetermined and hence consistent with the general concept of IF formation.

1. Introduction

Keratins are the epithelial subtype of intermediate filaments (IFs) that form – together with actin and microtubules – the filamentous cytoskeleton in most metazoan cells. There, IFs are found as single filaments (diameter classically described as about 10 nm) that can arrange into bundles. Approximately 70 genes in the human genome encode for different IF proteins (Hesse et al., 2001; Rogers et al., 2005; Szeverenyi et al., 2008) that form six different, tissue specific subtypes. In contrast to actin and microtubules all IFs are built of non-globular monomers that share a common tripartite structure: a conserved central α-helical rod domain flanked by a non-α-helical amino-terminal head domain and carboxy-terminal tail domain. Both head- and tail-domain vary in size (Fuchs and Weber, 1994; Steinert and Parry, 1985).

Three different assembly mechanisms are suggested for the different subtypes of IFs (Herrmann and Aebi, 2000; Kirmse et al., 2007) that strongly differ from the assembly mechanism of actin filaments and microtubules. Keratins belong to assembly group 1. The building blocks of keratins are so called coiled coil heterodimers, comprised of an acidic and a basic keratin monomer which are arranged in an antiparallel manner. Two such dimers associate to form a half-staggered tetramer (Herrmann and Aebi, 1998; Herrmann et al., 2007) followed by the lateral association of tetramers. Eventually, unit-length filaments (ULFs) are formed. By longitudinal annealing of ULFs and already elongated filaments, IFs are growing (Martin et al., 2015; Portet et al., 2009). The number of laterally associated dimers in different segments of the same filament can vary, a phenomenon that has been described as *polymorphism* (Herrmann et al., 1999). Hence, the diameter of IFs can differ – both within the same filament but also between different filaments.

Unlike actin filaments, where branching is a well described phenomenon (Mullins et al., 1998; Svitkina and Borisy, 1999) little is known about the bifurcation properties of IFs and to our knowledge branching of single keratin filaments has not been described in literature yet. In the present study we use different EM methods to demonstrate such branching. Quantitative analysis of cross sectional areas of the mother and the two daughter filaments revealed various thicknesses indicative for branchings with no obligatory number of subunits per mother/daughter arm.

* Corresponding author at: Central Facility for Electron Microscopy, Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany.
E-mail address: paul.walther@uni-ulm.de (P. Walther).

http://dx.doi.org/10.1016/j.jsb.2016.03.023
1047-8477/

2. Materials and methods

2.1. Cell culture procedures

The keratinocyte cell line SSC-25 (DSMZ, Germany, Braunschweig) was cultured as described previously (Fois et al., 2012).

2.2. Procedures for fluorescence microscopy

For analyzing the keratinocytes in the fluorescence microscope (Zeiss Axio Imager M1) they were seeded in 6 well plates and incubated at 37 °C and 5% CO_2 for one day. Before investigating them under the upright microscope the medium was replaced with isotonic bath solution after washing twice with warm PBS. The images were obtained with a Cool Snap EZ camera using the software VisiView (Visitron Systems).

2.3. Procedures for EM

High-pressure freezing and freeze substitution – Keratinocytes were seeded on glow discharged, carbon coated sapphire discs (3 mm in diameter, 160 μm thick, Engineering Office M. Wohlwend GmbH, Sennwald, Switzerland) for 48 h (37 °C, 5% CO_2). The cells were then high pressure frozen using a Wohlwend HPF Compact 01 high-pressure freezer (Engineering Office M. Wohlwend GmbH, Sennwald, Switzerland) as described by Buser and Walther (2008). Then the samples were freeze substituted by replacing the ice in the samples with substitution medium containing glutaraldehyde (3%), uranyl acetate (0.1%), and water (1.2%) in acetone. During freeze substitution the temperature was slowly increased from 183 to 273 K over a time period of 18 h. Afterwards the samples were kept at room temperature for 30 min before they were washed twice with acetone. Samples were embedded in epon (72 h, polymerization at 333 K) and sections were cut with an ultramicrotome (Leica Ultracut UCT ultramicrotome) using a diamond knife (Diatome, Biel, Switzerland). Section thickness was 70 nm for Fig. 1D, 240 nm for Fig. 2 and 475 nm for Fig. 3. The image in Fig. 1D was acquired with a JEOL-1400 (JEOL, Tokyo, Japan) at 120 kV acceleration voltage. The scanning transmission EM (STEM)-tomograms were acquired with a Titan (FEI, Eindhoven, The Netherlands) at 300 kV using the STEM mode as described by Höhn et al. (2011). For STEM-tomography the specimens were tilted from −72° to 72° with 1° increment for Fig. 2 and 2° increment for Fig. 3, respectively. Reconstruction of the tomogram from the tilt series and the 3D model was performed with IMOD software (Kremer et al., 1996).

Extraction of cells and critical point drying – For visualizing the three dimensional network of intermediate filaments by scanning EM (SEM) keratinocytes underwent a detergent treatment (extraction). Cells grown on sapphire discs (see above) were washed twice with phosphate-buffered saline (PBS; pH 7.3) for 5 min and then incubated in PBS with 2% Triton X-100 for 1 h at 281 K. After a washing step with PBS the cells were fixed with 2.5% glutaraldehyde (in PBS, phosphate buffer and 1% saccharose) for 1 h at room temperature. Then the cells were contrasted with OsO_4 (2% in PBS) for 1 h at room temperature and again washed with PBS. The samples were dehydrated in increasing concentrations of propanol for 5 min and then critical point dried with carbon dioxide as translation medium (Critical Point Dryer CPD 030, BalTec, Principality of Liechtenstein). Finally, the cells were coated with a 300 Hz layer of platinum (ca. 4.0 nm) using a Baf 300 (BalTec, Principality of Liechtenstein). A quartz crystal monitor controlled the thickness of the platinum layer.

SEM imaging – The extracted keratinocytes were investigated with a Hitachi S-5200 FE-SEM (Tokyo, Japan) by using an accelerating voltage of 5 kV and the secondary electron signal.

Keratin preparation for in vitro assembly and TEM – Keratin 8 and 18 were isolated from *Escherichia coli* and purified as described by Herrmann et al. (2004) and then dialyzed into 2 mM Tris-HCl buffer (pH 9.0). For filament assembly 20 mM Tris-HCl buffer (pH 7.0) was added in equal amounts and the protein concentration was adjusted to 0.5 g/l. Keratin was further diluted to 0.0125 g/l and at the same time the assembly process was stopped with 0.2% glutaraldehyde. For transmission electron microscopy (TEM) the protein solution was brought onto a glow discharged copper grid (300 lines/inch, Plano GmbH, Germany) coated with formvar® and carbon. The sample was negative stained with 2% uranyl acetate in water. Samples were observed with a Philips 400 TEM at an accelerating voltage of 80 kV.

Actin preparation for control experiments. Actin filaments have been assembled as followed:

Lyophilized muscle actin (Cytoskeleton #AKl99) was resuspended in distilled water as suggested by the manufacturer. Actin polymerization buffer (50 mM KCl, 25 mM MOPS at pH 7.0, 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM NaN_3) was used to polymerize actin at a concentration of 10 μM. To stabilize actin filaments phalloidin was added to polymerized actin at a 3:1 ratio. The final concentration of actin filaments on the silicon chips was of 0.5–1.0 μM. The filaments were attached onto glow discharged silicon chips and fixed with 2.5% glutaraldehyde for 1 h at room temperature. Samples were dehydrated and critical point dried and coated with 2 nm of platinum as described above.

Characterization of bifurcations in extracted keratinocytes and in vitro preparations – Y-shaped branches of individual keratin filaments were randomly chosen from different SEM extraction preparations (Fig. 4) and negative stainings of *in vitro* assembled filaments from isolated keratin 8/18 (Fig. 5). The diameter of each of the three branches was measured. Then the cross section area A of each branch was calculated assuming a circular cross section of the filaments. For this 4 nm were subtracted from the measured diameter in SEM samples, accounting for the platinum coating. The thickest branch was named "mother" (M) and the two others "daughter" (D) and the ratio r of the cross section areas was calculated according to Eq. (1), where d represents the measured diameter of the filaments.

$$r = \frac{(d_{D1}/2)^2\pi + (d_{D2}/2)^2\pi}{(d_M/2)^2\pi} \quad (1)$$

Assuming a simple longitudinal splitting of M in two D-filaments, with the sum of cross sectional areas of the D-filaments ($A_{D1} + A_{D2}$) being equal to the cross sectional area of M (A_M), a ratio of 1 is to be expected, whereas thicker D-filaments – requiring additional dimers in the D-filaments – shift the ratio to values larger than 1. D and M filaments of identical thicknesses would result in a value of 2.

3. Results

Fig. 1 shows the intermediate filament network in the keratinocyte cell line SCC-25 as it appears in living cells and after different preparations for EM. In Fig. 1A the GFP-labeled network of keratin filament bundles is clearly discernible in fluorescence microscopy in living cells. A similar arrangement of filament bundles is observed in SEM after cells underwent a detergent extraction procedure (Fig. 1B). Despite the entirely different methodological approach, the overall arrangement and thickness of filament bundles is not substantially altered. At higher magnifications (Fig. 1C) individual filaments can be visualized, some of