Threonine 150 Phosphorylation of Keratin 5 Is Linked to EBS and Regulates Filament Assembly and Cell Viability

Mugdha Sawant[1], Nicole Schwarz[1], Reinhard Windoffer[1], Thomas M. Magin[2], Jan Krieger[3], Norbert Mücke[3], Boguslaw Obara[4], Vera Jankowski[5], Joachim Jankowski[5,6], Verena Wally[7], Thomas Lettner[7] and Rudolf E. Leube[1]

A characteristic feature of the skin blistering disease epidermolysis bullosa simplex is keratin filament (KF) network collapse caused by aggregation of the basal epidermal keratin type II (KtyII) K5 and its type I partner keratin 14 (K14). Here, we examine the role of keratin phosphorylation in KF network rearrangement and cellular functions. We detect phosphorylation of the K5 head domain residue T150 in cytoplasmic epidermolysis bullosa simplex granules containing R125C K14 mutants. Expression of phosphomimetic T150D K5 mutants results in impaired KF formation in keratinocytes. The phenotype is enhanced upon combination with other phosphomimetic K5 head domain mutations. Remarkably, introduction of T150D K5 mutants into KtyII-lacking (KtyII$^{-/-}$) keratinocytes prevents keratin network formation altogether. In contrast, phosphorylation-deficient T150A K5 leads to KFs with reduced branching and turnover. Assembly of T150D K5 is arrested at the heterotetramer stage coinciding with increased heat shock protein association. Finally, reduced cell viability and elevated response to stressors is noted in T150 mutant cells. Taken together, our findings identify T150 K5 phosphorylation as an important determinant of KF network formation and function with a possible role in epidermolysis bullosa simplex pathogenesis.

Journal of Investigative Dermatology (2017) ■, ■–■; doi:10.1016/j.jid.2017.10.011

INTRODUCTION

Keratin intermediate filaments (KFs) constitute a major part of the epithelial cytoskeleton. They are obligatory heteropolymers of type I and type II keratin polypeptides. Each polypeptide consists of a conserved α-helical, approximately 310-amino acid-–long rod domain that is flanked by variable amino-terminal head and carboxy-terminal tail domains (Herrmann and Aebi, 2016; Loschke et al., 2015; Pan et al., 2013). The significance of KFs for structural scaffolding of epithelia is evident from the skin fragility observed in the autosomal dominant blistering disease epidermolysis bullosa simplex (EBS), which is caused by mutations of the type II keratin (K) 5 or type I K14 (Coulombe and Lee, 2012; Homberg and Magin, 2014; Szeverenyi et al., 2008). KF collapse into cytoplasmic granules is a characteristic feature of EBS, especially upon mechanical and other types of stress (Beriault et al., 2012; Chamcheu et al., 2011; Homberg et al., 2015; Russell et al., 2004). A still unresolved conundrum is why EBS-mutant keratins are able to form perfect 10-nm filaments in vitro (Herrmann et al., 2002) and are often part of normal-appearing KF networks in EBS-derived keratinocytes (Beriault et al., 2012; Morley et al., 2003) and even in epidermis of EBS patients (Anton-Lamprecht, 1994). These observations suggest that the mutations are not responsible for the deficiency in filament formation on their own but require additional factors.

Keratin granules have also been described in the context of increased keratin phosphorylation (reviews in Sawant and Leube, 2016; Snider and Omary, 2014). Phosphorylation targets almost exclusively the head and tail domains of keratins (Gilmartin et al., 1980; Ikai and McGuire, 1983; Sawant and Leube, 2016; Snider and Omary, 2014; Steinert, 1988) with a preference for the head domain of type II keratins (Liao et al., 1995; Yano et al., 1991). Type II keratins share the conserved and unique sequence motif LLS/TPL in their H1 head subdomain, which is a major target for phosphorylation (Toivola et al., 2002). Moreover, the H1 subdomain is essential for normal KF assembly (Hatzfeld and Burba, 1994; Wilson et al., 1992), and mutations in this domain have been identified in EBS patients (www.interfil.org). Phosphorylation of non-epidermal keratins was linked to multiple cellular dysfunctions in the context of diseases affecting the liver (Guldiken et al., 2015; Ku et al., 1998; Stumptner et al.,

[1]*Institute of Molecular and Cellular Anatomy, RWTH Aachen University, Aachen, Germany;* [2]*Institute of Biology and Translational Center for Regenerative Medicine, University of Leipzig, Leipzig, Germany;* [3]*Biophysics of Macromolecules, German Cancer Research Center, Heidelberg, Germany;* [4]*School of Engineering and Computing Sciences, Durham University, Durham, UK;* [5]*Institut für Molekulare Herz-Kreislaufforschung, RWTH Aachen University, Aachen, Germany;* [6]*School for Cardiovascular Diseases, Maastricht University, Maastricht, The Netherlands; and* [7]*EB House Austria, Research Program for Molecular Therapy of Genodermatoses, Department of Dermatology, University Hospital Salzburg, Paracelsus Medical University, Salzburg, Austria*

Correspondence: Rudolf E. Leube, Institute of Molecular and Cellular Anatomy, RWTH Aachen University, Wendlingweg 2, 52074 Aachen, Germany. E-mail: rleube@ukaachen.de

Abbreviations: EBS, epidermolysis bullosa simplex; FCS, fluorescence correlation spectroscopy; K, keratin; KF, keratin filament; KtyII, basal epidermal keratin type II; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ULF, unit length filament; WT, wild type

Received 4 July 2017; revised 11 September 2017; accepted 8 October 2017; accepted manuscript published online XXX; corrected proof published online XXX

FLA 5.5.0 DTD ■ JID1138_proof ■ 17 November 2017 ■ 3:39 pm ■ ce

2000; Zatloukal et al., 2000), pancreas (Liao and Omary, 1996), and colon (Zhou et al., 2006). Whether phosphorylation of epidermal keratins has similar effects on cellular physiology has not been examined in much detail.

The aim of this study was to resolve a potential link between EBS mutations, the occurrence of phosphorylation, and cellular physiology. Considering the shortcomings of other approaches such as the lack of specificity in drug-induced changes in phosphorylation (Feng et al., 1999; Liao et al., 1997) or the limited meaning of in vitro studies for the in vivo situation (Deek et al., 2016; Herrmann et al., 2002), we used a mutation-based strategy to investigate the effect of phosphorylation in the keratin type II head region in living cells.

RESULTS AND DISCUSSION

Phosphorylation of threonine 150 of K5 is linked to keratin aggregation in generalized severe EBS

It has been suggested that keratin phosphorylation is involved in granule formation of mutant keratin in EBS (Chamcheu et al., 2011; Woll et al., 2007). To directly test whether keratin phosphorylation is linked to granule formation, immunolocalization of keratin phosphoepitopes was performed on immortalized EBDM-4 keratinocytes carrying an R125C K14 mutation. The cells were derived from a patient with generalized severe EBS, previously referred to as Dowling Meara-type EBS (Fine et al., 2014). Using an antibody recognizing the T150 phosphoepitope of the conserved LLS/TPL sequence motif in the type II keratin K5 (Toivola et al., 2002) the strongest immunoreactivity was detected in granules (Figure 1b). Much weaker reactivity was seen in thick keratin filament bundles and only very weak to no reactivity was noted in thin filaments, as was the case in wild-type (WT) control keratinocytes of line hKC (Figure 1a and b). The fluorescence intensity patterns of the phosphoepitope-specific antibodies differed significantly from those observed with antibodies detecting keratins, irrespective of their phosphorylation status, which stained keratin bundles and granules at similar intensity and also clearly detected thin filaments (Figure 1a and b). Expression of YFP-tagged R125C K14 mutants in immortalized HaCaT keratinocytes also showed an enrichment of the T150 K5 phosphoepitopes in cytoplasmic granules (Figure 1c). Taken together, we conclude that T150 K5 phosphorylation is increased in granules that are formed in the presence of EBS mutant keratins. Immunoblotting of whole-cell lysates showed that the total level of K5 was reduced to 56% in EBDM-4 cells compared with hKC cells and that the ratio of phosphorylated to total K5 was approximately 1.5 times increased (see Supplementary Figure S1 online). The reduced level of keratins may be a consequence of increased keratin dynamics coupled with keratin degradation (Loffek et al., 2010; Werner et al., 2004; Windoffer et al., 2011).

Phosphomimetic keratin type II head domain mutations lead to increased granule formation in the presence of WT keratins

To delineate the potential role of T150 K5 phosphorylation in EBS skin fragility, the impact of phosphomimetic keratin mutation on KF network organization was studied. To this end, YFP-tagged WT K5 and phosphomimetic T150D K5 mutants were transfected into HaCaT keratinocytes. In both instances, a typical KF network was detected in most transfected cells, although granules were frequently observed next to KFs (Figure 2a and b). In some instances, the KF network was completely disrupted, leaving only granules (Figure 2c). Quantitation showed a slight decrease in the filament-only phenotype for the phosphomimetic mutant which was, however, statistically not significant (76% vs. 65%) (Figure 2d).

In addition to T150, multiple other potential phosphorylation sites are present in the K5 head domain (see Supplementary Figure S2a online and PHOSIDA database). To find out whether these other phosphorylation sites exacerbate the T150D-induced perturbation of KF network formation, further expression constructs were prepared containing the T150D mutation in all possible combinations with four other phosophomimetic mutations resulting in four double, six triple, four quadruple, and one quintuple mutants, which were transfected into HaCaT cells. Quantitative assessment showed that increasing the number of phosphomimetic sites correlated in general with a further decrease of the filament-only phenotype, suggesting that KF network formation was increasingly impaired (Figure 2d). Despite this overall tendency, certain sites had little effect or even improved the KF-network formation in some combinations (e.g., S35, S76).

To test whether the observed effects also apply to other keratins, we produced and tested a complementary set of mutants for the type II K8 (Figure 2e and see Supplementary Figure S2b and c). In this case, the phosphomimetic mutation S73D in the conserved LLS/TPL sequence motif was combined with four other phosphomimetic mutations of the head domain, again in all possible combinations. Transfection of the corresponding CFP-tagged fusion proteins showed very similar effects to those observed for K5.

Our observations in cultured cells are supported by published in vitro observations that showed that increasing the ratio of phosphomimetic K8 mutants to WT K8 reduces KF network connectivity (Deek et al., 2016). They are also in accordance with the observation that the impairment of in vitro KF assembly was proportional to the size of deletion in the K8 head domain (Hatzfeld and Burba, 1994).

Phosphomimetic T150 K5 mutation prevents keratin network formation in the absence of WT keratins

The low degree of phenotypic penetrance of the phosphomimetic mutants in HaCaT transfectants suggested that the phenotype was masked by endogenous WT keratins. This prompted us to use murine epidermis-derived keratinocytes lacking type II keratins (KtyII$^{-/-}$) (Kroger et al., 2013). Although WT K5 YFP and phosphomimetic T150D K5 YFP both integrated into the typical endogenous KF network of WT control keratinocytes, only K5 YFP was able to induce KF network formation in KtyII$^{-/-}$ cells, whereas T150D K5 YFP was not (Figure 2f, g, i, and j). Instead, strong diffuse fluorescence was detectable in the cytoplasm of all T150D K5 YFP-transfected KtyII$^{-/-}$ cells. In addition, small granules were visible throughout the cytoplasm. Occasionally, filamentous structures were seen in the cell periphery (Figure 2j).