

Hsp40 regulates the amount of keratin proteins via ubiquitin-proteasome pathway in cultured human cells

SHOTA YAMAZAKI, ASATO UCHIUMI and YOHTARO KATAGATA

Department of Biochemistry and Biotechnology, Faculty of Agriculture and Life Science,
Hirosaki University, Hirosaki 036-8561, Japan

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Abstract. Keratins represent important structural components of intermediate filament proteins. Their expression profiles are remarkably tissue-specific. Recent data have shown that keratins associate with many proteins including heat shock proteins (HSP). We recently identified cell-specific keratin and HSP expression. We aimed to gain further insight into the regulation of keratins by specific inhibition through knockdown of Hsp40 in human keratinocyte cells. Keratin-HSP interaction in HaCaT cell lysate was evaluated by immunoprecipitation followed by Western blotting. Immunofluorescence, was used to examine the co-localization of keratins and Hsp40. Hsp40 depletion led to an increase in the levels of keratin proteins (K5, K14, K10) and a decrease in keratin ubiquitination without influencing keratin gene expression. Our results demonstrate direct or indirectly association of Hsp40 and imply that expressed keratin proteins were regulated by Hsp40 depending on the ubiquitin-proteasome pathway in HaCaT. Furthermore, the K10 differentiation marker was increased by knockdown of Hsp40. The results presented in this study indicate that Hsp40 is related to the differentiation exchange of keratin pairs.

Introduction

Keratins are intermediate filament (IF) composition proteins. Keratins are major components of the cytoskeleton in most types of eukaryotic cells. The keratin subfamily, which is expressed preferentially in epithelial cells, has more than 20 members (K1-K20) that form obligate non-covalent heteropolymers of

at least one type I keratin (K9-K20) and one type II keratin (K1-K8) (1). One role that has been ascribed to various keratin filament networks of stratified squamous epithelia is to impart mechanical integrity to cells, without which the cells become fragile and prone to rupture (2). Disruption of the keratin IF network in epidermal keratinocytes via the targeted expression of dominant negative keratin mutants (3-5) or the introduction of null mutations (6) results in lysis of the targeted cell population whenever the skin of such mice is subjected to trivial mechanical trauma. Mutations in keratin genes, weakening the structural framework of cells, increase the risk of cell rupture and cause various human skin disorders (7,8). Interaction of keratin with HSP has been reported (9-12), but the role of this interaction remains unknown. Therefore, this report describes evidence for the *in vitro* association of Hsp40 with keratin and suggests a possible role of Hsp40 in the regulation of the protein quantity and expression pattern of keratin.

Materials and methods

Cell culture. The HaCaT cell line was cultured in Dulbecco's modified essential medium (DMEM with Eagle's salt and non-essential amino acids, without L-glutamine; Gibco Co., Ltd.) containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml kanamycin, and 2.5 µg/ml amphotericin B (13,14).

Immunoprecipitation. Cells were lysed on ice for 20 min in 10 mg/ml lysis buffer consisting of 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 12,000 rpm for 30 min. The HSPs were immunoprecipitated from cell lysates with anti-Hsp (Hsp40, Hsp60 and Hsp70) antibodies. Immunoprecipitates were analyzed by Western blotting with primary anti-keratin antibodies.

Western blot analysis. Proteins were extracted from several cultured cells using cell extraction buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA/1% Triton X-100/protease inhibitors (2 mM N-ethylmaleimide, 50 mg/ml aprotinin, 50 mg/ml leupeptin and pepstatin) at 48°C. After centrifugation, the soluble protein in the extract was quantified according to the method described by Bradford (15). Proteins were separated by SDS-PAGE using 10% gels and blotted

Correspondence to: Dr Yohtaro Katagata, Department of Biochemistry and Biotechnology, Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyocho, Hirosaki 036-8561, Japan
E-mail: katagata@cc.hirosaki-u.ac.jp

Abbreviations: WB, Western blot analysis; IP, immunoprecipitation; HSP, heat shock protein

Key words: Hsp40, keratin, ubiquitination, ubiquitin-proteasome pathway, cell differentiation

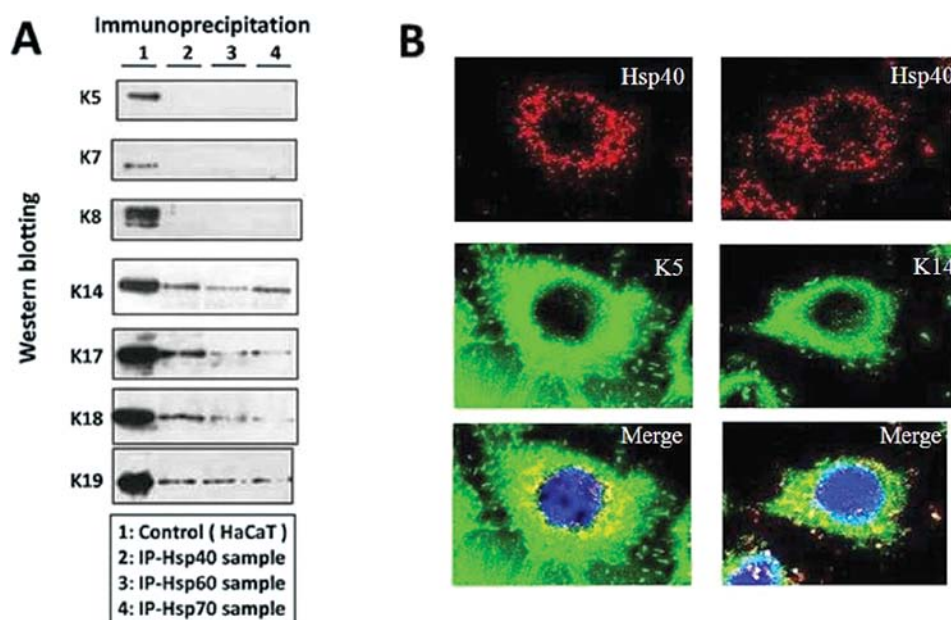


Figure 1. Co-sedimentation of heat shock proteins (HSPs) with keratins. We confirmed a relationship between keratins and HSPs. (A) As a control, HaCaT whole cell lysates (lane 1) and the immunoprecipitated samples with antibodies against the indicated proteins (lanes 2-4) were applied. The keratin-HSP interaction was evaluated by Western blotting. (B) Co-localization of K5/K14 keratins and Hsp40 was detected by immunofluorescence.

onto PVDF membranes. The membranes were then blocked in 5% non-fat dry milk TBST [10 mM Tris (pH 7.8), 150 mM NaCl, and 0.05% Tween-20] at room temperature for 1 h. The primary antibody was diluted at 1:1,000 in 0.5% BSA/TBST. The membrane was incubated at room temperature for 1 h with the following antibodies: anti-Hsp40 (Stressgen Biotechnologies Corp., Ann Arbor, MI), anti-Hsp60 and anti-Hsp70 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-K5 (Monosan; Sanbio B.V., NLD), anti-K14 (Progen Biotechnik GmbH, Germany), Ks 7.18 against K7 and Ks17.E3 (Progen Biotechnik GmbH), CAM 5.2 against K8/K18 (Becton-Dickinson, NJ, USA), KRT8 against K8, KRT18 against K18 and KRT19 against K19 (Abnova Corp., Taipei, Taiwan), anti-actin (Thermo, CA, USA), anti-ubiquitin (Stressgen Biotechnologies, Corp.), anti-involucrin Ab-1 (Thermo), KRT1 against K1 (Abnova Corp.), and VIK-10 against K10 (Genetex Inc., CA, USA). Membranes were washed three times for 5 min each with TBST. The membrane was then incubated with corresponding horseradish-peroxidase-conjugated secondary antibodies in 0.5% non-fat dry milk/TBST at room temperature for 1 h and washed four times for 15 min each with TBST. Antigen-antibody complexes were detected with chemiluminescence using an enhanced chemiluminescence (ECL) plus kit (Amersham Biosciences Corp., Buckinghamshire, UK). The complexes were then exposed to X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Immunofluorescence labeling and image analysis. Images were obtained with a fluorescence microscope (Olympus IMT-2; Olympus Corp., Tokyo, Japan). Cells were grown in a glass bottom dish (Iwaki Glass Co., Ltd., Tokyo, Japan) fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. To monitor Hsp40 and keratins, the cells were first immunostained with anti-vinculin and Alexa 488/568-conjugated secondary antibody (Molecular

Probes Inc.) and then the nuclei were counterstained with DAPI. Fluorescence microscopy images were obtained using a laser scanning confocal microscope (FV-1000; Olympus Co.).

siRNA-mediated knockdown of Hsp40. For RNA interference (RNAi) experiments, siRNA duplexes were synthesized using a Silencer siRNA Construction kit (Ambion Inc., Austin, TX, USA) in accordance with the manufacturer's protocol.

The siRNAs matching selected regions of Hsp40 sequence were synthesized using Ambion with siRNA Target Finder. The sequences were submitted to a BLAST search to ensure the target specificity. The siRNA sequence of Hsp40 was sense target sequence 5'-AACAAACGTCTGGTAGTAGTCTCCTGTCTC-3', antisense target sequence 5'-AAAGACTACTACCAGACGT TGCCTGTCTC-3'. The HaCaT cells were plated in 10 cm cell culture dishes and transfected using Lipofectamine™ RNAiMAX (Invitrogen Corp., Carlsbad, CA, USA), to give a final concentration of 100 pM. After transfection, the cells were incubated for 24 h and harvested for Western blot analysis.

Proteasome inhibition assay. The MG-132 proteasome inhibitor (Enzo Life Sciences, Inc.) at a concentration of 25 μ M was added to the media and the cells were incubated for 24 h. After incubation, the cells were harvested for Western blot analysis.

Results

Association of keratin with Hsp40 in HaCaT cells. We analyzed the *in vitro* association of HSP with keratins. In fact, HSPs were immunoprecipitated with anti-HSP (Hsp40, Hsp60 and Hsp70) antibodies. The associations of Hsp40, Hsp60 and Hsp70 were detected with anti-keratin antibodies by Western blotting (Fig. 1A). Type I keratins (K14, K17, K18, K19) were precipitated with HSPs, but Type II keratins (K5, K7, K8) did not form a precipitate. We then examined the co-localization of

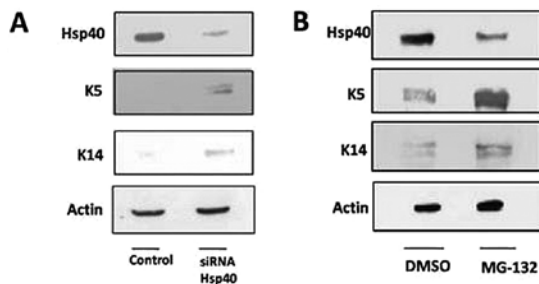


Figure 2. siRNA-mediated Hsp40 knockdown. (A) HaCaT cells were transfected at 24 h without control or Hsp40 siRNA. Transfection cells were prepared and processed for Western blotting with antibodies against the indicated proteins. (B) Effects of MG-132 were detected using Western blotting. Incubation with MG-132 for 24 h increased K5/ K14.

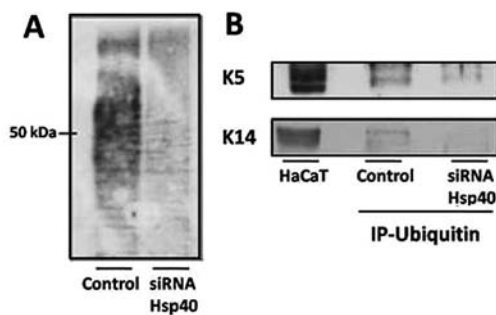


Figure 3. Influence of Hsp40 knockdown in HaCaT cells. Equal amounts of ubiquitination proteins were analyzed using Western blotting with ubiquitin antibody. (A) Ubiquitinated proteins were decrease by knockdown of Hsp40. (B) Furthermore, keratin ubiquitination detected by co-immunoprecipitation with ubiquitin antibody showed a decrease of ubiquitinated keratins in Hsp40 knockdown cells.

Hsp40 and keratins in HaCaT cells using immunofluorescence (Fig. 1B), which showed that K5/K14 and Hsp40 co-localized in the cytoplasm. These results indicate that HSPs directly or indirectly associate with keratins and especially that Hsp40 has a close affinity with K14. Therefore, in our subsequent experiments we chose to focus on K5/K14 keratins that are expressed in the basal cell layer and gradually decrease by cell differentiation.

Analysis of protein levels following siRNA-mediated knock-down of Hsp40. We previously established conditions using small inhibitory RNA (siRNA) to knockdown Hsp40 in cultured cells without influencing the levels of multiple control proteins. Additionally, we examined the influence of RNA expression using real-time PCR. Although no fluctuation of keratin expression was detected, the amount of keratins rose. Knockdown of Hsp40, led to an increase in K5/K14 protein levels (Fig. 2). HSPs are known to regulate the amount of proteins via proteasome-ubiquitin pathway. To quantify the association of K5/K14 with the ubiquitin-proteasome pathway, we inhibited the proteasome by MG-132 (proteasome-specific inhibitor) (Fig. 2B). We detected a similar increase of keratin protein levels in the proteasome-inhibited cells.

Hsp40 regulates keratins in protein level via ubiquitination. Although keratin expression was constant, the amounts of keratin

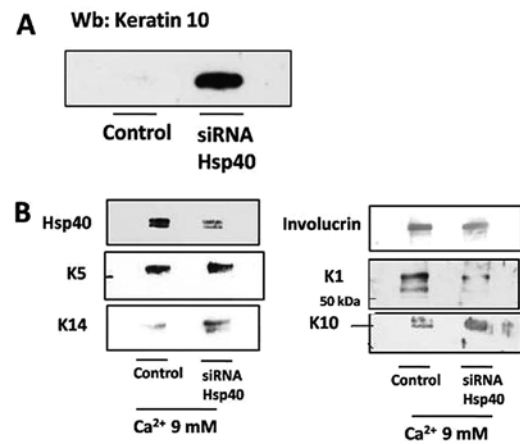


Figure 4. Unexpected expression of K10 and effects of Hsp40 on cell differentiation. (A) K10 is used as a differentiation marker and is only slightly expressed in HaCaT cells. However, Western blotting with anti-K10 antibody revealed excessive expression of K10 by Hsp40 knockdown. (B) Involucrin were used as a differentiation control. Treating the cells with 9 mM Ca²⁺ for 72 h prominently enhanced the protein of K1/K10. Hsp40 was knocked down 48 h after adding Ca²⁺. Protein expression was detected using Western blotting.

proteins were increased. The ubiquitin ligase C-terminus of the Hsc70 interacting protein (CHIP) mediates accumulation of mutant keratins with their ubiquitination (7). Therefore, the effects on keratin ubiquitination were confirmed in Hsp40 knockdown HaCaT. Protein ubiquitination was decreased by knockdown of Hsp40 (Fig. 3A). Then, we separated ubiquitinated K5/K14 from whole cell lysate by immunoprecipitation with anti-ubiquitin antibody, and detected keratin by Western blotting. Ubiquitinated K5/K14 proteins were remarkable reduced by siRNA transfection. These results showed that protein ubiquitination depends on Hsp40 in HaCaT cells.

Modulation of keratin during cell differentiation. We investigated effects of Hsp40 knockdown on other keratins. In contrast to control HaCaT cells, the level of K10 protein was increased in Hsp40 knockdown cells (Fig. 4A). Actually, K10 is a cell differentiation marker, and is expressed in the prickle cell layer (16). Therefore, we hypothesized that Hsp40 associates with keratin during cell differentiation. In order to demonstrate the effect of blockade of Hsp40 on cell differentiation, we examined the effects of Hsp40 knockdown on cell differentiation. Actually, Ca²⁺-dependent cell differentiation was detected by Western blotting (Fig. 4B). The levels of involucrin and K5 were equal to the control in knockdown cells. Involucrin was only detectable under high calcium conditions. K14 and K10 behaved according to their well known epidermal expression pattern, i.e. proliferating keratinocytes express K14 and differentiated keratinocytes switch to K10 expression. Compared with the control, K14 and K10 were increased in Hsp40 knockdown HaCaT cells.

Discussion

It is well known that HSP interacts with many proteins as a chaperone in cells (17,18). Additionally, we reported the HSP-keratin relation previously (12); recent reports indicate

that HSP homologue proteins associate with keratins. In this study, we investigated the HSP-keratin association and the role of Hsp40. Results show that Hsp40 has a closer affinity to keratin compared to other HSPs. Our results show that K14 was directly bound to Hsp40.

The amount of keratin was increased by knockdown of Hsp40 in HaCaT keratinocyte cells, although keratins expression was not significantly modulated. These results suggest that keratin regulation mediated by Hsp40 is protein-specific. Therefore, we searched keratin ubiquitination and degradation via the proteasome. Results show that keratin ubiquitination depends on Hsp40. Moreover, the amount of keratin was increased by proteasome inhibition. However, we detected K10, which was increased by knockdown of Hsp40. The K1 and K10 pair are known as cell differentiation markers. They are expressed in the epidermis except for the basal cell layer. These results suggest that during keratinocyte differentiation Hsp40 associates with the K5/K14 pair in exchange to the K1/K10 pair. Our results showed that K1/K10 were induced by Ca²⁺-dependent differentiation. In fact, K14 and K10 were significantly increased by knockdown cells. However, K1 tended to decrease. These imbalances of K5/K14, and K1/K10 expression in siRNA transfected cells may indicate the keratin-keratin interaction (18,19).

In conclusion, these results represent the indication that Hsp40 can regulate keratin protein levels in HaCaT keratinocyte cells. Furthermore, Hsp40 regulates the amount of keratin protein via the ubiquitin-proteasome pathway, and exchanges keratin pair expression. Moreover, Hsp40 may regulate the expression of non-specific keratins in a malignant tumor including non-epithelial cells (20,21). Cell migration depends on keratin expression (22). In the meantime, K5 or K14 mutation and accumulation caused epidermolysis bullosa simplex (23), for which Hsp40 may provide novel therapy approaches.

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