Sumoylation dynamics during keratinocyte differentiation

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Summary

SUMO modification regulates the activity of numerous transcription factors that have a direct role in cell-cycle progression, apoptosis, cellular proliferation, and development, but its role in differentiation processes is less clear. Keratinocyte differentiation requires the coordinated activation of a series of transcription factors, and as several crucial keratinocyte transcription factors are known to be SUMO substrates, we investigated the role of sumoylation in keratinocyte differentiation. In a human keratinocyte cell line model (HaCaT cells), Ca^{2+}-induced differentiation led to the transient and coordinated transcriptional activation of the genes encoding crucial sumoylation system components, including SAE1, SAE2, Ubc9, SENP1, Miz-1 (PIASxβ), SUMO2 and SUMO3. The increased gene expression resulted in higher levels of the respective proteins and changes in the pattern of sumoylated substrate proteins during the differentiation process. Similarly to the HaCaT results, stratified human foreskin keratinocytes showed an upregulation of Ubc9 in the suprabasal layers. Abrogation of sumoylation by Gam1 expression severely disrupted normal HaCaT differentiation, consistent with an important role for sumoylation in the proper progression of this biological process.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/1/125/DC1

Key words: Keratinocyte, Differentiation, SUMO, HaCaT, Ubc9

Introduction

Posttranslational modification of proteins is a rapid and efficient mechanism to modulate protein activity in response to a given stimulus. The small ubiquitin-like modifier, SUMO, has emerged as a complex and intriguing posttranslational modifier with a large variety of targets and a wide-range of effects on its substrates. Four SUMO isoforms (SUMO1-4) have been characterized in mammalian cells (Bohren et al., 2004; Su and Li, 2002), although SUMO4 expression is restricted to the kidney and other specific tissues suggesting a limited biological role for SUMO4. In contrast to SUMO4, SUMO1-3 are all widely expressed in mammalian cells (Xu and Au, 2005), though it is likely that these three proteins have at least partially distinct biological roles because SUMO1 shares only 50% identity with the closely related SUMO2 and SUMO3 (Saitoh and Hinchey, 2000). Consistent with the sequence divergence, distinct preferences for substrates (Guo et al., 2005; Manza et al., 2004; Rosas-Acosta et al., 2005b) and for specific de-sumoylating proteases (Melchior et al., 2003) have been observed for each SUMO type. Despite the above differences, all SUMOs are covalently attached to proteins via a series of enzymatic reactions and biochemical steps involving the same SUMO-specific enzymes (Johnson, 2004). The activating enzyme (E1), a heterodimeric protein composed of two subunits, SAE1 and SAE2 (Azuma et al., 2001), transfers SUMO to Ubc9, the only E2-conjugating enzyme (Desterro et al., 1997). The Ubc9-SUMO complex can directly interact with specific substrates, and this interaction leads to the formation of an isopeptide bond between SUMO and a lysine residue on its target (Okuma et al., 1999; Tatham et al., 2003). SUMO ligases (E3 enzymes) such as RanBP2 (Pichler et al., 2002), the PIAS protein family (Kotaja et al., 2002), and the polycomb protein Pc2 (Kagey et al., 2003) are not required for sumoylation in vitro, but when added to a sumoylation reaction they substantially increase the rate of this process and appear to play an essential role in vivo. Finally, there are specific SUMO proteases (SENP protein family) that are involved in both SUMO processing and de-sumoylation (Mossessova and Lima, 2000). The continuous interplay of the enzymes described above makes SUMO modification an active, reversible and dynamic process in mammalian cells.

SUMO modification usually exerts significant effects on its targets: it can increase protein stability (Desterro et al., 1998), affect protein-protein interactions (Seeler and Dejean, 2001), alter subcellular localization (Morita et al., 2005; Wilson and Rangasamy, 2001), and impact nuclear trafficking (Pichler and Melchior, 2002). To date, transcription factors (TFs) represent the largest group of SUMO substrates identified, and the most common effect associated with TF sumoylation is modulation of their transcriptional activity (Gill, 2003; Verger et al., 2003). In general, sumoylation results in the negative regulation of the activity of most TFs, including widely expressed factors such

as AP-1 (Bossis et al., 2005), Sp1/Sp3 (Ross et al., 2002; Spengler and Brattain, 2006), and C/EBP (Kim et al., 2002), though activation has been reported for some TFs, including TCF4 (Ihara et al., 2005) and IKaros (Arco et al., 2005). Recent studies have established multiple mechanisms for negative regulation of TFs by sumoylation, including recruitment of transcriptional co-repressors such as HDACs (Shiio and Eisenman, 2003; Yang and Sharrocks, 2004), sequestration in the cytoplasm (Morita et al., 2005; Salinas et al., 2004) or ubiquitylation and subsequent degradation (Ghioni et al., 2005).

Many of the TFs known to be SUMO targets, including pRB (Ledl et al., 2005), the p53/63/73 family (Ghioni et al., 2005; Melchior and Hengst, 2002), AP-2 (Eloranta and Hurst, 2002), and Sp1/Sp3 (Ross et al., 2002; Spengler and Brattain, 2006) are involved in regulating gene expression during cell-cycle progression and/or differentiation (Herwig and Strauss, 1997; Li and Kellems, 2003; Santini et al., 2001), suggesting that sumoylation could coordinate complex transcriptional programs in the cell. Several recent studies in metazoan systems support such a role for sumoylation in aspects of development and differentiation. Sumoylation is required for correct vulvar development in *C. elegans* (Leight et al., 2005; Poulin et al., 2005), is implicated in male germ cell maturation (Vigodner et al., 2006) and promotes differentiation of postsynaptic dendrites (Shalizi et al., 2006). Sumoylation is also critical for maintaining nuclear structure and chromosomal segregation during blastocyst development, with the absence of Ubc9 leading to an embryonic lethal phenotype (Nacerddine et al., 2005).

Skin is a complex and renewable organ for which little is known about the expression and function of the sumoylation system, although the presence of the SUMO1 protein in a mouse keratinocyte cell line has been reported (Zhong et al., 2000). The epidermis comprises multiple layers of differentiated keratinocytes that are continually regenerated from the replicative basal layer. In skin, keratinocytes differentiate vertically because of a Ca^{2+} gradient established throughout the different epithelial layers, increasing from the basal to the outermost layer of the epithelium (Menon et al., 1985; Vicanova et al., 1998). Ca^{2+} signaling drives proliferating keratinocytes out of the cell cycle and into a committed path of terminal differentiation, but the intermediate signaling transduction pathways that lead to this process are still poorly understood (Bikle et al., 2001; Lansdown, 2002; Tu et al., 2004). A network of keratin markers biochemically defines the keratinocyte stage within the epithelium structure (Eichner et al., 1986; Smith, 2003; Sun et al., 1985). Expression of keratin 5 (K5) and keratin 14 (K14) characterizes the basal proliferative phenotype restricted to the stem cell layer where the Ca^{2+} concentration is low (Schweizer and Winter, 1983). Early in differentiation, K5 and K14 are repressed and differentiation markers such as K1, K10 and involucrin begin to be expressed. Finally, keratinocytes complete terminal differentiation by producing the outer cornified layer of the skin characterized by markers such as filaggrin and involucrin (Candi et al., 2005; Eichner et al., 1986; Fuchs and Green, 1980). This pattern of markers permits discrimination between proliferating, differentiating and terminally differentiated keratinocytes. The well-coordinated expression of specific sets of TFs induces and represses these keratinocyte genes as the cells migrate through the epithelium layers, and a number of these crucial TFs are already known to be SUMO targets in other tissues (Girdwood et al., 2004; Verger et al., 2003), suggesting that sumoylation might play an important role in regulating epidermal differentiation.

Cultured keratinocytes, such as HaCaT cells, provide well-established differentiation models that can recapitulate many aspects of stratified epithelium (Boukamp et al., 1988; Schoop et al., 1999). HaCaTs are spontaneously immortalized and do not express exogenous transforming genes that could interfere with the natural process of sumoylation and differentiation. Moreover, HaCaT cells have the capacity to revert back and forth between the differentiated and the basal phenotype, and therefore are widely used to model keratinocyte differentiation in culture (Schoop et al., 1999). Here, we examine the sumoylation system in HaCaT cells during Ca^{2+}-induced differentiation. We show that the sumoylation system was transiently upregulated by Ca^{2+} signaling in HaCaT cells at both the RNA and protein level, whereas abrogation of sumoylation led to abnormal differentiation. These results suggest cross talk between the sumoylation system and the keratinocyte differentiation process that contributes to the normal program of morphological and biochemical changes during differentiation.

Results
HaCaT cells express the SUMO machinery
The SUMO system has been extensively studied, and the enzymes required for sumoylation are widely expressed in many tissues (Chen et al., 1998; Johnson, 2004). However, the sumoylation system remains largely unexplored in human keratinocytes, an important model for differentiation. To study the functional importance of this modifier in the context of keratinocyte differentiation, we first developed culture conditions to maintain HaCaT cells stably in either a basal or differentiated phenotype. HaCaT cells cultured in 0.03 mM Ca^{2+} were spindle-shaped and loosely packed (Fig. 1A). In addition, there was little or no expression of two differentiation markers, keratin 1 (K1) and involucrin (Inv), at the RNA (Fig. 1B) or protein (Fig. 1C) level, consistent with these cells remaining in a basal like state. By contrast, HaCaT cells maintained in 2.8 mM Ca^{2+} became more cuboidal, developed cell-cell tight junctions, and expressed both differentiation markers, K1 and Inv, at the RNA and protein level (Fig. 1A-C). Under both high and low $[Ca^{2+}]$ conditions, the cultures could be maintained for many passages without any subsequent changes in morphology or marker expression pattern. Therefore, these growth conditions allow HaCaT cells to be stably maintained in vitro in two alternative states resembling pre- and post-differentiated skin cells.

To characterize the sumoylation system in human keratinocytes, basal and stably differentiated HaCaT cultures were evaluated for expression of sumoylation components at both the RNA and protein levels. RT-PCR and quantitative RT-PCR (Q-PCR) analyses were used to detect transcripts for all the core SUMO components, including SUMO1, SUMO2, SUMO3, SAE1, SAE2 and Ubc9 (Fig. 2B,C), as well as the differentiation marker involucrin (Fig. 2A). Additionally, one SUMO ligase (Miz-1 or PIASxβ) and one SUMO protease (SENP1) were examined by RT-PCR. Consistent with the qualitative results shown in Fig. 1B, Q-PCR detected ~14-fold
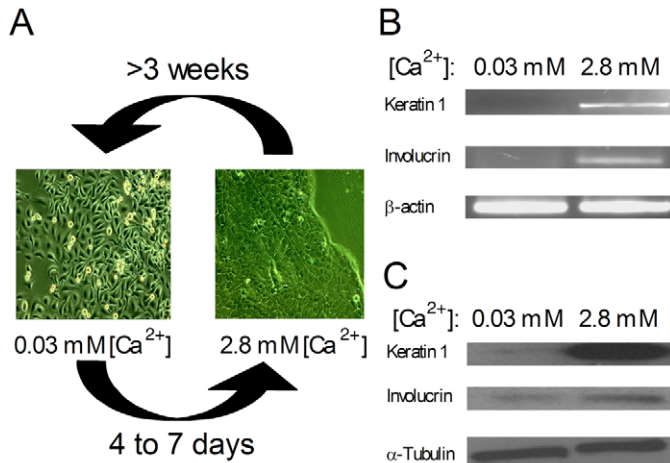
Fig. 1. HaCaT cell cultures exhibit phenotypic differences in response to Ca^{2+} concentration. (A) Phase-contrast microscopy of HaCaT cells maintained in low $[Ca^{2+}]$ medium (0.03 mM Ca^{2+}) or in high $[Ca^{2+}]$ medium (2.8 mM Ca^{2+}). Upon Ca^{2+} addition to the medium, basal HaCaT cells take 4-7 days to assume a complete differentiated state. Inversely, upon Ca^{2+} depletion, differentiated HaCaT cells take about 3 weeks to revert to a basal phenotype. (B) RT-PCR analyses of total mRNA harvested from basal (0.03 mM Ca^{2+}) and differentiated HaCaT (2.8 mM Ca^{2+}) cultures. (C) Immunoblot analyses showing expression of keratin 1, involucrin, and α-tubulin in total cell extracts harvested from basal (0.03 mM Ca^{2+}) and differentiated (2.8 mM Ca^{2+}) HaCaT cultures.

higher levels of involucrin mRNA in the high $[Ca^{2+}]$-maintained keratinocytes compared with the low $[Ca^{2+}]$ culture, confirming the differentiation state of these cells (Fig. 2A). By RT-PCR, transcripts for all of the SUMO component genes were detected in both the basal and differentiated HaCaT cells, indicating that the relevant genes for an active sumoylation system were actively transcribed in both cell populations (Fig. 2B). Although the HaCaT cells were maintained in two distinct phenotypic states, the transcript levels for the sumoylation components tested were similar under both culture conditions (Fig. 2C). Immunoblot analyses confirmed the expression of Ubc9 and SAE1 proteins, and the levels of each of these proteins were only slightly higher in the differentiated cells compared with the undifferentiated HaCaT cells (Fig. 2D). Next, we tested whether the sumoylation system was active in HaCaT cells as evidenced by the presence of sumoylated proteins. Immunoblotting of total cell extracts using a polyclonal anti-SUMO antibody showed that sumoylation occurred in both the basal and differentiated cell populations (Fig. 2E). Although the differences in expression levels of the sumoylation components were small, the sumoylation patterns in differentiated versus basal cells were slightly different: bands at ~40, 60 and 95 kDa were intensified in high $[Ca^{2+}]$ cells compared with basal cells (Fig. 2E, arrows). Since the basal and differentiated cell cultures were both equally proliferative and were generated from a single stock of HaCaT cells, we believe that these changes in sumoylation pattern reflect differentiation-related events rather than intrinsic differences in genetic background or growth capacity of the two populations of cells.

SUMO and Ubc9 are expressed in stratified human foreskin keratinocytes

To corroborate the HaCaT observations, the expression of SUMO and Ubc9 was evaluated in primary human foreskin keratinocytes (HFKs) that were stratified in organotypic cultures. Immunohistochemistry analyses were performed with an anti-SUMO polyclonal antiserum and a purified polyclonal antibody against Ubc9, and antibody specificity was confirmed by blocking experiments using purified SUMO1 or Ubc9 (Fig. 3). SUMO was detected in the nucleus and cytoplasm of keratinocytes found in all layers of the epithelium (Fig. 3C). However, as this antiserum crossreacts with SUMO2/3 (our unpublished observations), which SUMO types were present and whether or not there were changes in type expression in different layers is unclear. By contrast, Ubc9 was barely detectable in the basal layer of the epithelium where the cells remained relatively unstained (Fig. 3A, arrowheads). Strong anti-Ubc9 staining was detected in the intermediate differentiated layers just above the basal layer, and Ubc9 was present in both the nucleus and cytoplasm of the keratinocytes in this region (Fig. 3A, black arrows). Although Ubc9 could be detected in nuclei throughout the upper layers (Fig. 3A and data not shown), its overall expression faded as keratinocytes moved closer to the outer layer of the epithelium. It appears from these results that Ubc9 expression is low in basal cells, transiently increases with the initiation of differentiation in the suprabasal layers, and declines as terminal differentiation progresses. As Ubc9 is the only E2 enzyme for sumoylation, we speculate that the pool of sumoylated protein might be dramatically altered as well during this process. Interestingly, Ubc9 expression is maximal in the layers just below where keratin 1 expression commences (compare Fig. 3A with 3E). Overall, these analyses demonstrate that sumoylation components are present in stratified human skin epithelium and that Ubc9 levels change during differentiation. The similar observations in HaCaT cultures suggest that monolayer HaCaT cells are a good representative model of the sumoylation system in normal human keratinocytes.

Ca^{2+}-induced differentiation in HaCaT cells upregulates the sumoylation system

Although only minor differences in expression of the sumoylation components were detected between the two steady-state populations of HaCaT cells, the immunohistochemistry results suggested that more substantial changes in sumoylation might be taking place during the process of differentiation. To investigate this possibility we examined the sumoylation system in HaCaT cells undergoing active Ca^{2+}-induced differentiation. Addition of Ca^{2+} to the basal HaCaT cultures triggered a rapid response resulting in repression of the basal keratin markers, morphological changes (data not shown), and mRNA expression of differentiation markers such as K1 and Inv within 3-4 days (Fig. 4A,B). The Inv transcript detected by Q-PCR progressively increased from 48 to 144 hours following Ca^{2+} induction (Fig. 4A). Likewise, the K1 transcript, which was completely repressed in undifferentiated (0 hours) HaCaT cells, was detected by RT-PCR at 72 hours post Ca^{2+} induction, reaching a maximum expression by 120-144 hours (Fig. 4B). No K1 expression was detected in uninduced cells for up to 120 hours (Fig. 4B). Note, however, that the faint expression of K1 in uninduced cells by
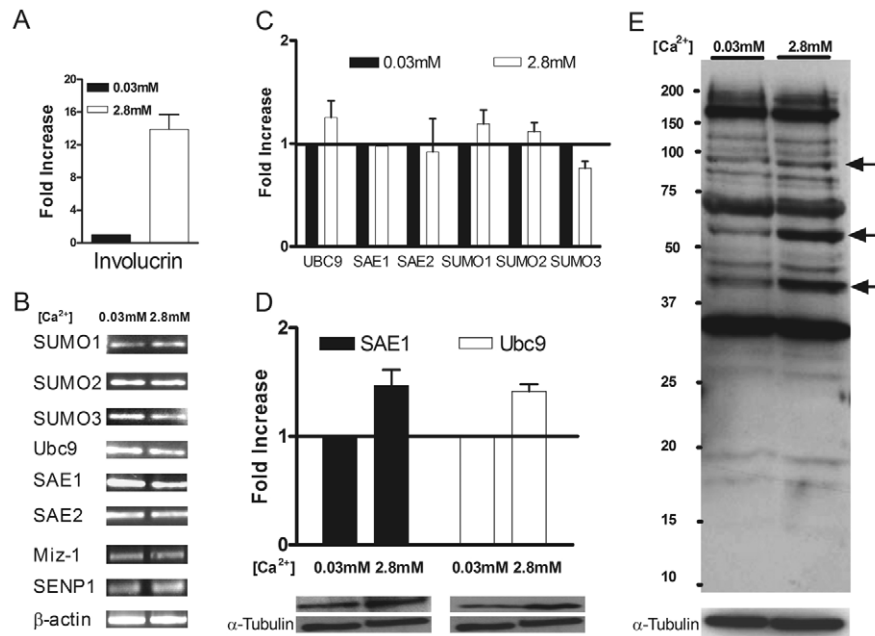
Fig. 2. Components of the sumoylation system are expressed in basal and differentiated HaCaT cells. RNA (A-C) or protein (D,E) were harvested from HaCaT cells maintained in either low [Ca^{2+}] (0.03 mM) or high [Ca^{2+}] (2.8 mM) medium. Harvesting and analysis were performed as described in Materials and Methods. (A) Quantitative RT-PCR of involucrin mRNA levels. (B) RT-PCR analysis of the indicated sumoylation system genes. (C) Quantitative RT-PCR analysis of the relative levels of Ubc9, SAE1, SAE2, SUMO1, SUMO2 and SUMO3 mRNAs. (D) Immunoblots for SAE1, Ubc9 and α-tubulin. SAE1 and Ubc9 blots were quantified by densitometry and normalized to α-tubulin. The graph shows the relative protein levels for HaCaT cells maintained in low and high [Ca^{2+}] medium, and the lower panels show representative SAE1 and Ubc9 immunoblots with their corresponding α-tubulin controls. (E) Anti-SUMO immunoblot of total cell extracts from HaCaT cells maintained in either low or high [Ca^{2+}] medium. Protein concentrations in the extracts were normalized to α-tubulin, and the samples were resolved by 10% SDS-PAGE. Arrows indicate sumoylated proteins whose levels appear higher in the high [Ca^{2+}] culture. Quantitative results in A, C and D are the average of at least three independent experiments.

144 hours was the result of the HaCaT cells reaching high confluency at this late time point (Capone et al., 2000). Based on K1 and involucrin expression, complete in vitro differentiation of HaCaT was reached within 144 hours after Ca^{2+} induction. Therefore, we chose to limit our study to a 6-day period during which we monitored the regulation of the sumoylation system while the HaCaT cells progressively differentiated. RT-PCR and Q-PCR showed rapid changes in the transcript levels for the genes encoding many of the sumoylation components (Fig. 4C,D). Transcripts for SUMO2, SUMO3, Ubc9 and SAE1 were upregulated twofold or more by 24 hours post-induction, with the SUMO2 and Ubc9 transcripts increasing fivefold or more at 144 hours post-induction (Fig. 4C). The final increase for SUMO3 and SAE1 was more modest, reaching a maximum increase of two- to threefold. The results for SAE2 were less clear, and the transcript levels did not consistently cross the twofold threshold until day 6. SENP1 transcripts detected by RT-PCR also gradually increased up to 144 hours, whereas Miz-1 (PIASxβ) transcripts were expressed maximally at 48 hours post induction and then declined (Fig. 4D). We conclude from these observations that there is a general upregulation of transcription of the genes for the sumoylation components accompanying the early stages of differentiation. Interestingly, the SUMO1 gene responded differently to Ca^{2+} induction than did the SUMO2 and SUMO3 genes (Fig. 4E). SUMO1 transcript levels did not increase significantly during the first

5 days post induction with Ca^{2+}, and even at day 6 the transcript levels were barely at the twofold threshold. Thus, SUMO1 gene expression during Ca^{2+}-induced differentiation appears to be regulated differently than the genes for the other SUMO components, though the biological significance of this result is not yet defined.

Transcriptional upregulation of sumoylation by Ca^{2+} is accompanied by increases in the corresponding proteins and overall sumoylation

Increase of specific transcripts during a biological process is not necessarily functionally important unless it is followed by a corresponding increase in protein expression. Immunoblot analyses of representative sumoylation components indicated that the Ubc9, SAE1 and SENP1 proteins levels remained relatively constant in uninduced cultures over the 6-day incubation period (Fig. 5B,C). By contrast, all three of these proteins were rapidly, but transiently, upregulated more than threefold during HaCaT cell differentiation (Fig. 5B,C). Maximum protein expression was reached at 48-96 hours post induction and then declined. Although the absolute increases for SAE1, Ubc9 and SENP1 were slightly different, the overall expression pattern was similar for all three enzymes (Fig. 5C). Interestingly, although protein expression levels declined 96 hours post induction, transcripts were still elevated and had not begun to decline during the time period examined (Fig. 4C). The explanation for this discordance is not known, but may
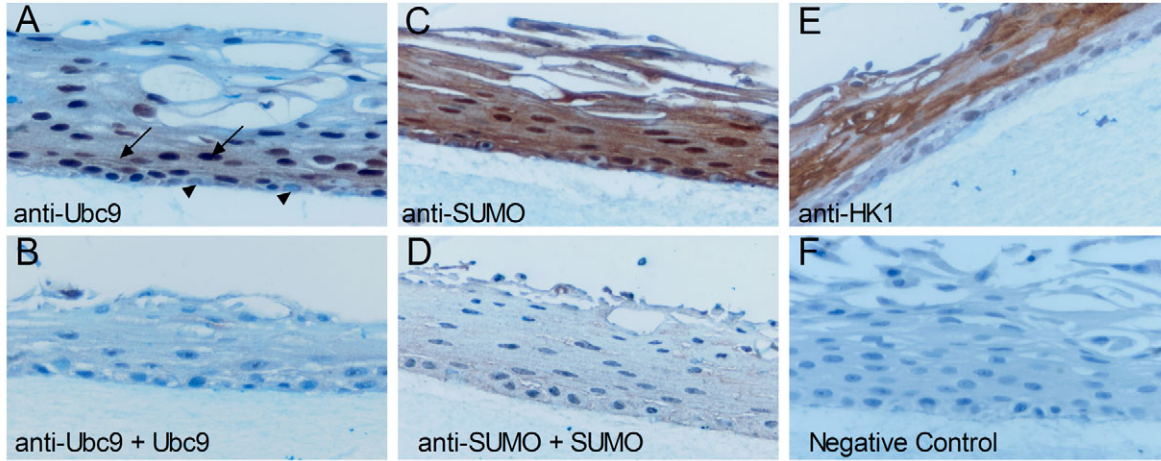
Fig. 3. Profile of SUMO and Ubc9 expression in organotypic cultures of human foreskin keratinocytes. Tissue sections were processed and evaluated for protein expression using antibodies to Ubc9 (A,B), SUMO (B,D), or human keratin 1 (E). For samples B and D, the respective purified proteins (25 μg Ubc9 or 7 μg SUMO1) were included in the primary antibody incubation step to block detection of the corresponding antigens. (F) Negative control where the primary antibody was absent. All sections were counterstained with hematoxylin. Arrows indicate cells expressing high levels of Ubc9. Arrowheads indicate cells in the basal layer exhibiting low levels of Ubc9 expression.

reflect the existence of a post-transcriptional regulatory mechanism that controls the level of protein expression for the different components of the sumoylation system during keratinocyte differentiation. Nonetheless, the combined results in Figs 2, 4 and 5 indicate that Ca^{2+} triggers a rapid and coordinated activation of transcription for many SUMO system genes leading to a corresponding accumulation of the SUMO system proteins, eventually followed by a decline to near basal protein levels. The observation in HaCaT cells that there is a transient upregulation of Ubc9 expression strongly correlates with the results from the Ubc9 immunohistochemistry data in the stratified HFKs (Fig. 3), which showed a transient increase in Ubc9 protein levels in the suprabasal layers that declined with further differentiation. Importantly, as in the stratified HFKs, the peak expression of Ubc9 in differentiating HaCaT

cells occurred just before expression of the K1 protein (Fig. 5A-C).

Next, we wanted to test whether the accumulation of sumoylation components increased the overall population of sumoylated targets. Total cell extracts of Ca^{2+}-induced HaCaT cells were collected every 24 hours up to 144 hours post induction and were analyzed by immunoblotting (Fig. 6). Overall sumoylation was clearly increased between 96 and 144 hours and discernible changes in the sumoylation of specific substrates were observed. During the differentiation process, several substrates (indicated by arrows) showed increased sumoylation, whereas a major sumoylated product at about 70 kDa decreased (arrowhead). In addition, new sumoylated bands appeared at about 45 and 95 kDa (arrows). No changes in substrate sumoylation were observed in
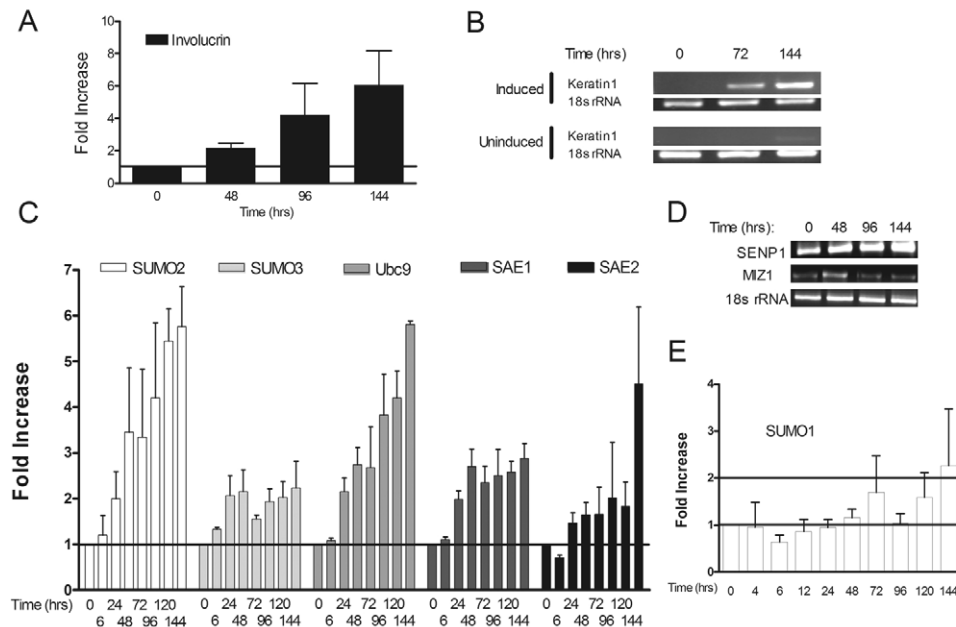

Fig. 4. Keratinocyte differentiation is accompanied by transcription upregulation of the sumoylation system. HaCaT cells maintained in low [Ca^{2+}] medium (Time 0) were induced to differentiate by replacing the medium with high [Ca^{2+}] medium. RNA was extracted at various times post Ca^{2+} induction as indicated. The extracted RNA was analyzed for expression of differentiation marker genes (A,B) or sumoylation system genes (C-E), and the mRNAs were detected either by quantitative RT-PCR (A,C,E) or RT-PCR (B,D). For the quantitative RT-PCR, the 0 hour time samples were set to a value of 1, and the values at other time points are relative to the 0 hour value. For the RT-PCR, 18S rRNA was used as the internal standard. The quantitative results in A,C and E were the average of at least three independent experiments.

parallel cultures maintained in low Ca^{2+} medium (data not shown). Enhanced sumoylation during HaCaT differentiation was even more pronounced when the samples were evaluated by 2D gel electrophoresis (unpublished observations). These results indicate that the increased expression of the sumoylation components leads to increased sumoylation activity, and that Ca^{2+}-induced differentiation of keratinocytes is accompanied by dynamic changes in the pattern of sumoylated proteins.
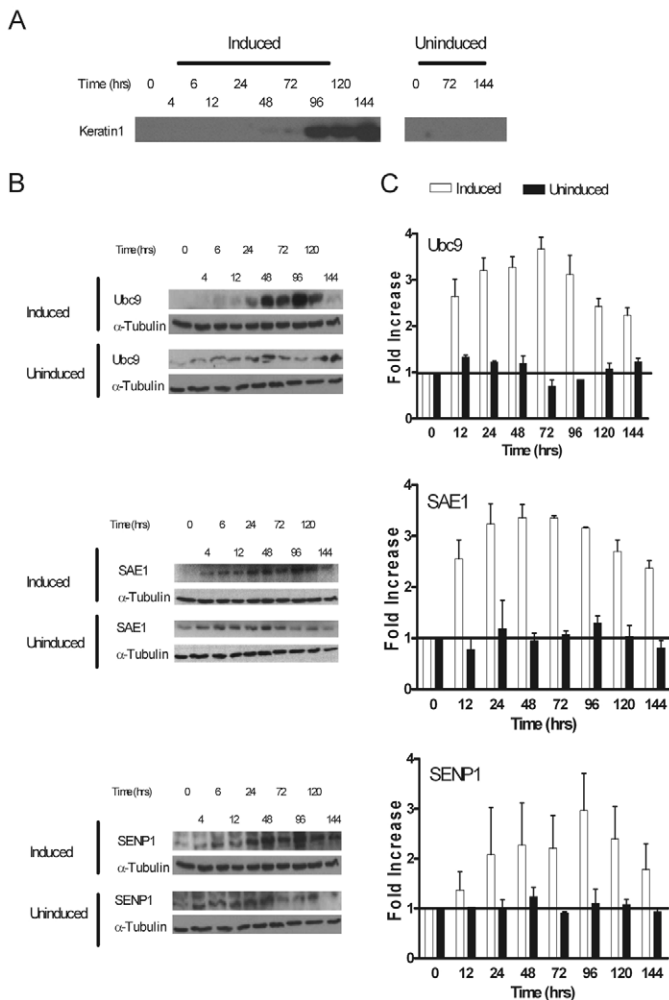

Fig. 5. Keratinocyte differentiation is accompanied by a transient upregulation of several sumoylation system proteins. Parallel HaCaT cell cultures were maintained either in low $[Ca^{2+}]$ medium (Uninduced) or switched from low $[Ca^{2+}]$ to high $[Ca^{2+}]$ medium (Induced). At the indicated times post Ca^{2+} induction, protein extracts were prepared from the paired cultures. The extracts were assayed for expression of K1 (A), Ubc9 (B), SAE1 (B), and SENP1 (B) by immunoblotting with specific antibodies. The induced and uninduced cell extracts were also evaluated for α-tubulin expression, and the immunoblots for α-tubulin are duplicated in each panel of B for comparative purposes. (C) The immunoblots in B were quantified by densitometry, and the values for Ubc9, SAE1, and SENP1 were normalized against α-tubulin. The 0 hour time values for both the induced and uninduced samples were set to 1, and the values for the later time points are relative to the 0 hour time. The quantitative results in C are the average of at least three independent experiments.

Inhibition of the sumoylation system perturbs the HaCaT cell differentiation process

To investigate the functional importance of sumoylation in the process of keratinocyte differentiation, we infected basal HaCaT cells with an adenovirus expressing Myc-tagged Gam1 (Ad-Gam1) and then exposed the cells to high $[Ca^{2+}]$ medium to trigger differentiation. Gam1 is known to inhibit sumoylation by inducing degradation of SAE1/2 and Ubc9 (Boggio et al., 2004). By 24 hours post-infection, Gam1 was detected in HaCaT cells infected with Ad-Gam1, but not in cells infected with Ad-GFP (Fig. 7B). In the Ad-GFP infected cells, the Ubc9 initially present increased 3.2-fold by 48 hours post Ca^{2+} induction, consistent with the response seen in the absence of viral infection (see Fig. 5B). By contrast, cells expressing Gam1 showed diminished Ubc9 levels by 24 hours and no detectable Ubc9 by 48 hours when Gam1 levels had reached maximal expression. A similar effect was seen in co-infections with Ad-Gam1 and Ad-GFP, indicating that Ad-GFP had no stabilizing effect on Ubc9. In contrast to these infection studies where the transgene could be delivered effectively into nearly 100% of the cells, alternative attempts to inhibit sumoylation via transfection approaches were unsuccessful owing to the poor transfection efficiency of HaCaT cells in low $[Ca^{2+}]$ medium (data not shown).

When examined microscopically, HaCaT cells expressing Gam1 exhibited a disruption of the normal morphological changes associated with differentiation. By 72 hours after Ad-
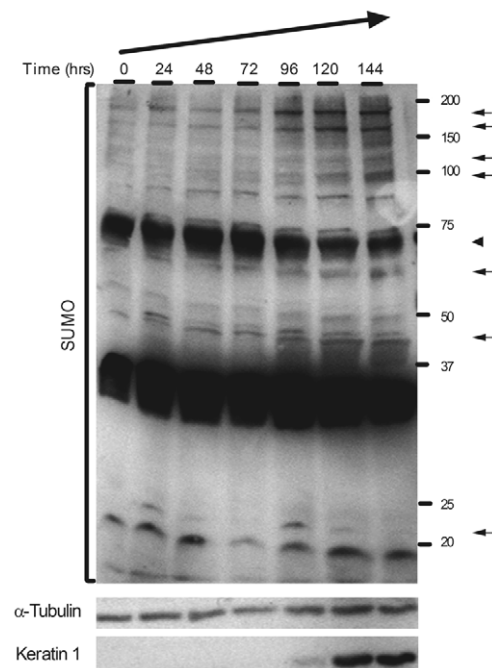

Fig. 6. The pattern of SUMO-conjugated substrates changes during HaCaT cell differentiation. Total cell extracts were prepared at the indicated times post Ca^{2+} induction and analyzed by immunoblotting using antibodies to SUMO1 (upper panel), α-tubulin (middle panel) or keratin 1 (lower panel). Protein concentrations in the samples were equalized based on the α-tubulin levels. The positions of molecular size markers are shown on the right. Arrows and arrowheads indicate bands whose quantity increased or decreased, respectively, during the time period examined.
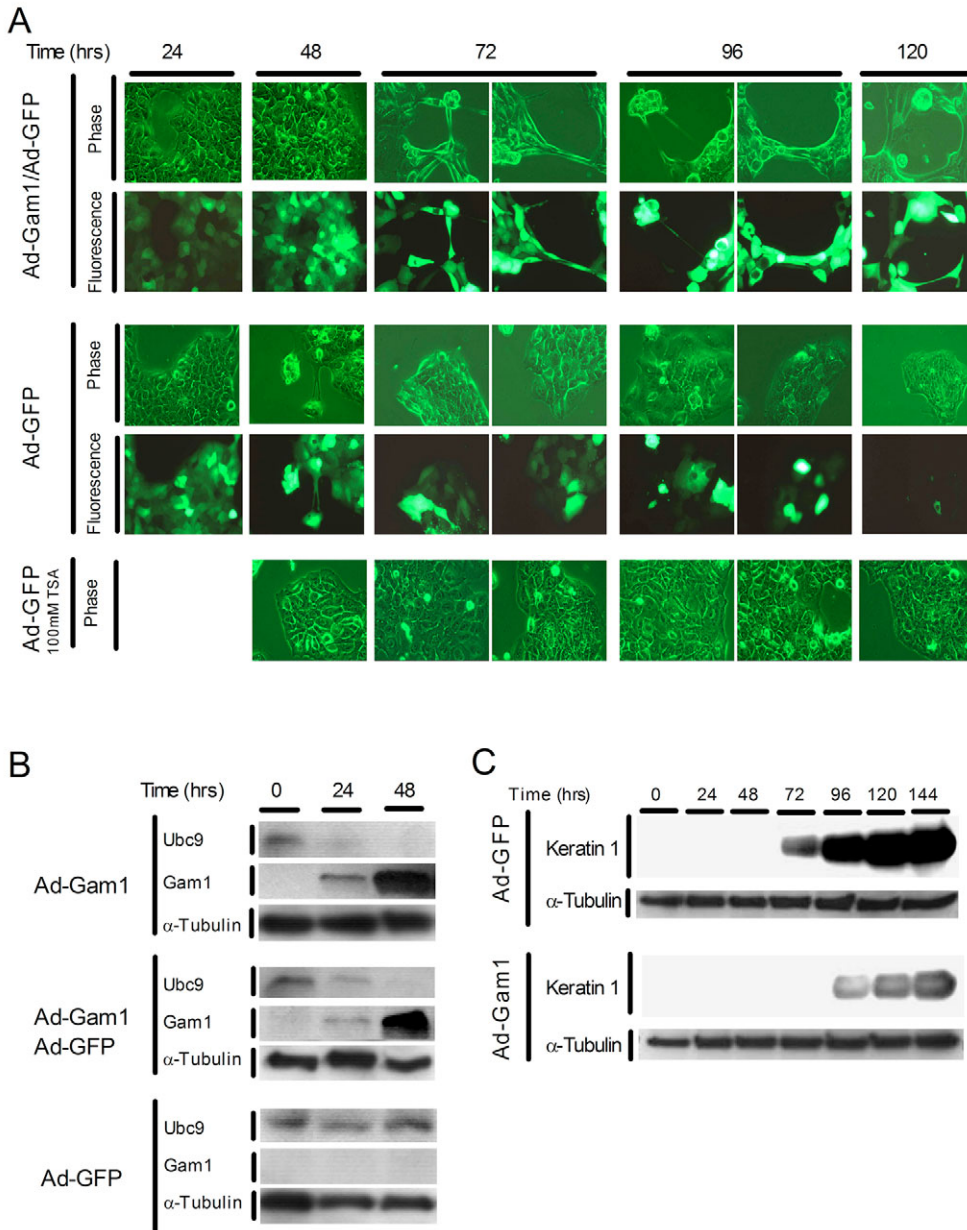
Fig. 7. Inhibition of sumoylation perturbs normal HaCaT cell differentiation. (A) HaCaT cells undergoing Ca^{2+}-induced differentiation exhibit abnormal morphology when expressing Gam1 protein. Shown are phase-contrast and fluorescence microscopy images of HaCaT cell cultures at 24-hour intervals. HaCaT cells were infected with Ad-Gam1/Ad-GFP (upper panel; MOI 150/150) or Ad-GFP alone (middle and bottom panels; MOI 300) and induced to differentiate by switching to high $[Ca^{2+}]$ medium. For the bottom panel, 100 mM TSA (an HDAC inhibitor) was added 24 hours post infection and induction and was re-added in fresh medium at 48-hour intervals. (B) Immunoblot analysis for Ubc9, Gam1, and α-tubulin in infected HaCaT cells. Cells were infected with the Ad-Gam1 (MOI 300), Ad-Gam1/Ad-GFP (MOI 150/150), or Ad-GFP (MOI 300) and induced to differentiate as in A. Cell extracts were prepared at the indicated times and immunoblotted. (C) Immunoblot analysis for keratin 1 and α-tubulin. Cells were infected with the indicated adenovirus constructs at an MOI of 300. Cell extracts were prepared and analyzed as in B.

Gam1/Ad-GFP co-infection and Ca^{2+} induction, when Ubc9 was absent, the differentiating HaCaT cells showed dramatic cellular rearrangements, including loss of tight cell-cell junctions and typical cuboidal shape (Fig. 7A). This abnormal morphology was not observed in cells infected with Ad-GFP alone at any time during the differentiation process (Fig. 7A). We also observed that GFP expression increased in the co-infected cultures throughout the experiment whereas in cells infected with Ad-GFP alone, the GFP expression gradually diminished and was almost undetectable 120 hours post induction. This observation was consistent with the known enhancing effect of Gam1 on transcription (Chiocca et al., 2002), and further demonstrated Gam1 activity in HaCaT cells. Identical morphological abnormalities were also observed in cultures infected with Ad-Gam1 alone (data not shown). Additionally, when basal cultures were infected with Ad-Gam1

without subsequent Ca^{2+} induction there was no change in cell morphology, whereas stably differentiated HaCaT cultures were susceptible to Gam1 (supplementary material Fig. S1). The insensitivity of the basal cultures to Gam1 confirms the fact that inhibition of sumoylation does not directly affect growth or morphology of the keratinocytes in the absence of differentiation. By contrast, the susceptibility of the stably differentiated cells to Gam1 further links differentiation state with a sumoylation requirement.

In addition to its inhibition of sumoylation, Gam1 is also known to inhibit HDAC activity (Chiocca et al., 2002). To exclude the possibility that the observed morphological effects on HaCaT cells could be due to HDAC inhibition, we conducted the Ca^{2+} induction in the presence of trichostatin A (TSA), a chemical inhibitor of HDACs (Yoshida and Horinouchi, 1999). Basal HaCaT cells were infected with Ad-
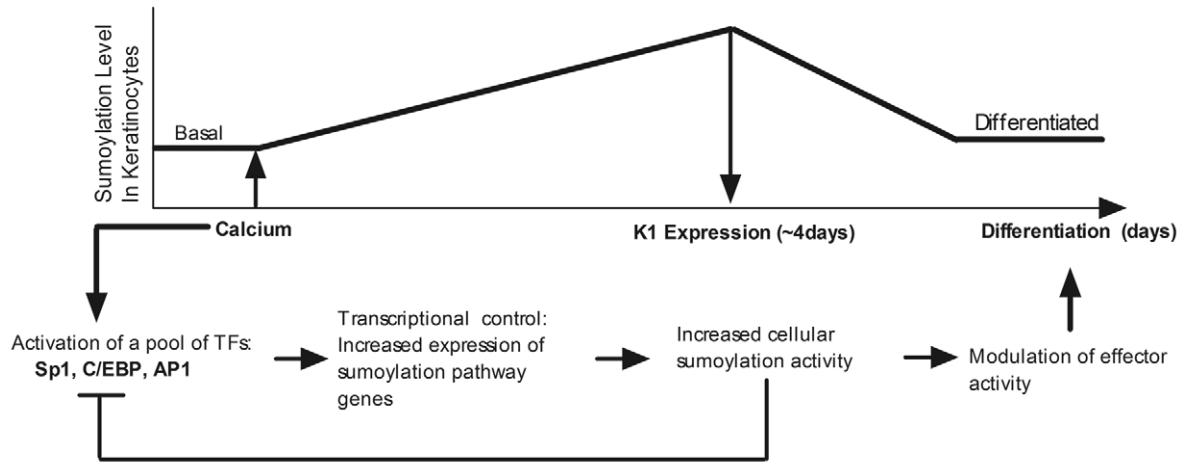
Fig. 8. Model for differentiation-dependent changes in the sumoylation system. The model proposes a negative-feedback mechanism to explain the transient increase in sumoylation observed during HaCaT cell differentiation. Initially, a pool of TFs, including Sp1, C/EBP and AP1, which are known to be directly stimulated by Ca^{2+}-induced differentiation, cause upregulation of the sumoylation system. As sumoylation activity increases, these TFs are in turn modified by SUMO conjugation, which decreases their transcriptional activity and leads to a decline in expression of the sumoylation pathway genes. A second feature of the model is that increased sumoylation is an active contributor to the differentiation process, through SUMO conjugation to downstream effectors of the differentiation signals. Additional details are provided in the text.

GFP, induced with Ca^{2+}, and then treated with 100 mM TSA at 24 hours after induction and infection. GFP expression was prolonged in the TSA-treated cultures compared with the untreated cells, consistent with TSA-mediated enhancement of transcription through HDAC inhibition (data not shown). However, no abnormal morphology was observed during differentiation in TSA, indicating that HDAC inhibition does not contribute to the Gam1 effect on differentiating keratinocytes (Fig. 7A).

In addition to keratinocyte morphology, the effect of Gam1 expression on the K1 differentiation marker was also evaluated. Gam1 caused a delay in and reduction of K1 expression in Ca^{2+}-induced cells (Fig. 7C). Although we cannot exclude the fact that an unknown, off-target effect of Gam1 might also contribute to both the defects in morphology and marker expression, the simplest explanation is that these defects are related to the dramatic inhibition of sumoylation. Altogether, the results presented here suggest that modification with SUMO might be a key event for the proper timing of the steps leading to keratinocyte differentiation, and that sumoylation may be an important regulator for this biological process.

Discussion

Recent studies on myoblast differentiation (Riquelme et al., 2006), embryonic development (Yamaguchi et al., 2005), genital tissue maturation (Vigodner et al., 2006) and synaptic formation (Shalizi et al., 2006) highlight sumoylation not only as a modifier of individual proteins but also as a global regulator of cellular development. In the present study, we investigated the role of sumoylation in another complex process, keratinocyte differentiation. Using an immortalized human keratinocyte line (HaCaT cells), we demonstrated that there was a coordinated transcriptional upregulation of many sumoylation system genes upon exposure to a high $[Ca^{2+}]$ environment, a well-established signal for induction of keratinocyte differentiation (Eckert et al., 1997b). In particular, gene expression for Ubc9, the sole conjugating enzyme for the

sumoylation process, was strongly activated. This transcriptional upregulation of Ubc9 and several other components of the sumoylation pathway led to increased expression of their respective proteins and to changes in the overall pattern of sumoylated substrates. Furthermore, in the absence of normal sumoylation, expression of a differentiation marker, K1 protein, was delayed, and normal keratinocyte morphogenesis was severely perturbed. In agreement with the HaCaT studies, both Ubc9 and SUMO proteins were expressed in organotypic cultures of normal human foreskin keratinocytes, consistent with an active sumoylation process in this stratified tissue. We also noted that Ubc9 immunostaining was most prominent in the intermediate layers of the epithelium just above the basal layer, suggesting increased accumulation of Ubc9 in early differentiation similar to that seen in Ca^{2+}-induced HaCaT cells. These combined results strongly suggest that sumoylation helps to orchestrate proper steps of keratinocyte differentiation and is therefore a key regulator in this biological event.

One of the salient observations of our studies was the coordinated transcriptional activation of multiple sumoylation genes following exposure of basal HaCaT cells to high $[Ca^{2+}]$ culture conditions. Mechanistically, such coordination could be achieved through common promoter elements responding to Ca^{2+}-induced TFs. High $[Ca^{2+}]$, both in vitro and in vivo, is known to trigger keratinocyte differentiation through multiple signaling pathways that activate a set of effector TFs (Fuchs, 1990; Sharpe et al., 1989), which includes NFAT, Sp1, C/EBP and AP1 (Eckert et al., 1997a; Santini et al., 2001). Although the promoters for the sumoylation component genes have not been experimentally characterized, bioinformatics analysis (www.genomatrix.de) of the putative promoter regions for the sumoylation genes tested in this study revealed the presence of binding sites for various combinations of the above TFs. There were single NFAT- and AP1-binding sites and three Sp1-binding sites on the SUMO2 promoter; five Sp1-binding sites on the Ubc9 promoter; three C/EBP-binding sites on the SAE1

promoter; and one Sp1-binding site on the predicted SAE2 promoter, consistent with all of these promoters being stimulated concurrently by Ca^{2+}-responsive TFs. Concordantly, SUMO1 transcription exhibited little or no change upon Ca^{2+} stimulation in HaCaT cells, and analysis of the SUMO1 putative promoter region did not reveal any binding sites for TFs directly activated by Ca^{2+}. Finally, the diversity of TF binding sites on the SUMO system promoters could also account for the quantitatively different transcriptional responses among the SUMO components that did respond to Ca^{2+} signaling. Detailed promoter studies will be needed to determine which, if any, of these predicted TF binding sites are directly influencing transcriptional expression of the sumoylation genes.

The observed upregulation of the sumoylation system appears to be a transient process associated with active differentiation, because overall transcript and protein levels for the sumoylation components vary only slightly between cells stably maintained in basal versus differentiated conditions (Fig. 2). Since the differentiating HaCaT cells remain normally proliferative, it is likely that the sumoylation changes reflect differentiation-dependent events and not changes related to growth state. We propose a simple model (Fig. 8) for downregulating the sumoylation response based on the known repressive effect that sumoylation exerts on the activity of most TFs (Gill, 2003). The relevant keratinocyte early differentiation transcription factors, SP1, C/EBP and AP1, are all known to be SUMO targets that are negatively regulated by sumoylation (Bossis et al., 2005; Eaton and Sealy, 2003; Spengler and Brattain, 2006; Terui et al., 2004). As sumoylation activity increases following transcriptional upregulation of the SUMO pathway genes, it is likely that the above TFs would become SUMO modified and repressed, leading to a decline in transcription of the sumoylation genes. This negative feedback system would dampen the sumoylation response as differentiation progresses resulting in a return to a basal level of sumoylation activity in terminally differentiated cells.

Our transcriptional results also suggest that there are different roles for SUMO2/3 versus SUMO1 in keratinocyte differentiation, because we observed activation of the SUMO2 and SUMO3 promoters, but not of the SUMO1 promoter. Additionally, we observed an increase in SENP1 protein levels during differentiation. SENP1 cleaves the precursor forms of all three SUMOs to produce their mature forms, but also exhibits preferential desumoylating activity for SUMO1-modified substrates compared with SUMO2 and SUMO3 conjugates (Xu and Au, 2005). The net result of the transcriptional and proteolytic changes may be an overall decrease in SUMO1 conjugates with a corresponding increase in SUMO2 and SUMO3 modified proteins. Although the biological significance of such a change in SUMO type utilization is not known, growing evidence supports distinct functions for SUMO2 and SUMO3 versus SUMO1. It is well established that SUMO1 has only 50% identity with SUMO2 and SUMO3, whereas SUMO2 and SUMO3 are closely related (Saitoh and Hinchey, 2000). In a recent proteomics study, we demonstrated significant differences between the array of targets modified by SUMO1 versus SUMO3, indicating that these two modifications are not equivalent with regard to substrate preference (Rosas-Acosta et al., 2005b). Other studies have shown SUMO type-specific differences in cellular

localization (Ayaydin and Dasso, 2004), response to external stimuli (Manza et al., 2004), and preference for SUMO ligases (Rosas-Acosta et al., 2005a; Tatham et al., 2005) and proteases (Gong and Yeh, 2006; Reverter and Lima, 2004), reinforcing the concept that the individual SUMOs have both common and unique biological activities. Intriguingly, there is evidence that SUMO1 is more specific for nuclear substrates whereas SUMO2 and SUMO3 targets are both nuclear and cytoplasmic proteins (Manza et al., 2004). Cytoplasmic events, such as formation of desmosomal junctions and specific cytoskeletal rearrangements, mark important aspects of keratinocytes differentiation (Hennings and Holbrook, 1983; Yin and Green, 2004). Preventing sumoylation by Gam1 expression during HaCaT cell differentiation led to a severe disruption of cell shape, implicating sumoylation as requisite for these morphological changes. A direct cytoplasmic role for SUMO2 and SUMO3 modification may contribute to the dramatic cytoskeletal changes that accompany keratinocyte differentiation.

In conjunction with a possible direct modification of cytoplasmic proteins, the observed increase in sumoylation activity following Ca^{2+} signaling likely contributes broadly to the keratinocyte differentiation process through effects on nuclear TFs (Fig. 8). A recent publication by Riquelme et al also demonstrated a sumoylation requirement during myogenesis (Riquelme et al., 2006): when the sumoylation system was inhibited, myoblast fusion-deficient phenotypes were observed. Although in their system they demonstrated an overall decrease of sumoylation during myogenesis, they showed that further decreasing sumoylation using a siRNA to Ubc9 dramatically affected overall myogenic differentiation and cellular fusion. Additionally, Shalizi et al. demonstrated the importance of sumoylation in a neuronal differentiation system. Using granule neurons, they showed that the MEF2 TF is activated through a Ca^{2+} signaling pathway in these cells, and that sumoylation of MEF2 is necessary to promote differentiation of dendritic claw structures (Shalizi et al., 2006). Thus, both these studies and our own strongly support an important role for sumoylation in cellular differentiation. Because inactivation of proliferation-enhancing TFs, such as p63 (Ghioni et al., 2005), has already been shown to be a prerequisite for keratinocyte differentiation, we propose that enhanced sumoylation facilitates this inactivation through direct modification of these or other targets. Ongoing proteomic studies in our laboratory are cataloging and identifying substrates whose sumoylation status changes during HaCaT differentiation (data not shown).

In conclusion, this study identifies sumoylation as a potential regulator in skin biology. Ca^{2+} signaling stimulates expression of the sumoylation system leading to changes in the sumoylation state of target proteins and allowing proper keratinocyte differentiation. Ca^{2+} signaling is also an important mediator of lymphocyte activation and differentiation (Freedman, 2006), oesteoblastic activity and bone formation (Henriksen et al., 2006), neuronal stem cell development (D'Ascenzo et al., 2006) and smooth muscle formation (Wamhoff et al., 2006). Although the intermediate signals are largely unknown for these processes, our work suggests that sumoylation may be modulated in each of these systems through Ca^{2+}-responsive transcription factors. Therefore, the interaction between Ca^{2+} signaling and the sumoylation system

may also be playing a crucial role for proper development of these and other tissues. Lastly, many skin diseases, such as psoriasis, skin cancer (Eckert et al., 2004) or infections resulting from microbial pathogens (Alfandari et al., 1999), hijack the normal process of keratinocyte differentiation and lead to abnormal skin formation. If dysregulation of sumoylation is contributing to an aberrant differentiation process, then modulating sumoylation might have therapeutic benefits for the treatment of these diseases.

Materials and Methods
Cell culture
HaCaT cells (kindly provided by Gary Bokoch, The Scripps Research Institute, La Jolla, CA) were cultured in Ca^{2+}-free DMEM (HyClone), with Ca^{2+}-depleted 10% FBS (Gemini Bioproducts), 4 mM L-glutamine (HyClone), and supplemented with calcium chloride to a final concentration of 0.03 mM (low $[Ca^{2+}]$ medium) or 2.8 mM final concentration (high $[Ca^{2+}]$ medium). FBS was Ca^{2+} depleted by incubation with Chelex 100 resin (Bio-Rad Laboratories) for 1 hour at 4°C according to the manufacturer's protocol. The Chelex was subsequently removed using a 50 ml Millipore 0.22 μm filter unit system (Millipore Corp.). To obtain cells exhibiting a basal keratinocyte phenotype, HaCaT cells were cultured in low $[Ca^{2+}]$ medium for at least 3 weeks and were maintained in the same culture conditions thereafter. Low $[Ca^{2+}]$ HaCaT cultures were never allowed to exceed 85% confluency. For stably differentiated HaCaT cells, the cultures were maintained in high $[Ca^{2+}]$ medium for at least 3 weeks before analysis. HaCaT cultures in either medium remained fully proliferative with similar doubling times (unpublished observations). Human embryonic kidney 293A (HEK293A) cells (Invitrogen Corp.) were maintained in DMEM supplemented with 10% FBS (Gemini Bioproducts). For the trichostatin A (TSA) experiment, HaCaT cells were treated with 100 mM TSA (Tocris Bioscience) 24 hours after infection with an adenoviral vector expressing GFP (Ad-GFP) and Ca^{2+} induction. HaCaT cells were observed up to 144 hours post Ca^{2+} induction.

RT-PCR and quantitative RT-PCR
All RNAs were extracted using the RNAqueous kit (Ambion). RNA concentration was measured using a spectrophotometer and 5 ng/μl aliquots were stored at –80°C until use. All primers were designed to overlap exon-exon junctions, therefore only amplifying the cDNAs targets and not the genomic sequences (primer sequences are provided in the supplementary material, Tables S1 and S2). The one-step RT-PCR mixture contained 50 ng RNA in a final reaction volume of 25 μl. The mixture also included 2.5 μl of 10× Taq DNA Polymerase buffer (Promega), 50 U MMLV reverse transcriptase, 1 U Platinum Taq (Invitrogen), 4 mM dNTPs, 20 U RNAse OUT (Invitrogen), 6 mM $MgCl_2$ and 0.4 μM of each primer. The one-step RT-PCR was performed for 20 minutes at 42°C for the reverse transcription step, followed by 90 seconds at 94°C, and 40 cycles of amplification (94°C for 30 seconds; 60°C for 30 seconds; 72°C for 60 seconds). Amplifications were performed in a PTC-200 Peltier Thermal Cycler machine (MJ Research). Amplified products were analyzed on 2.5% agarose gels and visualized with an Innotech Alphaimager system (Alpha Innotech). For the quantitative RT-PCR (Q-PCR), one step RT reactions were performed in a single well using 50 ng of harvested RNA in a 50 μl final volume. In addition to the RT components described above, reactions for Q-PCR contained 0.2 μM of LUX primers (designed with the Invitrogen custom primer software), 20 U RNAse OUT, 2 μl of Super Script III enzyme solution (which includes MMLV-RT, Platinum Taq, and dNTPs), and 1 μl Rox dye, all from Invitrogen. The LUX β-actin primer set (Invitrogen) was used to detect the internal control gene. The PCR reaction conditions for Q-PCR were the same as the RT-PCR described above. The Q-PCR plates were read with an ABI 7500 real-time PCR instrument (Applied Biosystems), and detection of the FAM or JOE label was recorded during the 72°C step. Results were graphed as the fold increase of the relative quantitative RQ values where $RQ=2^{(-\Delta\Delta Ct)}$. ΔCt was calculated as the average Ct for β-actin minus the average Ct for the gene of interest, with each sample being run in duplicate. Using the ΔCt values from each time point, the $\Delta\Delta Ct$ for each mRNA examined was calculated as the ΔCt of the time 0 sample minus the ΔCt of time x (in hours) after induction. The data shown are the average from at least three independent RNA preparations collected in separate experiments.

Affinity-purified polyclonal antibodies against Ubc9
Rabbit polyclonal serum 12741 was produced in-house using affinity-purified Ubc9 as the immunogen. A four immunization regime was followed, and 2 weeks after the final boost the rabbit was exsanguinated. To affinity purify anti-Ubc9 antibodies, 750 μg affinity-purified GST-Ubc9 were diluted in a final volume of 500 μl using 1× PBS, and the resulting dilution was dispensed on an 82-mm-diameter, 0.45-μm-pore-size Protran Nitrocellulose filter (Schleicher & Schuell). The membrane was dried for 30 minutes at room temperature, then re-wetted and blocked by incubation at room temperature for 30 minutes in 15 ml of 1× PBS supplemented with 1% BSA. The blocked membrane was incubated for 2 hours at room temperature with

10 ml of a solution containing 2.5 ml of rabbit polyclonal serum 12741 and 7.5 ml of 1× PBS supplemented with 10 mg/ml BSA and 0.05% Tween 20. The membrane was subsequently washed four times each with 15 ml of 1× PBS supplemented with 0.05% Tween 20, and four additional times each with 15 ml of 1× PBS alone. The bound antibodies were eluted by incubation with 2 ml of elution buffer (100 mM glycine, pH 2.5, 0.02% NaN_3) for 5 minutes at room temperature. The eluted antibodies were neutralized with 200 μl of 1.0 M Tris-HCl (pH 8.0), aliquoted, and stored at –70°C. The purity of the affinity-purified antibodies was determined by gel electrophoresis and immunoblotting, and its reactivity against Ubc9 was determined by immunoblotting using purified Ubc9 and unfractionated mammalian cell extracts.

Immunohistochemistry
Human foreskin keratinocytes were grown in organotypic raft cultures to form stratified epithelium as previously described (Lambert et al., 2005). All chemicals used for immunohistochemistry were from Biocare Medical (Concord, CA). Slides were deparaffinized in xylene and rehydrated according to the manufacturer's protocols. The following antibodies and dilutions were used: anti-SUMO serum 12783 (Rosas-Acosta et al., 2005a), 1:750; affinity purified polyclonal antibodies against Ubc9, 1:20; and polyclonal anti-human keratin 1 (anti-HK1, Covance, Berkeley, CA), 1:5000. Specificity control blocking experiments were performed by adding the corresponding purified proteins (25 μg of Ubc9 or 7 μg of SUMO1) to the cognate antibody dilution. The SUMO and Ubc9 proteins used in the experiments were purified as previously described (Rosas-Acosta et al., 2005a). After immunostaining, the slides were counterstained with hematoxylin.

Immunoblots and densitometry
Total cell extracts were prepared by adding a 1:1 (v/v) mixture of RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 15 mM $MgCl_2$, 1% NP40, 0.1% SDS, 1 mM DTT, 1:200 protease inhibitor cocktail and 10 mM N-ethylmaleimide) and 4× sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 8% SDS, 0.02% bromophenol blue, 4% β-mercaptoethanol) directly to the cells. The cells were shaken gently for 5-10 seconds, and the resulting lysate was collected by pipetting. Samples were heated at 95°C for 5 minutes and sonicated for 30 seconds using a Misonix sonicator 3000 (Misonix). Samples were resolved on 10% or 12.5% polyacrylamide gels and then transferred onto 0.45 μm Immobilon-P membranes (Millipore). The membranes were blocked for at least 15 minutes with 3% non-fat milk in TTBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.005% Tween 20), and incubated for 1 hour or overnight with the primary antibodies listed below at the indicated dilution: rabbit anti α-tubulin (Santa Cruz Biotechnology), 1:15,000; anti-Myc monoclonal antibody (Santa Cruz Biotechnology), 1:500; anti-RanGAP monoclonal antibody (Zymed/Invitrogen Corp.), 1:2500; rabbit serum 12783 against SUMO (Rosas-Acosta et al., 2005b), 1:1000; affinity-purified polyclonal antibodies against Ubc9, 1:500; anti-human K1 rabbit serum (Covance), 1:1000; anti-SAE1 sheep serum (Axxora), 1:2000; anti-SENP1 rabbit serum (Imgenex), 1:2000; and anti-involucrin rabbit serum (LabVision), 1:1000. After reaction with the primary antibodies, the membranes were incubated with Horseradish Peroxidase-conjugated antibodies (Santa Cruz Biotechnology) at 1:10,000 for 1 hour. The membranes were subsequently rinsed in TTBS, treated with the Western Lightning Chemiluminescence reagent (PerkinElmer Life and Analytical Sciences), and then visualized with X-ray film. Quantitative differences were determined by densitometry using an Innotech Alphaimager (Alpha Innotech) and were normalized to the α-tubulin signal. Quantitative results are the average of at least three separate experiments.

Virus production and infection
Myc-tagged Gam1 adenoviral DNA (Ad-Gam1) was kindly provided by Matt Cotten (GPC-biotech, Munich, Germany). The Ad-Gam1 DNA was transfected into HEK 293A cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. Cells were lysed by freeze-thaw and the supernatant was collected. The initial viral stock was subsequently amplified with two rounds of infection on HEK 293A cells and the final freeze-thaw supernatant collected as the high-titer stock. Adenovirus expressing GFP (Ad-GFP) was kindly provided by G. Davis (Texas A&M Health Science Center, College Station, TX) and amplification was performed as above. Titer was assessed by the limiting-dilution method (Qbiogene Adenovirus Manual, version 1.4) using HEK 293A cells plated at $1×10^6$ cells/well on six-well plates. For adenovirus experiments, HaCaT cells maintained in low $[Ca^{2+}]$ medium were plated at $5×10^6$ in T75 flasks 20 hours before infection. Each culture was infected at an MOI of 300 in 3 ml of Ca^{2+}-free medium supplemented with 8 μg/ml polybrene (Fisher Scientific). Three hours after infection the medium was removed, cells were trypsinized, and released cells were resuspended in 10 ml of high $[Ca^{2+}]$ medium to induce differentiation. The resulting cells suspension was split among the wells of a six-well plate at a ratio such that the cells in each well would achieve approximately 80% confluency by the time of collection. Cells were maintained in high $[Ca^{2+}]$ medium and harvested at various times post plating by direct lysis in the wells using the 1× RIPA:4× sample buffer mixture described above. Proteins were analyzed by immunoblotting as described in the previous section. Cells were visualized by phase contrast and fluorescence

microscopy at a magnification of 200× using an Olympus IX70 microscope. Images were captured digitally using a Qcolor3 camera (Olympus).

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