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HPV E6 Proteins Target Ubc9, the SUMO Conjugating Enzyme

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Abstract

The human papillomavirus oncogenic protein, E6, interacts with a number of cellular proteins, and for some targets, E6 directs their degradation through the ubiquitin-proteasome pathway. Post-translational modification with ubiquitin-like modifiers, such as SUMO, also influences protein activities, protein-protein interactions, and protein stability. We report that the high risk HPV E6 proteins reduce the intracellular quantity of the sole SUMO conjugation enzyme, Ubc9, concomitant with decreased host sumoylation. E6 did not significantly influence transcription of Ubc9, indicating that the effects were likely at the protein level. Consistent with typical E6-mediated proteasomal degradation, E6 bound to Ubc9 *in vitro*, and required E6AP for reduction of Ubc9 levels. Under stable E6 expression conditions in differentiating keratinocytes there was a decrease in Ubc9 and a loss of numerous sumoylated targets indicating a significant perturbation of the normal sumoylation profile. While E6 is known to inhibit PIASy, a SUMO ligase, our results suggest that HPV E6 also targets the Ubc9 protein to modulate host cell sumoylation, suggesting that the sumoylation system may be an important target during viral reproduction and possibly the subsequent development of cervical cancer.

Keywords

SUMO; Ubc9; human papillomavirus; E6; HaCaT; proteomics

1. Introduction

Papillomaviruses infect skin epithelium and are responsible for a wide variety of skin abnormalities ranging from skin warts to cervical carcinoma (DiMaio and Liao, 2006; Weaver, 2006). Expression of the two viral oncogenic proteins, E6 and E7, hijacks the normal process of keratinocyte differentiation and maintains differentiated keratinocytes within the cell cycle (McMurray et al., 2001). The high risk HPV E6 proteins form a trimeric complex with p53 protein and E6AP, an ubiquitin ligase, (Ro et al., 2006). This association enhances p53 degradation through the ubiquitin-proteasomal route, resulting in loss of the p53-mediated cell cycle arrest and apoptotic functions (Doorbar, 2006). Furthermore, E6 has also been shown to associate with and enhance degradation of other important cellular proteins, including the PDZ domain proteins (Matsumoto et al., 2006),

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E6TP1 (Gao et al., 2001), procaspase 8 (Filippova et al., 2007), and PTPN3 (Jing et al., 2007). The combined action of E6 through degradation of tumor suppressors and other cellular regulatory proteins reduces apoptotic responses and contributes to keratinocyte immortalization.

The ubiquitin system is part of a large superfamily that includes ubiquitin and all the other ubiquitin-like modifiers, among them SUMO, a small protein of about 12 kD (Kroetz, 2005). SUMO, like ubiquitin, is conjugated to cellular proteins using a series of cellular enzymes including SAE1/SAE2, an heterodimeric activating enzyme; Ubc9, the only known conjugating enzyme; and several ligases (including the PIAS proteins, RanBP2, and Pc2) which determine substrate specificity and help to enhance the rate of conjugation (Hay, 2005). Sumoylation has a broad range of effects on target proteins such as influencing the conjugate's sub-cellular localization, activity, or binding partners (Geiss-Friedlander and Melchior, 2007; Wilkinson and Henley, 2010). In the case of transcription factors, a major class of protein targeted by sumoylation, SUMO addition generally reduces their transcriptional activities (Gill, 2005).

In addition to its pleiotropic roles in cellular biology, SUMO also has been shown to play an important role in viral infections (Boggio and Chiocca, 2006; Deyrieux A. and Wilson, 2009). Many viral proteins are SUMO modified and have their activities influenced by SUMO; for example, Ad-E1B (Lethbridge et al., 2003), HHV-6 (Gravel et al., 2004), HCMV-IE1/IE2 (Hofmann et al., 2000), EBV-Z (Adamson, 2005), and HPV E2 (Wu et al., 2009; Wu et al., 2008) are all SUMO modified and this affects their stability and/or transactivation activity. While it is clear that many viral proteins are themselves SUMO modified, viral modulation of host sumoylation is less well studied. Viral modulation of the sumoylation system can be thought of as any change to the sumoylation system or its targets that would benefit the virus. This could be a redistribution of the sumoylation machinery, an overall decrease or increase in sumoylation, or alteration in the sumoylation status of specific substrates. Several reports demonstrated that herpesvirus immediate early proteins such as ICP0 (Bailey and O'Hare, 2002), IE1 (Muller and Dejean, 1999), and Z protein (Adamson and Kenney, 2001) could reduce the sumoylation of specific SUMO targets such as PML or SP100. Z protein appears to act simply as a substrate competitor (Adamson, 2005) and ICP0 appears to recruit a SUMO protease to the PML bodies (Bailey and O'Hare, 2002), but other mechanisms may also be involved. Subsequently, GAM1, an early gene product from the CELO avian adenovirus, was shown to decrease overall host sumoylation (Boggio et al., 2004). GAM1 binds to the sumoylation activating enzyme (SAE1/2) and recruits ubiquitin ligase activity leading to proteasomal degradation of the activating enzyme (Boggio et al., 2007). Loss of SAE1/2 also destabilizes the conjugating enzyme, Ubc9, resulting in cessation of sumoylation and accumulation of unmodified substrates which may lead to an enhancement of transcription (Boggio and Chiocca, 2005). More recently, HPV E7 was shown to block pRb sumoylation through protein interaction (Ledl et al., 2005), and HPV E6 was shown to inhibit activity of a SUMO ligase, PIASy (Bischof et al., 2006). In contrast to inhibition of sumoylation, the K-bZIP protein of Kaposi's sarcoma-associated herpesvirus (KSHV) has a SUMO2/3 ligase activity that enhances sumoylation of K-bZIP and its binding partners (Chang et al., 2010). Similarly, the Ebola Zaire VP35 protein undermines the innate immune system by enhancing sumoylation of IRF7 which reduces its transcriptional activity and decrease interferon expression (Chang et al., 2009). In this case VP35 has no SUMO ligase activity, but recruits the sumoylation machinery to IRF7 through VP35's ability to interact with both Ubc9 and SUMO ligase, PIAS1. Thus, it is clear that both DNA and RNA viruses have developed means to interfere with or influence sumoylation in order to create a cellular environment that favors the viral life cycle. In this study, we have extended these observations by showing that high risk HPV E6 oncoproteins are capable of targeting the sumoylation system through reducing Ubc9 levels. The resultant

decreased sumoylation of certain host proteins may be mechanistically important for viral reproduction and/or transformation.

2. Materials and methods

2.1. Cell culture

Human embryonic kidneys 293A cells (Invitrogen Corp), C33A cervical carcinoma cells, and HeLa cells were maintained in low sodium bicarbonate DMEM with 10% FBS. The medium was replaced every two to three days, and cells were subcultured before reaching 90% confluency. H1299 and K3 cells were maintained as previously described (Kuballa et al., 2007). The HaCaT, HaCaT E6, and parental cell line HaCaT FRT/TR#8 were maintained in calcium-free DMEM supplemented with 10% Benchmark FBS (Gemini Bio; serum was treated with Biorad chelex to remove calcium from serum prior to adding to media), 4 mM glutamine, and either 0.03mM calcium (low) or 2.38mM calcium (high) depending on induction conditions. The HaCaT SNAP-SUMO and HaCaT SNAP-SUMO/16E6 cells were maintained in the same medium except that 10% tetracycline screened FBS (HyClone; chelex treated as for the Benchmark serum) was used in place of Benchmark FBS. To maintain selection for the various transgenes the cell lines were propagated continuously in antibiotics as follows: the HaCaT E6 line was maintained in 400 µg/ml G418; the HaCaT FRT/TR#8 line was maintained in 100 µg/ml zeocin and 10 µg/ml blasticidin; the HaCaT SNAP-SUMO line was maintained in 100 µg/ml hygromycin and 10 µg/ml blasticidin; and the HaCaT SNAP-SUMO/16E6 line was maintained in 100 µg/ml hygromycin, 10 µg/ml blasticidin, and 400 µg/ml of G418. To induce the expression of SNAP-SUMO cells, tetracycline was added to the medium at a concentration of 1 µg/ml 48 hours prior to harvesting the sample. Media and antibiotics were changed daily after tetracycline induction to ensure fresh tetracycline for induction. All cells were grown at 37°C in a 5% CO₂ environment.

2.2. Plasmids and expression constructs

The expression vectors for SUMO1 and Ubc9 (HA-tagged) have been described previously (Rosas-Acosta et al., 2005b). For *in vivo* expression of other proteins, plasmids were provided by Dr. P. Angeletti (C1-GFP HPV16-E6; (Vaeteewoottacharn et al., 2005), Dr. Z.-M. Zheng (pGFP-HPV6-E6; (Tao et al., 2003)), Dr Q. Wei (pGFP-HPV18-E6; (Wei, 2005)), and Dr. J. Mymrk (pEBG-16E7; (Bernat et al., 2003)). The vector for *in vitro* expression of HPV 18E6 (pSP64-18E6; (Thomas et al., 2001)) was provided by Drs. L. Banks. The Flag-p53 plasmid was obtained from Addgene.

2.3. Transfections

Eighteen hours prior to transfection, 293A cells were plated at 9×10^5 cells/well in a 6-well plate format, and plasmid DNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol with the amount of reagent reduced to only 8 µl per well. Transfection mixtures were prepared in DMEM without serum then added to each well containing cells in 500 µl of complete medium. An additional 2 ml of complete medium was added to the wells at 5 hours after transfection. 293A cells were collected 32 hours post transfection. C33A were plated at 2.5×10^6 cells/well in the 6-well plate format and were transfected as for 293A cells. For the HeLa siRNA transfections, 2×10^5 cells were transfected with 0.125 pmoles of E6 siRNA or the Negative Universal Control siRNA using Lipofectamine 2000 according to the manufacturer's protocol.

2.4. Protein collection and immunoblots

For the 293A and C33A transfection studies total cell extracts were obtained by adding 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 min and sonicated for 15 seconds using a Misonix sonicator 3000 (Misonix). Samples were resolved on 10% or 15% SDS polyacrylamide gels and then transferred to 0.45 μm Immobilon-P membranes (Millipore). The membranes were blocked for at least 1 min with 3% non-fat milk in TTBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.005% Tween 20), and incubated for 45 min or overnight with the primary antibodies listed below at the indicated dilution: rabbit serum 12783 against SUMO (Rosas-Acosta et al., 2005a) at 1:1000 and goat anti-GST antibody (Amersham Biosciences) at 1:5,000. Antibodies from Santa Cruz Biotechnology included rabbit anti α-tubulin, 1:10,000; mouse HA antibody, 1:10,000; mouse anti-GFP, 1:10,000; and mouse anti-HPVE2, 1:5,000. The anti-Flag antibody was from Sigma-Aldrich and was used at a 1:2000 dilution. After reaction with the primary antibodies, the membranes were incubated with Horseradish Peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) at 1:10,000 for 1 hour. The membranes were then rinsed in TTBS, treated with the Western Lightning Chemiluminescence reagent (PerkinElmer Life and Analytical Sciences), and exposed to X-ray film.

The effects of HPV 16E6 on p53 and Ubc9 levels in parental HaCaT cells and the various derived cell lines were examined by plating 5×10^5 cells and harvesting 48 hours later with boiling 4 X SDS sample buffer followed by passage through a 27 gauge syringe ten times. For K1 samples the cells were plated so that at 96 hours post differentiation in 6 cm dishes the cells would be 80–85% confluent. Samples for K1 analysis were harvested similar to the samples for Ubc9 and p53. All samples then were boiled and loaded onto 8% (p53 and K1) or 12% (Ubc9) SDS polyacrylamide gels. The gels were transferred to 0.2 μm PVDF membrane at a constant 1.2 Amps. The membranes were blocked overnight with 5 % nonfat dry milk (NFD) and then probed with either anti-p53 (Santa Cruz Biotech) at 1:1,000, anti-K1 at 1:1,000, or anti-Ubc9 antibodies at a 1:500 dilution in 5 % NFD milk. Tubulin served as a loading control using anti-tubulin (Santa Cruz Biotech) at a 1:2,000 dilution. Secondary antibodies were used at a 1:2,000 dilution and detection was done with the SuperSignal West Pico or West Femto detection kit (Pierce) using an Alpha Innotech imager at a medium sensitivity/medium resolution setting.

2.5. Protein expression and purification

GST and GST-Ubc9 were expressed using pGEX-5X-1-based expression plasmids as previously described (Rangasamy and Wilson, 2000). Twelve ml of overnight pre-cultures were inoculated into 500 ml of 2XYT broth supplemented with 100 μg/ml of ampicillin. Cultures were incubated at 37°C until they reached an $OD_{600}=1.0$ followed by induction by addition of isopropylthiogalactoside to 0.5 mM. Induced cultures were incubated at 25°C for 4 hours then harvested by centrifugation. After collection, the cells were lysed in lysis buffer (phosphate buffered saline, pH 7.3, 5 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 15 mg/ml lysozyme). The lysate was kept on ice for 30 min followed by sonication three times at 36 watts. After sonication, Triton X-100 was added to the lysates to a final concentration of 1% followed by centrifugation at $10,000 \times g$ at 4°C for 30 min. The supernatant was incubated with 600 μl of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) at 4°C for 1 hour. The glutathione-Sepharose beads were washed four times with phosphate buffered saline, pH 7.3. Bound GST proteins were eluted with 20 mM reduced L-Glutathione (Sigma) in 100 mM Tris-HCl, pH 8.0, 120 mM NaCl. The eluted proteins were then adjusted to 10% glycerol and stored at -70°C .

2.6. In vitro GST pull-down assays

The HPV 18E6 protein was expressed from a pSP6 plasmid using the SP6-coupled rabbit reticulocyte lysate system in the presence of [³⁵S] cysteine. The HPV E7 protein was expressed from a pSG5 vector using the T7-coupled rabbit reticulocyte lysate system in the presence of [³⁵S] methionine according to the manufacturer's instructions (Promega). Luciferase and bovine papillomavirus E1 were *in vitro* translated with the T7 system for use in the binding assays as a negative and positive control for Ubc9 interaction, respectively. For the GST pull-down assay, GST (8 ug) or GST-Ubc9 (8 ug) were pre-incubated with glutathione-Sepharose beads for 1 hour at room temperature in binding buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 0.05% Tween 20, 0.5% bovine serum albumin). Five µl of ³⁵S-labeled protein was then added, and incubation was continued for another 2 hours. The beads were washed twice with binding buffer and another three times with wash buffer (10 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.05% Tween 20) supplemented with 200 mM NaCl, 300 mM NaCl, or 150 mM NaCl, respectively. Labeled proteins bound on the beads were eluted by heating at 95 °C in 10 µl of SDS-sample buffer (150 mM Tris-HCl, pH 6.8, 12% SDS, 30% glycerol) and subsequently analyzed by electrophoresis on a 12.5% SDS-polyacrylamide gel. Radiolabeled products were visualized by autoradiography.

2.7. Creation of a Stable SNAP-SUMO Cell Line

The SNAP tag sequence was amplified from the pSS26m mammalian source plasmid (Covalys) using the forward and reverse primers: 5'-ATCGATAAGCTTGATATCACCATGGACAAAGACTGC-3' (T_m 62.2°C) and 5'-TATAAGCTTGCCAGCCAGGCTTGCCAGTC-3' (T_m 69.9°C). The resulting PCR product was then gel purified (Qiagen) and cloned into the pGEM-T Easy shuttle vector (Promega) to generate pGEM-SNAP. Chemically competent DH5α cells were transformed with the resulting plasmid, plated on LB plates containing 50 µg/ml of ampicillin, and incubated overnight followed by miniprep (Sigma Aldrich) isolation of the plasmid DNA. The pGEM-SNAP DNA and the pcDNA5FRT/TO/His-S-SUMO3 vectors (Rosas-Acosta et al., 2005b) were digested by HindIII for three hours. The digestion products were then gel purified, ligated using the Quick Ligation Kit (NEB) according to manufacturer's instructions, transformed into DH5α cells, and selected on LB plates containing 50 µg/ml of ampicillin. Colonies that were positive for the insert were then sequenced with the both forward (5'-GAAAACCGCCCTGAGCGGAAATCC-3' [T_m 62.6°C]) and reverse primers (5'-TCGCACCCAGACAGTTCCAGCTT-3' [T_m 62.9°C]) to ensure proper orientation. A plasmid exhibiting proper sequence and orientation was designated pcDNAFRT-TO-SNAP-His-S-SUMO. Final plasmid DNA was isolated and purified via a cesium chloride gradient.

The parental cell line, HaCaT FRT/TR#8, contains a single copy of the Flp-In T-REx integration cassette. The parental cells were plated at 30% confluency on 10 cm tissue culture plates and allowed to adhere overnight at 37°C and 5% CO₂. The following day 2.6 µg of pcDNAFRT-TO-SNAP-His-S SUMO DNA plus 21.4 µg of the Flp recombinase plasmid pOG44 were transfected via Lipofectamine 2000 (Invitrogen) into the HaCaT FRT/TR#8 cells to generate the HaCaT SNAP-SUMO line. The cells containing the inserted SNAP-His-S-SUMO cassette were selected with hygromycin and blasticidin. After two weeks of selection cells were pooled and stocks were frozen in liquid nitrogen. To ensure that the SNAP-tagged SUMO was expressed and functional for conjugation to substrate proteins, 4 × 10⁵ HaCaT SNAP-SUMO cells were plated on 6 cm plates and allowed to adhere overnight. Tetracycline was added the following day to induce expression of the SNAP-tagged SUMO, and induction was carried out for 48 hours. Cells were then lysed in 4 X SDS sample buffer and passed through a 27 gauge syringe ten times. Samples were electrophoresed on 8% SDS gels, transferred to PDVF membrane, and probed with S protein conjugated to HRP (Novagen) at a 1:1,000 dilution to visualize free and conjugated SNAP-

SUMO. Images were captured on Alpha Innotech Fluor Chem HD2 camera and Alpha Ease software. The HaCaT SNAP-SUMO cells were also checked against the parental HaCaT FRT/TR#8 line and against normal HaCaTs for cell cycle distribution and morphological characteristics (details of the production and characterization of the parental HaCaT FRT/TR#8 and the HaCaT SNAP-SUMO lines will be presented elsewhere).

2.8. 2D gel electrophoresis of proteins

HaCaT SNAP-SUMO and HaCaT SNAP-SUMO/16E6 cells (2.0×10^5) were plated on 6 cm dishes and allowed to adhere overnight. The next day the medium was changed from basal medium to high calcium to induce differentiation. Forty-eight hours prior to harvesting the samples, tetracycline was added to the medium to induce expression of SNAP tagged SUMO. After 96 hours of differentiation cells were labeled for 30 min with SNAP-Cell TMR-Star (NEB), washed and incubated in fresh medium for 30 min, and then processed immediately for analysis. Cells were washed twice with cold 1 X PBS and lysed in 2-D sample buffer containing 8 M urea, 4% CHAPS, 0.5% Pharmalytes pH 3–10, and 40 mM DTT. Samples were centrifuged at $12,400 \times g$ for two minutes to pellet cellular membranes and debris. Protein concentrations in the supernatants were calculated using the 2-D Quant Kit (GE Lifesciences) per the manufacturer's directions. Samples containing 200 μ g of protein were cleaned by methanol chloroform precipitation. The pellet was resuspended in 150 μ l of 2-D sample buffer and the samples were cup loaded onto pH 3–11 NL strips that were rehydrated overnight in rehydration buffer containing 8 M urea, 2% CHAPS, 0.5% IPG buffer, and 0.002% bromophenol blue. The first dimension was run on an Ettan IPGphor3 as follows: STP 500V for 3 hours, GRD 1000 V for 1 hour, GRD 8000 V for 2.5 hours, and STP 8000V for 0.5 hour for a total of 14 kVh on average. After isoelectric focusing the strips were equilibrated first in SDS equilibration buffer containing 6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue and 10 mg/ml DTT. This was followed by equilibration in the same SDS equilibration solution containing 25 mg/ml iodoacetamide in place of DTT. The strips were then sealed on top of a 6% polyacrylamide gel with an agarose sealing solution containing SDS Running buffer, 0.5% agarose, and 0.002% bromophenol blue. The second dimension was then run overnight at 15 Amps.

2.9. Imaging of 2D Gels

For fluorescent gels the samples were imaged with a Typhoon Trio variable mode imager using the 532 nm laser and the 580 nm Band Pass filter at 800 PMT at high sensitivity. Silver stained 2-D gels were stained with SilverQuest silver staining kit (Invitrogen) and imaged using the Alpha Innotech FluorChem HD2 imager with the Alpha Ease FC software.

2.10. Analysis of 2D gels

SNAP-labeled gels were analyzed using the Melanie 7 software. Gels were separated into two classes (HaCaT SNAP-SUMO and HaCaT SNAP-SUMO/16E6) with four gels each per group. Spots were matched within each group and then between the classes. Matched gels were then visually scrutinized to ensure the matches were accurate. To determine differences in protein levels between the two classes ANOVA analysis was performed. Any spot with a p value ≤ 0.05 was considered significant.

3. Results

3.1. High risk HPV E6 proteins target Ubc9

Previous work by our laboratory demonstrated that the sumoylation system plays a role in keratinocyte differentiation (Deyrieux et al., 2007). In addition, it is well established that

papillomaviruses disrupt the normal process of keratinocyte differentiation through the actions of E6 and E7 (Hebner and Laimins, 2006), though the mechanisms through which differentiation is disrupted are not completely defined. It has already been reported the 16E6 binds a SUMO ligase, PIASy, and inhibits its ligase activity (Bischof et al., 2006), but a broader exploration of E6 interactions with the sumoylation system has not been performed. Therefore, we wanted to determine if the E6 oncoproteins could target other components of the sumoylation system, as modulation of sumoylation might contribute to viral perturbation of keratinocyte properties. Quantitative RT-PCR examination of sumoylation system RNAs indicated that E6 had little or no effect at the transcriptional level (Supplemental Fig. S1). However, during co-transfection experiments with E6 and individual sumoylation components in 293A cells we observed that high risk HPV16 and 18 E6 proteins reduced protein levels of the SUMO conjugating enzyme, Ubc9, in a dose-dependent fashion. Parallel transfections with EGFP or 18E7 expression vectors had no effect on Ubc9 (Fig. 1A and Supplemental Fig S2), ruling out a non-specific transfection-related basis for the Ubc9 reduction. For these initial experiments, EGFP-E6 fusions were used so that the various E6 forms could be detected equivalently using anti-GFP. Immunoblots of total cell extracts probed with anti-GFP antibody revealed that both high risk HPV16 and 18 E6 proteins were expressed predominantly in the E6* form (Fig. 1B), suggesting that the E6* form might be sufficient for this activity, but this has not yet been explored in detail. In the blot shown there is no full-length 16E6 detectable, but in other transfections with this clone a faint full-length form is detectable. No effect on the SUMO activating enzyme, SAE, was observed with either 16E6 or 18E6 (data not shown).

Given that Ubc9 is the sole conjugation enzyme in the sumoylation pathway, we reasoned that E6 reduction of Ubc9 might also affect overall sumoylation. To examine the effect of E6 on total sumoylation, 293A cells were co-transfected with Ubc9 and SUMO1 with or without E6 co-transfection. Both 16E6 (Fig. 1C) and 18E6 (not shown) expression decreased overall levels of many sumoylated protein, consistent with the Ubc9 reduction having a functional impact on the cellular environment. Identical results were observed in C33A cells (not shown) and H1299 cells (see Fig. 3), so the ability of E6 to target Ubc9 appears to be a general property not restricted to a particular cell type.

To further investigate an effect of E6 on Ubc9 at the protein level, a GST pulldown experiment was performed to evaluate possible interaction between E6 and Ubc9. *In vitro* produced 18E6 effectively bound GST-Ubc9, but not GST alone (Fig. 1D). Similar interaction was seen with 16E6 and Ubc9 (Supplemental Fig. S3). These results indicate that high risk E6 can form a complex with Ubc9, though whether or not the interaction is direct or requires other factors present in the translation mix has not been determined.

The experiments performed in Figure 1 were conducted with EGFP-tagged E6 to facilitate detection and comparison of different E6 types. However, the presence of the large EGFP tag might alter E6 activity and somehow contribute to the observed reduction of Ubc9. To rule out artifactual effects, an untagged 16E6 was tested in parallel with the EGFP-16E6 (Fig. 2A), and the untagged E6 was just as effective as EGFP-E6 in reducing Ubc9. As the untagged E6 could not be detected in immunoblots (data not shown), its expression and functionality were confirmed by the ability to reduce p53 (Fig. 2B). Consequently, the EGFP tag does not appear to influence the E6 effects on Ubc9. Taken together, the results in Figures 1 and 2 support a role for high risk HPV E6 in down regulation of Ubc9 leading to reduced cellular sumoylation, and 16E6 was chosen for subsequent study.

3.2. E6 Requires E6AP to Target Ubc9

High risk E6 proteins have a well-established mechanism for reducing intracellular levels of specific proteins by targeting them for proteasomal degradation (Ghittoni et al., 2010). This

process involves E6 forming a complex with the target protein and the ubiquitin ligase known as E6AP. Since we showed in Fig. 1D that E6 can form a complex with Ubc9, we next asked if Ubc9 reduction is dependent E6AP. Parallel E6 transfections were performed into H1299 cells (the E6AP positive parental line) and K3 (a stable derivative of H1299 exhibiting reduced E6AP levels due to constitutive expression of an E6AP siRNA). As previously reported for these paired cell lines (Kuballa et al., 2007), high risk E6 proteins show reduced ability to degrade p53 in the K3 line compared to H1299 (Fig. 3A). Similarly, while the H1299 parental line shows both reduced Ubc9 expression and decreased sumoylation in the presence of exogenous 16E6, no effect on Ubc9 or sumoylation is observed in the E6AP-defective K3 line (Fig. 3B). We conclude from these results that Ubc9 reduction is likely due to proteasomal degradation mediated by E6 binding to Ubc9 and recruitment of E6AP. However, the degradation of E6 targets typically is prevented by proteasome inhibitors, but our attempts to rescue Ubc9 with common inhibitors were only partially successful (data not shown), so the mechanism for Ubc9 reduction still remains uncertain. Interestingly, low risk 6E6 had no effect on Ubc9 or sumoylation in either the H1299 or K3 cells (Fig. 3B), even though it was effectively expressed in both cell lines (not shown). The results with 6E6 suggest that Ubc9 degradation is a property restricted to high risk E6 proteins, though we have not yet explored this with other low risk E6 proteins.

3.3. Physiological levels of HPV E6 proteins are sufficient to reduce Ubc9 levels

Our transfection results illustrate an overall decrease in Ubc9 and sumoylation associated with exogenously expressed high risk HPV E6. To validate these results without over expression of E6, we examined E6 effects on Ubc9 and sumoylation in cell lines stably expressing E6. Initially we used HeLa cells expressing HPV 18E6, and we reduced E6 levels by siRNA knockdown (Fig. 4) or by exogenous expression of E2 to repress E6/E7 transcription (not shown). Knockdown of E6 was confirmed by the significantly higher levels of p53 in the E6 siRNA treated sample compared to the control siRNA. E6 siRNA treated cells also demonstrated increased levels of Ubc9 and an increase in overall sumoylated proteins (Fig. 4), suggesting that constitutive E6 expression in HeLa cells is sufficient to reduce Ubc9 and negatively impact sumoylation. Similar effects on sumoylation were seen by repressing E6 expression in HeLa cells via transfection of E2 while introduction of E2 into E6 negative 293 cells had no effect on sumoylation levels (data not shown).

One caveat to the HeLa experiments is that E6 reduction in this cell type leads to growth suppression and to apoptosis (Desaintes et al., 1997; Desaintes et al., 1999), and these pleiotropic effects might be modulating the sumoylation system indirectly rather than by preventing E6 mediated effects on Ubc9. To avoid this complication and develop cell lines amenable to more thorough analysis of sumoylation we constructed E6 expressing HaCaT cell lines. HaCaT cells are spontaneously immortalized human keratinocyte cells that our lab has previously established as a suitable model for studying sumoylation in keratinocytes (Deyrieux et al., 2007). A HaCaT cell line expressing HPV 16E6 was generated by infection with the LXSNI6E6 system (Halbert et al., 1991). The presence of HPV 16 E6 in the resultant polyclonal cell culture was not detectable by direct immunoblotting (data not shown), so E6 expression was verified through indirect markers including abnormalities of morphology, delayed differentiation marker expression, as well as diminished levels of p53. Normal HaCaTs exhibit steadily increasing levels of K1 up to 144 hours following induction of keratinocyte differentiation with calcium (Fig 5A). The HaCaT cell line derived by LXSNI6E6 infection lacks this response and from a biochemical standpoint fails to differentiate properly. The effects of high risk E6 expression was also seen when looking at the morphology of these cells. Normal HaCaTs displayed a rapid differentiation response to calcium exposure and showed changes in morphology within 24–48 hours where the cells

became more cuboidal and densely packed (Fig 5D). In contrast, the HaCaT E6 cells showed little or no alteration in their morphology after induction of differentiation (Fig. 5E). Finally, decreased levels of p53 are a characteristic hallmark of cells expressing high risk HPV. It was reported previously that p53 in HaCaT cells is unresponsive to E6 (Cho et al., 2001; Magal et al., 1998), so levels of exogenous Flag-tagged p53 were tested after transfection into parental HaCaTs and the HaCaT E6 culture. Flag-p53 was significantly reduced in amount in the E6 expressing cells compared to normal HaCaTs (Fig. 5B), and similar results were seen with the HaCaT SNAP-SUMO/16E6 cell line (Supplemental Fig. S4). Based on this combination of p53 reduction and differentiation impairment we conclude that the HaCaT E6 and HaCaT SNAP-SUMO/16E6 lines are expressing functional 16E6 protein.

To examine the effect of E6 on Ubc9 in HaCaT cells, we transfected both the parental HaCaT line and the HaCaT E6 line with HA-tagged Ubc9 followed by immunoblotting; blots were probed with an anti-Ubc9 antibody which detects both the endogenous Ubc9 and the slightly larger HA-Ubc9. Both endogenous and transfected Ubc9 were reduced in the HaCaT E6 cells compared to the parental HaCaTs (Fig. 5C). This is consistent with the 293A, C33A, and HeLa cell results and confirms E6 targeting of Ubc9 in four different cell types. While we have not directly measured E6 levels or quantitatively compared relative levels of E6 between the transfected cells and the stable cell lines, these combined results suggest that E6 mediates significant effects on endogenous Ubc9 at levels of E6 that would be achievable during infection and transformation.

3.4. Stable expression of E6 in HaCaT cells reduces sumoylated proteins

The next question was whether or not the decrease in endogenous Ubc9 seen in the HaCaT E6 line was sufficient to produce a decrease in sumoylated targets similar to that caused by over expression of E6 in 293 and C33A cells. To observe specific proteins whose sumoylation status changes due to HPV 16E6 expression we constructed a pair of novel HaCaT lines, HaCaT SNAP-SUMO and HaCaT SNAP-SUMO/16E6, which allow fluorescent detection of individual sumoylated target proteins using a 2D gel electrophoresis approach. A detailed description and characterization of the HaCaT SNAP-SUMO line will be presented elsewhere (manuscript submitted). The resultant HaCaT SNAP-SUMO cell line expresses a SNAP-tagged SUMO3 under the control of a tetracycline inducible promoter for regulated expression; SUMO3 was chosen as the SUMO moiety for these studies based on our previous observations that SUMO2/3 undergoes dynamic changes during keratinocyte differentiation while SUMO1 remains more constant (Deyrieux et al., 2007). The SNAP-SUMO fusion protein is efficiently utilized by the sumoylation system and is attached to target proteins, allowing the target proteins to be detected fluorescently on 1D or 2D gels. Since the fluorescence is not quenched by denaturation of the SNAP moiety, cellular extracts can be prepared under stringently denaturing conditions to preserve the SUMO linkage to target proteins. A parallel HaCaT SNAP-SUMO/16E6 line was also constructed by infecting the HaCaT SNAP-SUMO line with LXSNI6E6.

To determine the suitability of the HaCaT SNAP-SUMO and HaCaT SNAP-SUMO/16E6 cell lines for examination of E6 effects on sumoylation, it was necessary to confirm that both lines behaved like their parental HaCaT counterparts during growth and differentiation. Supplemental Figure S4 shows that HaCaT SNAP-SUMO cells behave like normal HaCaTs. The SNAP-SUMO cell line showed a similar induction of K1, and the typical differentiation-related morphological changes appeared, albeit about 24 hours later than observed in HaCaTs. Similarly, the HaCaT SNAP-SUMO/16E6 cell line showed no K1 expression indicating an inability of the cells to differentiate properly. P53 was also reduced in the HaCaT SNAP-SUMO/16E6 cells, which is consistent with what we observed in HaCaT E6 cells and confirms E6 expression (Supplemental Fig. 4B). Finally,

immunoblotting for endogenous Ubc9 showed a decrease in Ubc9 levels in the HaCaT SNAP-SUMO/16E6 cells compared to the HaCaT SNAP-SUMO (Supplemental Fig. 4C), similar to what was observed for HaCaT versus HaCaT E6 in Fig. 5. These results demonstrate that the HaCaT SNAP-SUMO and the HaCaT SNAP-SUMO/16E6 lines retain the appropriate and expected properties of the parental HaCaT cells and are a suitable model to study the relationship between E6 and sumoylation.

3.5. E6 expression in the HaCaT SNAP-SUMO cells reduces sumoylation of specific target proteins

High risk HPV E6 expression induces specific changes in protein levels for certain cellular proteins as shown previously in 2D gel analysis of total cellular proteins (Merkley et al., 2009; Richard et al., 2010), and our results in 293 and HeLa cells indicate general changes in sumoylation also occur as a response to E6 expression. To examine the effect of E6 on sumoylation in differentiating keratinocytes we conducted 2D gel electrophoresis on total protein from the HaCaT SNAP-SUMO and HaCaT SNAP-SUMO/16E6 lines. Cells were plated and differentiation was induced for 96 hours with SNAP-SUMO induction started 48 hours prior to harvesting samples to allow SNAP-SUMO expression and incorporation into target substrates. At one hour prior to harvest SNAP substrate was added the medium to label the SNAP-tagged sumoylated proteins for thirty minutes followed by a wash and incubation in media to allow unbound SNAP substrate to leech from the cells. After labeling the cells were harvested and total cell extracts were analyzed on 2D gels. Four independent experiments were performed for each cell line, and representative gels are shown in Fig. 6. Silver staining to detect total protein showed a complex spot pattern with the majority of spots falling in the middle of the pH range and showing distribution between 50 and 150 kDa (Fig 6A). To visualize sumoylated proteins, the 2D gels were imaged on a Typhoon Trio variable mode imager (GE Health Sciences) which detects the fluorescent signal from the SNAP-SUMO moiety. Inspection of the fluorescent images (Fig. 6B) revealed fewer spots than for the silver stained gels with a spot distribution skewed towards the higher molecular weight and lower pH range. This distribution is consistent with a subpopulation of cellular proteins modified by addition of one or more of the acidic SNAP-SUMO moieties. Images were then analyzed using Melanie 7.0 to quantitate, match, and detect changes in individual spots. Statistical analysis of spot changes was performed using Anova (Supplemental Table S1), and p values ≤ 0.05 were considered significant. Table 1 depicts the summary results from the Melanie analysis. Interestingly HaCaT SNAP-SUMO/16E6 cells showed a decrease in both the average number of total sumoylated spots (180 versus 210) and the number of matched spots per set (169 versus 203) compared to HaCaT SNAP-SUMO cells. While this difference in total spot count was not statistically significant at the $p \leq 0.05$ value, it was consistent with a down regulation of sumoylation by E6. Stronger evidence for an E6 effect on sumoylation was apparent in the spot-by-spot comparison of the matched population sets. There were 63 unique spots in the HaCaT SNAP-SUMO samples that were not present in the HaCaT SNAP-SUMO/16E6 samples, indicating a substantial decrease in the sumoylated protein population in the E6 expressing cells. (Among these 63 unique spots only 32 were statistically significant; spots that were apparently unique but not statistically different between the populations were present in very low quantities and/or had high variance which precluded accurate statistical analysis.) Interestingly, there were also 30 spots unique to the E6 cells, though only 9 spots were identified as statistically different between the two sample groups; the significance of these E6-dependent new spots is uncertain, but they may represent cellular proteins only expressed and/or sumoylated in the presence of E6. For the 141 spots common to both samples a spot value distribution plot indicated that 87% of the HaCaT SNAP-SUMO/16E6 spots had values $\pm 50\%$ of the parental HaCaT SNAP-SUMO sample (Supplementary Fig. S4), and only 3 out of the 141 common spots showed a statistical difference in spot value.

The high degree of consistency in values for common spots between the HaCaT SNAP-SUMO and HaCaT SNAP-SUMO/16E6 demonstrates the overall equality of these two populations and reinforces the interpretation that spots lost in the HaCaT SNAP-SUMO/16E6 line reflect a specific E6 effect on sumoylation of certain host proteins.

4.0 Discussion

Human papillomaviruses express two oncogenic proteins, HPV E6 and HPV E7, which in combination are responsible for altering the cellular environment during viral infection to create a proliferative state in terminally differentiating keratinocytes that normally have exited the cell cycle. Here we demonstrate that E6 protein decreases the protein level of the sole SUMO conjugating enzyme, Ubc9, with a concomitant reduction in some sumoylated cellular proteins. This effect on Ubc9 and sumoylation was manifested by E6 proteins from both high risk types 18 and 16, while low risk HPV6 E6 showed little or no activity. The physical mechanism by which high risk E6 proteins can decrease the intracellular levels of Ubc9 is not at the gene expression level, indicating that E6 is acting post-transcriptionally to reduce Ubc9. It is well established that HPV E6 proteins bind to E6AP, an ubiquitin ligase, and this association mediates degradation of many cellular partners of E6 via the ubiquitin-proteasome pathway (Munger and Howley, 2002). A similar degradative process for Ubc9 seems likely based on the ability of E6 to bind Ubc9 and the requirement of E6AP for Ubc9 reduction, even though Ubc9 protein levels were poorly rescued *in vivo* by the use of proteasome inhibitors. While there are other recent reports of the failure of proteasome inhibitors to rescue E6 targets effectively, such as Bax (Magal et al., 2005), Dlg (Kuballa et al., 2007), and p53 (Camus et al., 2007), the basis for this failure remains unclear and studies are in progress to further evaluate the mechanism by which E6 affects Ubc9 protein levels.

To gain further insight into the cellular consequences of E6 mediated reduction of Ubc9, we developed a method for looking at endogenously sumoylated proteins in a 2-D gel system using SNAP-tag technology to fluorescently label sumoylated proteins (details of this methodology will be presented elsewhere). Using a paired set of SNAP-SUMO HaCaT lines, with or without 16E6 expression, we observed an E6-dependent reduction in Ubc9 and a loss of specific sumoylated targets, while the majority of sumoylated substrates were unaffected. Our current results do not allow us to distinguish between proteins that are simply no longer expressed in the E6 positive cells versus proteins that remain but whose sumoylation is diminished. Additionally, E6 is also known to inhibit the SUMO ligase, PIASy (Bischof et al., 2006), so it is possible that some of the selective loss of sumoylated proteins seen in the HaCaT SNAP-SUMO E6 line is due to reduced sumoylation of PIASy targets. However, given the limited number of PIASy targets and the significant reduction in Ubc9, we believe that decreased sumoylation due to lower Ubc9 levels accounts for at least some of the observed loss of sumoylated proteins. How this selective loss of some sumoylated targets occurs has not yet been determined, but several possibilities exist. First, this selective loss may be a quantitative effect if the reduced levels of Ubc9 are only sufficient to catalyze sumoylation of those target proteins with an adequate affinity for Ubc9 or whose abundance is above some threshold limit. Alternatively, there could be uneven reduction in Ubc9 levels related to co-localization with E6. For example, regions where E6 co-localizes with Ubc9 could have severely reduced amounts of Ubc9 so sumoylation of substrates in those subcellular regions would be most impacted. In contrast, regions that have low E6 levels may have adequate Ubc9 levels and normal sumoylation. Further study will be needed to evaluate the possible mechanistic basis for the selective reduction in sumoylated proteins.

An important implication of our results is that the E6-mediated reduction of Ubc9 and the subsequent effect on sumoylated target proteins is somehow beneficial to the viral life cycle.

While the specific functional outcomes resulting from loss of these sumoylated targets is not yet known, the sumoylation system has been tied to a variety of cellular processes that the virus may want to modify to its advantage. For example, sumoylation of transcription factors generally leads to decreased activity, so reducing sumoylation of certain transcription factors could provide an additional viral mechanism to up regulate transcription of both viral and requisite host genes. Additionally, reducing sumoylation could contribute to creating a proliferative environment in differentiating keratinocytes that are normally nonreplicative. The balance between proliferation and differentiation in keratinocytes can be altered by changes in activity of specific regulatory transcription factors, such as p63, which is known to be negatively regulated by sumoylation (Ghioni et al., 2005). We previously showed that the overall level of sumoylation increases during keratinocyte differentiation (Deyrieux et al., 2007), suggesting that proliferative activity would decrease due to increased sumoylation and subsequent reduced activity of pro-proliferative factors such as p63. Therefore, reducing Ubc9 via E6 could decrease sumoylation of pro-proliferative factors leading to their increased activity and thus help restore proliferative capacity in terminally differentiating cells. A related consequence is that the effects of E6 on the sumoylation pathway may contribute to the observed perturbation of keratinocyte differentiation in HPV infected cells. HaCaT cells blocked for sumoylation by GAM1 expression display altered morphological features and an overall decrease in K1 levels (Deyrieux et al., 2007), suggesting that sumoylation is important for the normal differentiation process. Thus, the decreased sumoylation promoted by high risk E6 proteins may account, at least in part, for the altered differentiation seen in the HaCaT E6 and HaCaT SNAP-SUMO E6 lines. Although the differentiation impairment associated with GAM1 expression in HaCaT cells was more severe than observed in E6-expressing cells, this may simply reflect additional properties of GAM1 as well as a more modest impact on sumoylation resulting from stable expression of low levels of E6 compared to transient high level expression of GAM1. Finally, it is important to note that transcription factors are not the only possible sumoylation targets that could be affected by E6. There are numerous SUMO substrates that are not transcription factors (Rosas-Acosta et al., 2005b; Vertegaal et al., 2006), and reduced sumoylation of some of these other targets could enhance viral fitness through mechanisms such as impairing either apoptosis or the host immune response. Identification of specific proteins whose sumoylation is affected by E6 will be necessary in order to explore the functional consequences of the E6 reduction in Ubc9 levels. Initial attempts to identify individual spots by mass spectrometry have been unsuccessful due to the low amounts of total protein in the fluorescent spots, so development of enrichment methods is in progress.

In conclusion, it has become clear that SUMO is a major regulator of many biological processes (Kerscher et al., 2006) and that manipulation of the sumoylation system by viral proteins is functionally important to a variety of DNA and RNA viruses. We now demonstrate for the first time that the high risk HPV E6 proteins can down regulate sumoylation through apparent degradation of the SUMO conjugating enzyme, Ubc9. Thus, we have identified a new E6 function that may have a significant impact on the host cell environment through pleiotropic effects on the assortment of sumoylated host cell proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

High risk E6 proteins decrease Ubc9 levels. (A) 293A cells were transfected with HA-Ubc9 (1.0 ug) and increasing amounts of the plasmid DNAs indicated on the right side of the blots (lane 1: 0.5 ug; lane 2: 1.5 ug; lane 3: 3.0 ug). All the E6 proteins were GFP tagged and the E7 protein was HA tagged. At 48 hours post-transfection the cells were harvested, lysed, and immunoblotted with anti-HA to detect HA-Ubc9 (top 4 panels) and anti-tubulin (bottom panel) to ensure equal sample loading. (B) The E6 proteins in part A were visualized by immunoblotting using anti-GFP. E7 expression was verified on a separate blot (not shown). (C) 293A cells were transfected with mixtures containing 1.0 ug of the HA-Ubc9 plasmid DNA, 1.0 ug of the SUMO plasmid DNA, and 1.5 ug of EGFP or 16E6 plasmid DNA as indicated. At 48 hours post-transfection the cells were harvested, lysed, and immunoblotted with anti-SUMO (upper panel), anti-GFP (middle panel), and anti-tubulin (bottom panel). In this particular gel the GFP and GFP-E6 (middle panel) migrated similarly, but see 1B for a more representative depiction of their modest molecular weight difference. (D) GST pulls down assay were performed using purified GST and GST-Ubc9 protein generated from *E.*

coli. The high risk HPV 18E6 protein was *in vitro* translated and radiolabeled in TNT rabbit reticulocyte lysates. *In vitro* translated BPV E1 protein (E1) and luciferase (Luc) were used as positive and negative Ubc9 binding controls, respectively. The Input samples represent 50% of the amount of radiolabeled material used for each binding assay.

Figure 2.

Untagged E6 is effective at reducing Ubc9 and sumoylation levels. (A) 293A cells were transfected with 1.0 ug of HA-Ubc9 plasmid DNA and 1.5 ug of plasmid DNA encoding EGFP, EGFP-16E6, or untagged 16E6 as indicated. At 48 hours post-transfection the cells were harvested, lysed, and immunoblotted with anti-HA to detect Ubc9 (upper panel) and with anti-tubulin (lower panel). (B) Samples from part A were immunoblotted with anti-p53. Lanes marked C were transfected with the EGFP plasmid.

Figure 3.

E6 proteins require E6AP for reduction of Ubc9 and sumoylation levels. (A and B) E6AP normal (H1299) and deficient (K3) cells were transfected with 1.5 ug of pcDNA (M lanes), 16E6, or 6E6 DNA as indicated. Samples in (A) also were transfected with 1.0 ug of p53 plasmid. Samples were harvested at 48 hours and immunoblotted with anti-p53 (A upper panel), anti-tubulin (A lower panel), anti-SUMO (B upper panel), or anti-Ubc9 (B lower panel).

Figure 4.

E6 affects endogenous Ubc9 and sumoylation. HeLa cells were transfected with siRNA against 18E6 (E6 lane) or with a universal negative control (NUC) siRNA. At 24 hours post-transfection cells were lysed and the extracts immunoblotted with anti-tubulin, anti-p53, anti-Ubc9, and anti-SUMO as indicated for the panels.

Figure 5.

E6 prevents differentiation and reduces Ubc9 in HaCaTs. HaCaT and HaCaT E6 cells were immunoblotted for K1 and tubulin expression (A). (B) HaCaT cells (- lane) and HaCaT E6 cells (+ lane) were transfected with a Flag-p53 expression vector then immunoblotted with anti-Flag and anti-tubulin. (C) As in (B) except that cultures were transfected with an HA-Ubc9 expression vector rather than a p53 vector. Cell extracts were immunoblotted with anti-Ubc9 to detect both endogenous and exogenous Ubc9 and with anti-tubulin. HaCaT (D) and HaCaT E6 (E) were induced with calcium, and the cultures were examined by phase contrast microscopy at 24 hour intervals for 6 days. The HaCaT E6 cells show less morphological evidence of differentiation over this time period.

Figure 6.

Differentiating HaCaT SNAP-SUMO cells and HaCaT SNAP-SUMO/16E6 cells were induced for SNAP-SUMO and labeled with SNAP substrate as described in Materials and Methods. After labeling, cells were lysed and subjected to 2-D electrophoresis. Total protein was visualized by silver staining (A) and sumoylated proteins were visualized by fluorescent detection (B). For all gels the pH range of the isoelectric focusing is shown across the horizontal dimension and the position of molecular weight markers is shown in the vertical dimension. Spots in (A) represent total cellular proteins while spots in (B) are sumoylated proteins.

Table 1

Spot Analysis of HaCaT SNAP-SUMO and HaCaT SNAP-SUMO-16E6 Matched Gels

SPOT CHARACTERISTICS	HaCaT SNAP-SUMO^a	HaCaT SNAP-SUMO-16E6^a
Average spot number per gel	210	180
Spots matched per set	203	169
Number of unique spots	63 ^b	30 ^b
Number of statistically significant spots (p< 0.05)	32	9

^a Each sample was repeated four times for analysis^b All unique spots not significantly different. See text for details