Published in final edited form as:

Virus Res. 2010 June ; 150(1-2): 135–137. doi:10.1016/j.virusres.2010.02.011.

Common Importin Alpha Specificity for Papillomavirus E2 Proteins

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Abstract

Papillomaviruses infected keratinocytes and their reproduction is tied to differentiation of the skin. The E2 protein of papillomaviruses is a multifunctional early protein that binds specifically to the viral DNA to regulate genome transcription, replication, and segregation. All of these are nuclear events that require specific transport of E2 into the host nucleus. Nuclear localization signal (NLS) sequences have been mapped for several E2 proteins, and these sequences resemble motifs that interact with cellular transport adaptor molecules termed alpha importins. To determine which importins could carry E2 proteins, in vitro binding studies were performed with three different E2 proteins and the five ubiquitous alpha importins. The E2 proteins preferentially interacted with alpha importins 3 and 5, and showed very weak or no interaction with the other three widely expressed alpha importins ($\alpha 1$, $\alpha 4$, and $\alpha 7$). While all five alpha importins appear to be constitutively expressed in keratinocytes, during differentiation of a human keratinocyte line (HaCaT) we observed a specific increase in expression of alphas 3 and 5. This differentiation-specific increase in $\alpha 3$ and $\alpha 5$ expression suggests that preferential usage of these two importins by E2 may facilitate E2 nuclear uptake during terminal differentiation.

Keywords

Papillomavirus; E2; Early Proteins; Importins; Nuclear Transport

RESULTS & DISCUSSION

The papillomavirus E2 proteins are critical early regulatory factors that function in viral replication, transcription, and genome segregation (Hamid et al., 2009). All of these functions require nuclear localization of E2, and previous reports have identified E2 protein nuclear localization signals (NLS) for both human (Blachon et al., 2005; Klucevsek et al., 2007; Zou et al., 2000) and bovine papillomavirus (Skiadopoulos and McBride, 1996) that are rich in basic residues. Basic amino acid-rich NLS sequences typically interact with importin alpha adaptor molecules in conjunction with the importin beta transport carrier. Surprisingly, the number and location of NLS sequences varies with high-risk HPVs having a single NLS in the C-terminal DNA binding domain and the low-risk HPVs having 3 NLS

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CONFLICT OF INTEREST

The authors have no commercial interests that would constitute a conflict of interest with this work.

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sequences, one each in the N-terminal, hinge, and C-terminal domains. Additionally, the HPV16 NLS sequence is located within an alpha helix rather than the extended conformation more classically seen for NLS elements that bind importin alphas. All these features suggest that there may be significant differences in how various E2 proteins interact with the nuclear transport system.

There are six alpha importins described in human cells ($\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, and $\alpha 7$), and we recently examined the interaction of these alpha importin with another papillomavirus early protein, E1 (Bian et al., 2007). E1 preferentially interacted in vitro with importins 3 and 5, to a lesser extent with $\alpha 4$, and did not bind to $\alpha 1$ or $\alpha 7$; expression of $\alpha 6$ is restricted to testis (Kohler et al., 1997; Umegaki et al., 2007) and was not examined in this study. To evaluate differences in importin alpha interaction by E2 types we utilized the same in vitro GST-pulldown assay developed for the E1 protein (Bian et al., 2007). The five relevant alpha importins were expressed as His-tagged fusions in *E. coli* and purified by affinity chromatography; the purified proteins are shown in Fig. 1A. Representative low-risk (HPV11), high-risk (HPV16), and bovine papillomavirus E2 proteins were expressed as GST fusions and purified from *E. coli*. The purified E2 proteins or GST alone were incubated with the purified importins, collected on glutathione-Sepharose beads, washed, and eluted for analysis by SDS-PAGE. Similar to the results for E1 protein, none of E2 proteins interacted detectably with importins $\alpha 1$ or $\alpha 7$ [Fig. 1B; the functionality of the $\alpha 1$ or $\alpha 7$ proteins was verified previously (Bian et al., 2007)]. Consistent with differences in their NLS content, the three tested E2 proteins showed somewhat different patterns of binding to importins $\alpha 3$, $\alpha 4$, and $\alpha 5$. HPV 11 E2 showed similar binding to both alpha importins 3 and 5 with only weak interaction with $\alpha 4$. In contrast, HPV 16 E2 bound strongly to $\alpha 5$, but only weakly to both alphas 3 and 4. Similar to 16E2, BPV E2 bound most effectively to $\alpha 5$, weakly to $\alpha 3$, and not detectably to $\alpha 4$. However, while there were clear differences in the specific patterns of interactions, there nonetheless was a hierarchy of binding preference shared by all three tested E2 proteins: $\alpha 5 \geq \alpha 3 > \alpha 4$.

Our observation that both papillomavirus E1 and E2 proteins interact most effectively with importins $\alpha 5$ and $\alpha 3$ raised the question of the biological basis for this effect, and suggested that there might be preferential expression of these two alpha importins in keratinocytes. Previous studies have shown tissue-specific differences in expression of alpha importins, though skin was not examined (Kamei et al., 1999; Tsuji et al., 1997). More recently, expression of alpha importins 1, 3, 4, 5, and 7 was shown in epithelial lines by immunoblotting (Kohler et al., 2002), and expression of importins 1, 3, 4, and 5 was shown in normal human epidermal keratinocytes by Northern blots (Umegaki et al., 2007). To confirm and extend these results we examined HaCaT, HeLa, and 293A cells by immunoblotting for importin alpha protein expression (Fig. 2). Alphas 1, 3, and 4 were detected in all three lines while $\alpha 5$ was detected in HaCaT and 293A cells, but not HeLa cells. However, $\alpha 5$ detection is weak in both HaCaT and 293A and may simply be below the threshold in HeLa cells. Alpha 7 was not tested due to lack of an effective antibody, but has previously been detected in HeLa and HaCaT cells (Kohler et al., 2002). Thus, our results combined with previous studies on monolayer cultured keratinocytes suggest that the preferential utilization of alphas 5 and 3 by papillomavirus E1 and E2 proteins is not due to the absence of other importin alpha types in keratinocytes. One caveat to this conclusion is the fact that stratified epithelium has not yet been examined, and there could be difference in expression profiles in multilayered skin compared to monolayer cultures.

While the above results indicate that all of the five ubiquitous alpha importins are expressed in keratinocytes, the effect of differentiation on expression levels in this cell type has not been examined. In other cell types differentiation clearly alters the relative levels of specific alpha importins (Kohler et al., 2002), so we examined the effect of differentiation on

importin expression in keratinocytes using HaCaT cells. HaCaTs are immortalized human keratinocytes that retain normal characteristics and differentiation behavior (Deyrieux et al., 2007) and are widely used as a keratinocyte model. Using antibodies that are highly specific for the individual importins (Fig. 3A), we evaluated importin alpha expression over a 6-day period following calcium-induced differentiation of HaCaT cells (Fig. 3B). Human keratin 1 (HK1) expression was used as the marker for differentiation and is clearly visible by day 3 after induction with a peak at day 5. During this differentiation period, no change was detected in $\alpha 1$ or $\alpha 4$ levels, but $\alpha 3$ and $\alpha 5$ both exhibited an approximately 3-fold increase in protein levels. It is intriguing that the two alpha importins that preferentially associate with the E1 and E2 proteins are the two whose expression levels are differentiation dependent. If similar changes in importin expression occur during differentiation of stratified epithelium, then it may be advantageous for papillomavirus nuclear proteins to target these specific importins to ensure adequate nuclear localization during productive replication in the higher layers of the epidermis.

In summary, we have shown that papillomavirus E2 proteins have a binding preference for a subset of the five alpha importin types expressed in keratinocytes. The E2 preference for importins $\alpha 5$ and $\alpha 3$ is similar to that seen previously for the E1 protein (Bian et al., 2007). We also showed that importins $\alpha 5$ and $\alpha 3$ are the only two alpha importins whose expression increases during differentiation in a keratinocyte monolayer culture system, and their preferential usage by papillomavirus early proteins may be related to this differentiation-dependent increase in expression.

Acknowledgments

This work was supported by a grant from the National Cancer Institute (R01 CA089298) to V.G.W.

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Fig. 1.

In vitro binding of papillomavirus E2 protein to alpha importins. (A) Five His-tagged alpha importins were expressed in *E. coli* and purified as previously described (Bian et al., 2007). The purified proteins were visualized by immunoblotting with anti-His antibody. (B) The three GST-E2 proteins (2 ug each) were each incubated with the indicated alpha importins (1 ug each), captured on glutathione-Sepharose, eluted, and the bound importins visualized by immunoblotting with anti-His. These quantities yield an approximately 1.5:1 (E2/importin) molar ratio. The GST lane shows the result for importin alpha 3 incubated with GST alone. The other four importins also showed no binding to GST alone (data not shown).

Fig. 2. Importin alpha expression in established cell lines. Equal amounts of total cell extract from the three cell lines indicated were immunoblotted using alpha importin-specific antibodies and anti-tubulin.

Fig. 3. Differential importin α expression during HaCaT differentiation. (A) Sets of purified α importins (lanes 1–4) were immunoblotted with the individual anti-importin antibodies (indicated on the right of each panel) to confirm the specificity of the antibodies. (B) Monolayer cultures of HaCaT cells were maintained in low calcium medium (Uninduced) or induced to differentiate (Induced) by calcium addition as previously described (Deyrieux et al., 2007). At the indicated times whole cell extracts were immunoblotted with the specific anti-importins from (A), with anti-human keratin 1 (HK1) to verify differentiation, and with anti-tubulin to verify equal loading. (C) The experiment shown in (B) was quantitated by image analysis of the blots as previously described (Deyrieux et al., 2007). Data shown are the average of three independent repeats of the experiment.