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HPV16 infection of HaCaTs is dependent on β4 integrin, and α6 integrin processing

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ABSTRACT

Our understanding of human papillomavirus (HPV) is still evolving. To further study the field, our laboratory has focused on determining the role of integrins in the initial steps of viral endocytosis into HaCaT cells. Our and others' previous findings have shown that α6 is necessary for infection. Here we show that α3 and β1 were dispensable, and we identified integrin α6β4 complex as necessary for infection in HaCaTs. β4 knock down resulted in a significant decrease in HPV16 PsV infection and perhaps most importantly resulted in defective post-translational α6 processing. We showed that the unprocessed α6 does not localize to the cell surface. We propose that the α6β4 complex is necessary for the formation of an endocytic complex that results in the signaling transduction events necessary for initial endocytosis.

Introduction

Papillomaviridae family consists of non-enveloped, 55 nm diameter, circular dsDNA viruses. To date, more than 150 human papillomavirus (HPV) types are identified by DNA sequencing and these types are commonly divided into five genera based on genotyping similarities (Doorbar, 2006; Doorbar et al., 2012). Infections occur in epithelial cells and can either cause benign warts on skin and mucosa (low-risk HPVs) or lead to cervical cancer (Scheffer et al., 2013). Out of the 15 identified oncogenic HPV types, HPV16 is the most common etiologic agent associated with cervical cancer (Laniosz et al., 2009; Schiffman, 2007). Although this disease-mediating agent has been under study for more than 40 years, the details of the infection process have not been clearly identified.

Research to identify the biological receptor(s) for HPVs has led to identification of some of the key players in HPV infection. The viral particle consists of the virally encoded L1 and L2 capsid protein. Many laboratories have shown data supporting that the initial HPV binding is L1 mediated, and occurs through heparan sulfate proteoglycans (HSPGs). Data have shown that removing the heparan sulfate glycosaminoglycans reduces infection; furthermore, addition of a soluble analog of heparan sulfate competes for HPV binding and thus blocks infection (Abban and Meneses, 2010; Giroglou et al., 2001; Johnson et al., 2009; Joyce et al., 1999). This initial binding has been suggested to occur in the extracellular matrix or at the cell surface. Upon binding of the virus to the HSPGs, a conformational change occurs. This change has been shown to be mediated by host cell protein cyclophilin B and results in the exposure of a hidden N-terminus of L2 (Bienkowska-Haba et al., 2009). This exposed region of L2 can be cleaved by members of the proprotein convertase family of peptidases, furin and/or PC 5/6 (Richards et al., 2006; Seidah and Prat, 2012). The cleavage of the L2 N-terminus is necessary for infection and is hypothesized to facilitate the transfer of the virus capsid to a secondary receptor on the keratinocyte plasma membrane (Day et al., 2008; Selinka et al., 2007). Search for this secondary receptor initially led to integrin α6 using HPV6b L1 viral like particles (VLP) (Evander et al., 1997). Using HPV16 pseudovirions, we confirmed the initial binding of viral particles to α6 and showed that siRNA-mediated silencing of integrin α6 decreases infection in HaCaTs (Abban and Meneses, 2010). In addition to the role of α6 integrin, the role for tetraspanins has been proposed by Dr. Florin's group; Dr. Ozbun's group has discussed the presence of an endocytic complex consisting of HPV16 PsVs, HSPGs and EGF; Annexin A2 and its S100A10 subunit have been identified as L2-specific receptor by Dr. Kast's group and they have further been characterized by Dr. Ozbun's group; and most recently Drs. DiMaio and Atwood's group have suggested the role of a retromer as necessary for infection and trafficking of incoming virus (Scheffer et al., 2013; Surviladze et al., 2012; Woodham et al., 2012; Dziduszko and Ozbun, 2013; Lipovsky et al., 2013).

In the aforementioned studies of Drs. Florin and Ozbun, the two common molecules seem to be the integrin α6 and tetraspanin

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151 (Scheffer et al., 2013; Surviladze et al., 2012). Integrins are transmembrane receptors that have no intrinsic catalytic or enzymatic activity. To date, 18α and 8β integrin subunits have been identified—each of which is a single pass type I transmembrane protein—making up 24 identified integrin complexes. Upon ligand binding, integrins can mediate outside-in signaling with their ability to transduce signals through proteins that dock on their cytoplasmic tails, and also by regulating their ligand-binding affinity they can mediate inside-out signaling events (Takada et al., 2007). The proposed secondary HPV receptor integrin α6 can form complexes with either β1 or β4 integrins (Kligys et al., 2012). The possible roles of β subunits as a direct binding partner of HPV virions during infection were investigated, and these studies lead to the conclusion that neither β1 nor β4 showed any direct binding to viral particles (Evander et al., 1997; Huang and Lambert, 2012; Yoon et al., 2001).

In a previous study, we showed the importance of α6 in infection and we profiled the integrin subunits in human adult keratinocyte cell line (HaCaT). Our data showed that β1, α2, β2, α3, β4, β6 and α6 were present on HaCaT's cell surface (Abban and Meneses, 2010). Because integrins function as obligate heteromers, we addressed what complexes were formed in HaCaTs. We focused on four integrin subunits based on the profiling data: α3, α6, β1 and β4. These integrins have been implicated in wound healing (Goldfinger et al., 1999; Margadant et al., 2009). We identified several integrin complexes, and α6β4 was found to be involved in HaCaT infection by HPV. Our data showed that the level of β4 was directly correlated to the level of infection and for proper α6 integrin processing.

Results

Assessment of possible integrin complexes on HaCaTs

Integrin function requires a pairing of an α and a β subunit into a heterodimer. Based on our previous integrin profiling we addressed what heterodimers are found on HaCaTs. Using standard immunoprecipitation experiments with the α subunits as bait we showed that α3 was able to pull down β1 but not β4 (Fig. 1A, lanes 6 and 12) while α6 was able to pull down β4 but not β1 (Fig. 1B, lanes 6 and 9). We confirmed the presence of α6β4 and the lack of α3β4 complex by the reverse pulldown (Fig. 1C, lanes 8 and 14) These pulldowns showed that integrin complexes α3β1 and α6β4 were formed in HaCaTs.

Integrin β4, but not α3 or β1 are required for HPV16 pseudovirion infection

Having identified two heterodimeric integrin complexes, we tested the biological significance of the various integrins. HaCaT cells were treated with integrin specific siRNAs, subsequently incubated with HPV16 pseudovirion, and infection levels assessed by flow cytometry (GFP pseudogenome 8fwb plasmid was used in viral particles). The protein level of α3, β1, and β4 were reduced by 81%, 99%, and 96% compared to untreated HaCaTs (Fig. 2 western blots A, D, G and the measurements B, E, H). The necessity of α6 integrin data have been previously published by our group (Abban and Meneses, 2010). We did not observe any changes in infection in either α3 or β1 knockdown cells, but saw a 90% decrease in infection in β4 knockdown cells. Taken together with our previous α6 work, these data suggested that the necessary integrin complex for HPV16 infection in HaCaTs is α6β4.

β4 Integrin is necessary for α6 integrin detection at the cell surface

We used confocal microscopy and flow cytometery to test the status of α6 and β4 integrins in β4 knockdown cells. As shown in Fig. 3A, control siRNA treated HaCaT cells have β4 and α6 integrin staining (Fig. 3A red and green, respectively; Fig. 3C lanes 1–2). By contrast, the level of α6 is minimal in β4 knock down cells (Fig. 3B red for β4, green for α6; Fig. 3C lanes 3–4). Flow cytometry analysis showed that the cell surface levels of β4 and of α6 are greatly decreased. Fig. 3D and E shows the shift to the left of both β4 and α6 staining indicative of a decrease in the surface expression of both integrins (Fig. 3D and E, compare the black trace-untreated vs. gray-β4 siRNA treated HaCaTs). Data are presented in graphical form in 3F (black-control cells, gray-β4siRNA treated cells, isotypic IgG controls were used in these experiments). These experiments showed that the detectable levels of both β4 and α6 within cells and at the cell surface is greatly reduced in β4 knockdown cells.

In β4 knock down HaCaTs, α6 integrin does not form a complex with β1

The observed decrease in levels of β4 by microscopy and flow cytometry were consistent with our western blot analysis of β4 knockdown cells. It was surprising to see a comparable loss of α6 expression. Based on the literature, one would theorize that in the absence of β4, α6 would pair with β1. Unexpectedly, our results showed that no such compensation occurs. The western blot of β4 knockdown cells clearly showed α6 expression but an immunoprecipitation with α6 in these samples did not immunoprecipitate β1 (Fig. 4). Thus, α6 does not complex with β1 in the absence of β4 in these experiments.

α6 processing is affected in the absence of β4 but not in the absence of β1 or α3 protein

Analysis of α6 on the western blot of the β4 knockdown HaCaTs showed an α6 band at 140 kDa. This size was inconsistent with a processed α6 protein. A comparison of lanes 5, 6 to lanes 1–4 in Fig. 5A (control cells vs. β4 knockdown cells) shows that, in β4 knockdown cells the predominate α6 band detected is 140 kDa as compared to the 120 kDa band detected in control cells. The data lead us to conclude that lack of β4 protein results in the loss of α6 processing which results in its inability to be properly express at the cell surface and thus contributes to the loss in infection. The level of α6 processing was not disrupted in β1 or α3 knockdown cells, which did not have a loss of infection (Fig. 5 B and Fig. S4). α3 siRNA knockdown for experiment in Fig. S4 is shown in Fig. 2A. These final data are supportive although correlative with the importance of α6 processing.

Discussion

The many improvements in the production of papillomavirus particles have allowed for a rapid development of data regarding the process of HPV virus binding, entry, trafficking and thus, onset of infection. In our laboratory we have taken advantage of the pseudovirion production technique that results in a viral particle containing an easily detectable reporter gene/protein, GFP. We used these pseudovirions to transduce the target cell model HaCaTs. In a previous work, we sought to determine if the finding that HPV16 VLPs bound to α6 integrin meant that α6 integrin was indeed a biological receptor for HPV16, i.e., absence of α6 integrin would result in a loss of infection (Evander et al., 1997; Yoon et al., 2001). We showed that α6 integrin was indeed crucial for HPV16 PsV infection in HaCaT cells (Abban and Meneses, 2010). We also characterized the integrin subunits that were expressed in HaCaTs with the thought that perhaps other integrin subunits played a role in HPV16 infection. We showed that the integrin subunits β1,