Human papillomaviruses (HPVs) are small, non-enveloped DNA viruses that infect differentiating epidermal or mucosal epithelial cells (zur Hausen, 2002). Over 100 types of HPV have been identified; approximately 15 of these are considered high risk and are associated with the vast majority of cervical cancers (Woodman et al., 2007). HPV type 16 (HPV16) is the predominant oncogenic type and alone is responsible for over 50 % of cervical cancers worldwide (Forman et al., 2012). For a productive infection, HPVs must bind to and enter host epithelial cells and then uncoat and translocate the viral genome to the nucleus. Although endosomal acidification is believed to be important, the mechanism of HPV genome uncoating is poorly defined. We have previously observed degradation of the major capsid protein L1 at early times post-infection (p.i.) (Campos et al., 2012), suggesting that L1 proteolysis may contribute to capsid disassembly. As HPV capsids are known to undergo pH-dependent disassembly in the endosomal/lysosomal compartment (Schelhaas et al., 2012; Smith et al., 2008), lysosomal proteases probably participate in L1 degradation and genome uncoating. Recent work has suggested that two lysosomal proteases, cathepsin L (CatL) and cathepsin B (CatB), may be important for HPV16 infection (Dabydeen & Meneses, 2009).

CatL and CatB are acid-dependent endo/lysosomal cysteine proteases that function in a variety of cellular processes, including protein maturation and turnover, as well as in regulation of the immune response (Conus & Simon, 2010; Turk et al., 2012). Several viruses also depend on these cathepsins for productive infection. For example, filoviruses, reovirus, Ebola virus and adeno-associated virus all require CatL or CatB activity for disassembly or proteolytic activation of their capsid proteins (Akache et al., 2007; Ebert et al., 2002; Kaletsky et al., 2007; Misasi et al., 2012). In this paper, we tested the hypothesis that CatL and CatB are necessary for productive infection by HPV16.

To examine the role of CatL and CatB in HPV16 infection, wild-type or cathepsin-deficient mouse embryonic fibroblasts (MEFs) were infected with HPV16 pseudovirions containing the pGL3 luciferase reporter plasmid (HPV16–luc). At 24 h p.i., cells were washed once with PBS and lysed in 1× Reporter Lysis Buffer (RLB; Promega). Luciferase activity was measured using Luciferase Assay Reagent (Promega) according to the manufacturer’s instructions. One CatL-deficient cell line and two different CatB-deficient cell lines, CatB.1 and CatB.2, were tested. Infectivity was assessed by comparing the normalized luciferase level produced in each knockout cell line with that of their cognate wild-type cell line. At 24 h p.i., CatL/−/− cells had higher infection levels compared with the cognate wild-type MEF line (Fig. 1a). Similar results were observed at 48 h.p.i. (data not shown). No differences were observed in viral binding between wild-type and CatL-deficient MEFs (Fig. 1b, c), although CatL/−/− cells had slightly lower levels of viral entry compared with wild-type cells (Fig. 1d, e). These data suggested that CatL is not required for HPV16 infection in MEFs.

Interestingly, the two CatB-deficient cell lines produced contradictory results. CatB.1/−/− cells exhibited significantly lower levels of infection when compared with their cognate wild-type cells at both 24 h (Fig. 1a) and 48 h (data not shown) p.i. This finding is consistent with previous work that indicated that CatB expression is important for HPV16 infection of MEFs (Dabydeen & Meneses, 2009). In contrast, no reduction in infectivity was observed in CatB.2/−/− cells. The CatB.2/−/− cells instead displayed higher levels of infection than wild-type cells at 24 h (Fig. 1d, e).
1a) and 48 h (data not shown) p.i. Both CatB-deficient cell lines had higher levels of viral binding than wild-type cells (Fig. 1b, c). However, neither of the CatB$^{-/-}$ cell lines displayed a significant difference in viral entry when compared with their cognate wild-type cell lines (Fig. 1d, e), suggesting that the different infection levels of CatB.1$^{-/-}$ and CatB.2$^{-/-}$ did not result from changes in viral entry. Enzyme activity assays (Fig. 1f, g) confirmed that both the CatB.1$^{-/-}$ and CatB.2$^{-/-}$ lines are deficient for CatB. Thus, the difference between CatB.1$^{-/-}$ and CatB.2$^{-/-}$ infection levels could not be explained by a reversion of phenotype in one of the cell lines.

To resolve the conflicting data observed in the two CatB$^{-/-}$ MEF lines, we examined HPV16 infection in HaCaT cells by knocking down CatL or CatB levels with small interfering RNA (siRNA). HaCaTs are immortalized human foreskin keratinocytes (Boukamp et al., 1988) and thus represent a more physiologically relevant cell line for studying HPV than MEFs. For each knockdown, ~60 000 HaCaT cells were
transfected with 50 nM siRNA using Lipofectamine RNAiMax (Invitrogen). At 40 h post-transfection, the cells were infected with HPV16–luc and luciferase activity was measured at 24 h p.i. Parallel siRNA-treated wells were lysed directly in SDS-PAGE sample buffer and subjected to Western blotting to confirm target knockdown at both the start and end of infection (Fig. 2a–d). Cells treated with CatL- or CatB-specific siRNA exhibited higher levels of luciferase activity compared with cells treated with a scrambled siRNA control (Fig. 2e). The siRNA results indicated that neither CatL nor CatB was required for HPV16 infection in HaCaT cells. Indeed, these results suggested that CatL and CatB may function to restrict HPV16 infection of human keratinocytes.

The role of CatB was also tested by infecting cells in the presence of CA074Me (EMD Millipore), a cell-permeable pharmacological inhibitor of CatB activity. HaCaT cells were infected with HPV16–luc in combination with increasing concentrations of CA074Me for 24 h, at which time infection was measured by luciferase assay. Control cells were infected in the presence of DMSO vehicle alone. CA074Me treatment promoted HPV16 infection in a dose-dependent manner (Fig. 3). Thus, pharmacological inhibition of CatB activity promoted HPV16 infection of HaCaT keratinocytes.

The lysosomal proteases CatL and CatB are important for caspoid disassembly of many viruses and have been implicated previously in HPV16 infection. However, we demonstrated that these cathepsins are not necessary for productive infection by HPV16. We observed higher levels of HPV infection in the CatL$^{-/-}$ and CatB.2$^{-/-}$ cell lines compared with wild-type MEFs (Fig. 1). Knocking down cathepsin expression with siRNA (Fig. 2) or blocking CatB activity with a pharmacological inhibitor (Fig. 3) also resulted in increased HPV infectivity of HaCaT cells. Combined, these results demonstrate that CatL and CatB are not required for HPV16 infection. Rather, they indicate that CatL and CatB most likely limit infection by HPV16.

Surprisingly, infections with two separate CatB$^{-/-}$ cell lines gave contradictory results (Fig. 1). The higher levels of infection observed in the CatB.2$^{-/-}$ MEFs were in good agreement with the siRNA knockdown and pharmacological inhibition data in HaCaT keratinocytes. These findings suggest that the CatB.1$^{-/-}$ cell line may have undergone a secondary mutation that restricts HPV16 infection independently of CatB. The nature of this possible mutation is unknown but does not appear to affect HPV16 binding or entry (Fig. 1b–e). Whilst these two lines came from different immediate sources (CatB.1$^{-/-}$ and CatB.2$^{-/-}$ cells were kind gifts from Dr Terry Dermody, Vanderbilt University, TN, USA, and Dr Bonnie Sloane, Wayne State University, MI, USA, respectively), the original source of both lines was from Dr Christoph Peters (T. Dermody and B. Sloane, personal communication), whose laboratory generated the original CatB knockout mice (Deussing et al., 1998).

Whilst our data indicate that CatL and CatB function to restrict HPV16 infection, previous work has suggested that these cathepsins actually promote HPV infectivity (Dabydeen & Meneses, 2009). These conflicting observations may result from variations in the experimental systems used. In the previous study, the CatB inhibitor CA074Me was found to marginally block HPV16 infection of HEK293 cells, a human embryonic kidney cell line. In contrast, we observed a dose-dependent increase in infection when the more physiologically relevant HaCaT keratinocytes were treated with CA074Me. Dabydeen & Meneses (2009) also observed lower rates of HPV infection in CatB$^{-/-}$ MEFs, which is consistent with our findings in the CatB.1$^{-/-}$ cell line. Indeed, the source of the MEFs used in their previous studies was the same as the CatB.1$^{-/-}$ cells used here. However, as discussed above, comparisons with CatB-deficient HaCaT keratinocytes suggest that the CatB.1$^{-/-}$ cell line displays an aberrant phenotype that makes it unsuitable for studying HPV.

The mechanism by which CatL and CatB restrict HPV16 infection is currently not known. One possibility is that, in
the presence of CatL and CatB, the HPV capsid is degraded too quickly, resulting in lower levels of infection. Compared with other viruses, HPV infection kinetics are remarkably slow; virions undergo a prolonged residence in the acidic endo/lysosomal compartment, and genome uncoating does not occur until approximately 8 h post-inoculation in vitro (Day et al., 2004; Schelhaas et al., 2012). This long incubation period may be necessary for the virion to undergo conformational changes that allow successful translocation of L2 or the genome to the nucleus. Thus, removing CatL and CatB may delay non-productive degradation to allow more capsids to reach this infectious conformation. Alternatively, eliminating CatL and CatB may upregulate infection-promoting host factors that are normally suppressed or inactivated when these cathepsins are present.

Our work has demonstrated that, contrary to prior reports, neither CatL nor CatB is required for infection. Instead, CatL and CatB may actually limit HPV16 infection of keratinocytes, as higher levels of infection were observed in the absence of these cathepsins. However, the host cell contains many other cathepsins and proteases that may be important for capsid uncoating and L1 degradation. Further work is needed to identify which, if any, of the host-cell proteases are involved in capsid disassembly during HPV infection.

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