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Study of infectious virus production from HPV18/16 capsid chimeras

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ABSTRACT

Using the HPV18 genome as the backbone, we exchanged the HPV18 L2 or L1 genes with those of HPV16. The intertypical exchange of HPV18 L1 with the HPV16 L1 produced genomes that efficiently replicated and produced infectious virus. Genomes containing an intertypical exchange of HPV18 L2 for the HPV16 L2 failed to produce infectious virus in multiple independently derived cell lines. Using chimeric constructs of individual capsid proteins, we identified a type-specific domain at the N-terminus of the HPV18L1 capsid protein, which interferes with its ability to cooperate with the HPV16 L2 protein to form infectious viral particles. Deletion of this domain allows for the cooperation of the HPV18 L1 protein and HPV16 L2 protein and production of infectious progeny. In addition, cooperation of this N-terminal HPV18 L1 deletion mutant protein with the wild-type HPV18 L2 protein efficiently replicates infectious virus but changes occur in the viral structure.

Introduction

The life cycle of human papillomaviruses (HPV) has evolved to be intimately connected to the differentiation program of host epithelial tissues (Meyers et al., 1992; Meyers et al., 1997; Taichman and LaPorta, 1986). The development of the organotypic (raft) epithelial culture system has allowed for the development on an in vitro culture system capable of reproducing the complete HPV life cycle, including the propagation of infectious viral particles (Alam et al., 2008; McLaughlin-Drubin et al., 2004; McLaughlin-Drubin and Meyers, 2004; McLaughlin-Drubin et al., 2003; Meyers et al., 1992; Meyers et al., 1997; Sen et al., 2004). The raft culture system has been used to study in detail the steps in the HPV life cycle (Alam et al., 2008; Bedell et al., 1991; Bodily et al., 2006; Bodily and Meyers, 2005; Frattini et al., 1996; Grassmann et al., 1996; Mayer and Meyers, 1998; Ozbun and Meyers, 1996; Ozbun and Meyers, 1997; Ozbun and Meyers, 1998a,b; Ozbun and Meyers, 1999b; Sen et al., 2004). In addition, two recent publications have shown that the replication and maturation of native virus in differentiating host tissue differs in significant aspects from particles made using pseudovirus/quasivirus technologies (Conway et al., 2009a,b).

Chimeric viruses are commonly used to compare genes from one virus with the homologous genes from a related virus to determine the similarities and differences of those genes. A chimeric virus system can be used to assign a particular viral phenotype to a specific gene or sequence. Another use of a chimeric virus system is to discover the commonalities of related viral genes. In this article, we used chimeric HPVs to test the hypothesis that, although the HPV18 and HPV16 capsid genes have a high amount of sequence homology, there are type-specific domains affecting the interaction of the two capsid proteins during virion morphogenesis.

Previously, we designed experiments to study whether the non-structural genes from one HPV type could function with the structural genes of another HPV type. In that study, we replaced the L1 and L2 capsid protein open reading frames (ORFs) from HPV type 18 (HPV18) with the L1 and L2 capsid protein ORFs from HPV16 (Meyers et al., 2002). The resulting HPV18/16 chimeric virus was able to carry out the complete viral life cycle culminating in the production of infectious virus after introduction into keratinocytes that were allowed to terminally differentiate and stratify in raft culture. Antiserum raised against HPV16 virus-like particles (VLPs) and not HPV18 VLPs specifically neutralized the HPV18/16 chimeric virus (Meyers et al., 2002). This study established the use of a viable chimeric virus replication system to study HPV genetics and confirmed the ability of the nonstructural genes of HPV18 to function with the structural genes of HPV16.

To extend these studies, we replaced either the L1 capsid ORF of HPV18 with that of HPV16 L1 or the L2 capsid ORF of HPV18 with that of HPV16 L2. Chimeric HPV18 containing the HPV16 L1 ORF were able to behave similar to wild-type HPV18 in that they completed the viral life cycle in terminally differentiating raft tissue with the production of infectious chimeric viral particles. However, while chimeric HPV18

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containing the HPV16 L2 ORF immortalized primary keratinocytes was maintained in an episomal state, induced a transformed phenotype in raft culture, amplified viral DNA (vDNA), and HPV18 L1 and the HPV16 L2 capsid gene expression appeared normal in raft cultures, we were unable to detect the production of infectious virus from this chimera. In an effort to begin to narrow down the region of the HPV18 L1 or HPV16 L2 capsid gene responsible for the interference of production of infectious particles, we analyzed chimeric exchanges of only half of the L1 or L2 capsid gene ORF of HPV18 with that of HPV16. These studies identified the N-terminus of the L1 capsid protein of HPV18 to be inhibitory to the production of infectious chimeric virus containing the HPV16 L2 capsid gene.

Results

L2 and L1 chimeric viruses

Previously, to investigate whether the nonstructural genes of one HPV type could cooperate with the structural genes of a second HPV type during the complete viral life cycle, we constructed a recombinant plasmid consisting of the URR and the nonstructural early gene ORFs of HPV18 and the structural late gene ORFs of HPV16 (Meyers et al., 2002). This chimeric virus behaved similar to wild-type HPV18 or HPV16 maintaining 50–100 episomal genomes copies in infected cells; on differentiation in the organotypic raft culture system, late viral functions, including capsid gene expression and infectious virus propagation, were observed (Meyers et al., 2002). These data demonstrated that the nonstructural genes of HPV18 function with the structural genes of HPV16, allowing the complete HPV life cycle to occur. In the present study, we hypothesized that the structural genes of one viral type contain domains that may affect intertype interactions during the viral life cycle and virion morphogenesis.

To test this hypothesis, it was necessary to create chimeric mutant viruses exchanging either the L2 or the L1 ORF of HPV18 for the L2 or L1 ORF of HPV16. An initial problem with creating these chimeric mutants is that the L2 and L1 ORFs of HPV18 and HPV16 overlap; therefore, direct exchanges of one of the ORF would affect the other ORF (Fig. S1). To overcome this situation, we first created mutant viruses using PCR technology to individually amplify each ORF, introducing appropriate restriction enzyme sites and cloning the amplimers into the pHPV18L1/L2Δ plasmid that lacks the L2 and L1 ORFs (Meyers et al., 2002) (Fig. S1). This created two mutant viral genomes; one wild-type for HPV18 except that the L2 and L1 ORFs do not overlap but are now separated by an unique *Hind*III site, the second is similar to our previous reported HPV18/16 chimera (Meyers et al., 2002), except that the HPV16 L2 and L1 ORFs do not overlap but are now separated by an unique *Hind*III site (Fig. S1). We named these mutants HPV18-L2(18)L1(18) and HPV18-L2(16)L1(16). Our main goal was to determine if capsid proteins from two different HPVs could cooperate to produce infectious virus, and if not, then what domain(s) of the protein is responsible. However, in creating these mutant genomes, there was the possibility that the tandem duplication of the putative L1 splice acceptor would interfere with expression of the capsid protein and therefore the ability of these mutants to produce infectious virus. Therefore, we first analyzed the mutants for their ability to correctly express the capsid proteins and produce infectious virus.

Continuously infected cell lines were made using our standard electroporation protocol previously reported (Conway et al., 2009a,b; McLaughlin-Drubin et al., 2004; McLaughlin-Drubin and Meyers, 2004; McLaughlin-Drubin and Meyers, 2005; McLaughlin-Drubin et al., 2003; Meyers et al., 2002; Meyers et al., 1997). Upon differentiation in raft culture, these cell lines were tested for late viral functions of viral genome amplification by Southern blotting, capsid gene expression by Western blotting, and propagation of infectious progeny by a limited dilution RT–PCR titering assay. Wild type and mutant viruses had a similar phenotype including viral titers when late viral functions were analyzed (Fig. 1). This suggested that the physical separation of the L2 and L1 ORFs had no significant effect on the viral life cycles. We concluded that construction of mutant viruses having their capsid gene ORFs separated did not introduce any adverse effects in the capsid protein expression and the production of infectious virus. All mutant genomes for this section and throughout this report were analyzed by restriction digestion and sequenced to ensure they were correct. Here and throughout the article, three or more independently derived virus-infected cell lines were tested, and the results were always found to be similar.

We then proceeded to create two chimeric mutants by exchanging the HPV18 L2 or L1 ORF for its counterpart from HPV16 (Fig. S1). We named these mutants HPV18-L2(18)L1(16) and HPV18-L2(16)L1(18). Cell lines continuously infected with each mutant were made as described (Conway et al., 2009a,b; McLaughlin-Drubin et al., 2005; McLaughlin-Drubin et al., 2004; McLaughlin-Drubin and Meyers, 2004; McLaughlin-Drubin et al., 2003; Meyers et al., 2002; Meyers et al., 1997). Upon differentiation in raft culture, these cell lines were tested for late viral functions of vDNA amplification by Southern blotting, capsid gene expression by Western blotting, and propagation of infectious progeny by Limited Dilution RT-PCR titering assay. Both mutant viruses were similar to wild-type in their ability to amplify their genomes and express their capsid genes, however, when infectivity was measured by Limited Dilution RT-PCR titering, HPV18-L2(18)L1(16) was capable of infectious virus synthesis similar to wild-type, but the chimera HPV18-L2(16)L1(18) was not (Fig. 1). Three individual continuously infected cell lines using three separate batches of primary foreskin keratinocytes were originally made for each of the mutants. To rule out the possibility that the particular genetic background of the three batches of primary foreskin keratinocytes were responsible for the lack of infectious virus production, we created 17 more HPV18-L2(16)L1(18) continuously infected cell lines for a total of 20 cell lines. Infectious virus was undetectable in 19 of the 20 cell lines after growth in raft culture (Table 3). Following growth in raft culture, we were able to detect infectious virus with only 1 of the 20 cell lines, but detection was at

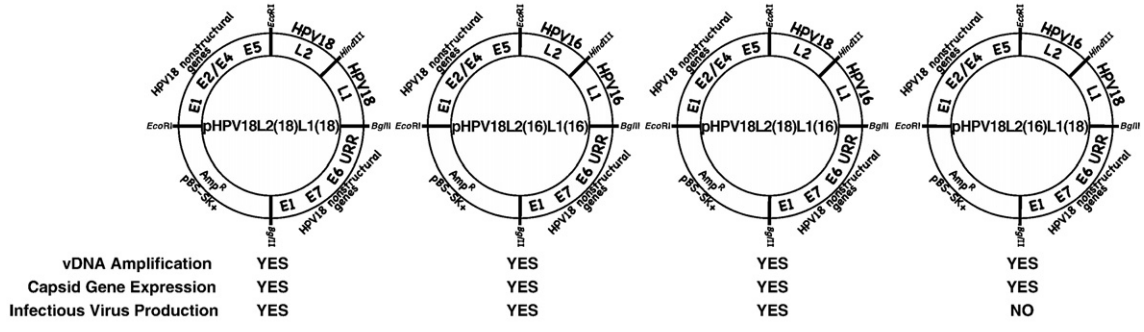

Fig. 1. Late functions of viral mutants with nonoverlapping L2 and L1 ORFs. Late viral life cycle functions were analyzed for the four mutant HPV18 viruses containing nonoverlapping L2 and L1 ORFs; HPV18-L2(18)L1(18), HPV18-L2(16)L1(16), HPV18-L2(18)L1(16), and HPV18-L2(16)L1(18).

the lowest limit of our ability to detect infectious virus having a titer of 20 (Table 3). This shows that the chimeric HPV, HPV18-L2(16)L1(18), was extremely inefficient at propagating infectious virus. We concluded that the low levels of infectious virus were a consequence of cooperation between the HPV18 L1 protein and the HPV16 L2 protein.

The separation of the L2 and L1 ORFs in these two chimeric mutants could potentially affect the splicing patterns of the mRNAs encoding the late proteins. Therefore, the inability of HPV18-L2(16)L1(18) to propagate infectious virus could be due to a lack or decrease in capsid protein expression. We therefore compared capsid protein expression of the mutant viruses to wild-type virus. For these analyses, we included three separate isolates of the replication deficient mutant, HPV18-L2(16)L1(18) (Figs. 2–4). Two different HPV18 L1-specific mAbs, H18.E20 and H18.L9 (Figs. 2–3), that cross-react with the HPV16 L1 protein and one HPV16 L2 mAb that cross-reacts with the HPV18 L2 protein, H16.4B4 (Fig. 4), were used. A replication-competent chimeric HPV18/CRPV virus (HPV18 with the capsid genes of CRPV) was used as a negative control. All HPV18/HPV16 L2/L1 chimeric viruses, including the three separate isolates of the replication incompetent virus HPV18-L2(16)L1(18), expressed similar levels of L1 (Figs. 2–3) and L2 (Fig. 4) as compared to wild-type HPV18. The physical separation of the two overlapping structural ORFs did not interfere with capsid protein expression and therefore the lack of infectivity observed with the HPV18-L2(16)L1(18) mutant virus is not due to reduced capsid protein expression. At least three separate batches were used for the Western analyses, and the results were the same for each batch. Differentiating epithelium infected with HPV18-L2(16)L1(18) produced assembled virion-like particles with benzonase protected encapsidated viral genomes at levels similar to wild-type levels (data not shown). Therefore, the lack of infectivity was not due to the inability to form stable viral particles.

We concluded from these studies that part or all of the HPV18 L1 and/or the HPV16 L2 proteins were unable to mutually cooperate to efficiently produce infectious virus. This led us to begin to characterize the region(s) of these two proteins responsible for this inability to cooperate.

L2 and L1 half and half chimeric viruses

Many secondary structures and functional domains have been mapped in the L1 and L2 proteins (Fig. 5). It was not readily apparent which structural or functional domain may account for the inability of L1 and L2 to cooperate in the chimeric mutant HPV18-L2(16)L1(18). Consequently, we decided to characterize potential inhibitory domains by making HPV18 and HPV16 L2 or L1 chimeras containing the N-terminal half of a protein from one viral type and the C-terminal half of a protein of the other viral type. We chose to use a homologous sequence in the middle of the L2 and L1 proteins of HPV18 and HPV16 for the chimeric junction, allowing for the maintenance of sequence and structure around the junction sites (see Materials and methods; Fig. S2; Fig. 5). In total, at least three independently derived cell lines for each of the eight chimeric mutant HPV genomes were created (Fig. S2).

Multiple continuously infected cell lines were made with each of the eight mutant genomes and their abilities to transit the viral life cycle and replicate infectious viral stocks were studied. When grown in raft culture, all cell lines were able to differentiate, and their morphologies were within the parameters we have observed for tissues infected with

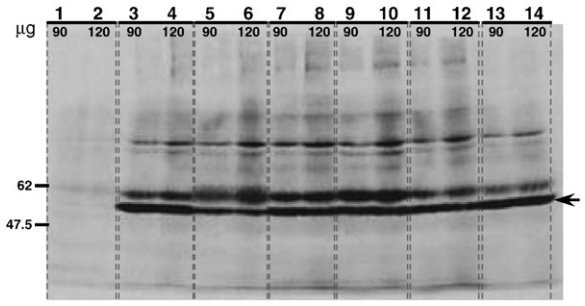

Fig. 3. Western blot of HPV L1 capsid protein expression with the L1-specific mAb H18.L9. H18.L9 monoclonal antibody was used to detect L1 protein expression from chimeric HPV-infected raft tissues. H18.L9 was raised against HPV18 L1 protein but cross-reacts with HPV16 L1 protein. Protein isolated from chimeric HPV18/CRPV virus-infected raft tissues, expressing the CRPV L2 and L1 capsid proteins was used as a negative control. Protein isolated from wild-type HPV18-infected raft tissues was used as a positive control. Protein isolated from virus-infected raft tissues were loaded, 90 or 120 µg to each well as labeled. Lanes 1 and 2: HPV18/CRPV-infected tissues; lanes 3 and 4: wild-type HPV18-infected tissues; lanes 5 and 6: HPV18-L2(18)L1(18)-infected tissues; lanes 7 and 8: HPV18-L2(18)L1(16)-infected tissues; lanes 9 and 10: HPV18-L2(16)L1(18)-infected tissues (cell line #1); lanes 11 and 12: HPV18-L2(16)L1(18)-infected tissues (cell line #2); and lanes 13 and 14: HPV18-L2(16)L1(18)-infected tissues (cell line #3). Note that lanes 9–14 represent three individually derived cell lines of the same mutant, HPV18-L2(16)L1(18). Molecular weight markers are on the left. The arrow indicates the position of the L1 protein.

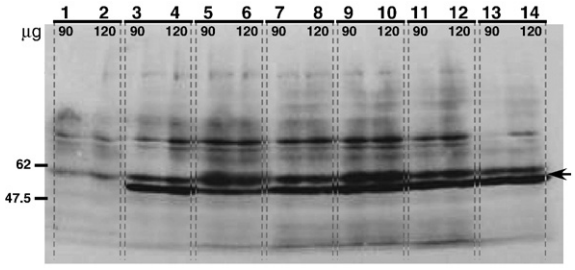

Fig. 2. Western blot of HPV L1 capsid protein expression with the L1-specific mAb H18.E20. H18.E20 monoclonal antibody was used to detect L1 protein expression from chimeric HPV-infected raft tissues. H18.E20 was raised against HPV18 L1 protein but cross-reacts with HPV16 L1 protein. Protein isolated from chimeric HPV18/CRPV virus-infected raft tissues, expressing the CRPV L2 and L1 capsid proteins was used as a negative control. Protein isolated from wild-type HPV18 infected raft tissues was used as a positive control. Protein isolated from virus-infected raft tissues were loaded, 90 or 120 µg to each well as labeled. Lanes 1 and 2: HPV18/CRPV-infected tissues; lanes 3 and 4: wild-type HPV18-infected tissues; lanes 5 and 6: HPV18-L2(18)L1(18)-infected tissues; lanes 7 and 8: HPV18-L2(18)L1(16)-infected tissues; lanes 9 and 10: HPV18-L2(16)L1(18)-infected tissues (cell line #1); lanes 11 and 12: HPV18-L2(16)L1(18)-infected tissues (cell line #2); and lanes 13 and 14: HPV18-L2(16)L1(18)-infected tissues (cell line #3). Note that lanes 9–14 represent three individually derived cell lines of the same mutant, HPV18-L2(16)L1(18). Molecular weight markers are on the left. The arrow indicates the position of the L1 protein.

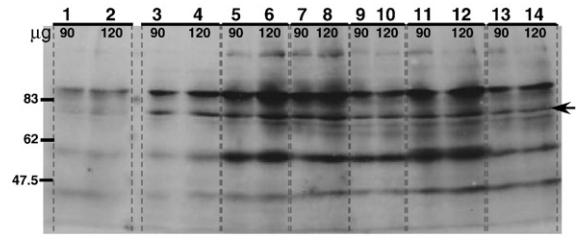

Fig. 4. Western blot of HPV L2 capsid protein expression with the L2-specific mAb H16.4B4. H16.4B4 monoclonal antibody was used to detect L2 protein expression from chimeric HPV-infected raft tissues. H16.4B4 was raised against HPV18 L2 protein but cross-reacts with HPV16 L2 protein. Protein isolated from chimeric HPV18/CRPV virus-infected raft tissues, expressing the CRPV L2 and L1 capsid proteins was used as a negative control. Protein isolated from wild-type HPV18-infected raft tissues was used as a positive control. Protein isolated from virus-infected raft tissues were loaded, 90 or 120 µg to each well as labeled. Lanes 1 and 2: HPV18/CRPV-infected tissues; lanes 3 and 4: wild-type HPV18-infected tissues; lanes 5 and 6: HPV18-L2(18)L1(18)-infected tissues; lanes 7 and 8: HPV18-L2(18)L1(16)-infected tissues; lanes 9 and 10: HPV18-L2(16)L1(18)-infected tissues (cell line #1); lanes 11 and 12: HPV18-L2(16)L1(18)-infected tissues (cell line #2); and lanes 13 and 14: HPV18-L2(16)L1(18)-infected tissues (cell line #3). Note that lanes 9–14 represent three individually derived cell lines of the same mutant, HPV18-L2(16)L1(18). Molecular weight markers are on the left. The arrow indicates the position of the L2 protein.

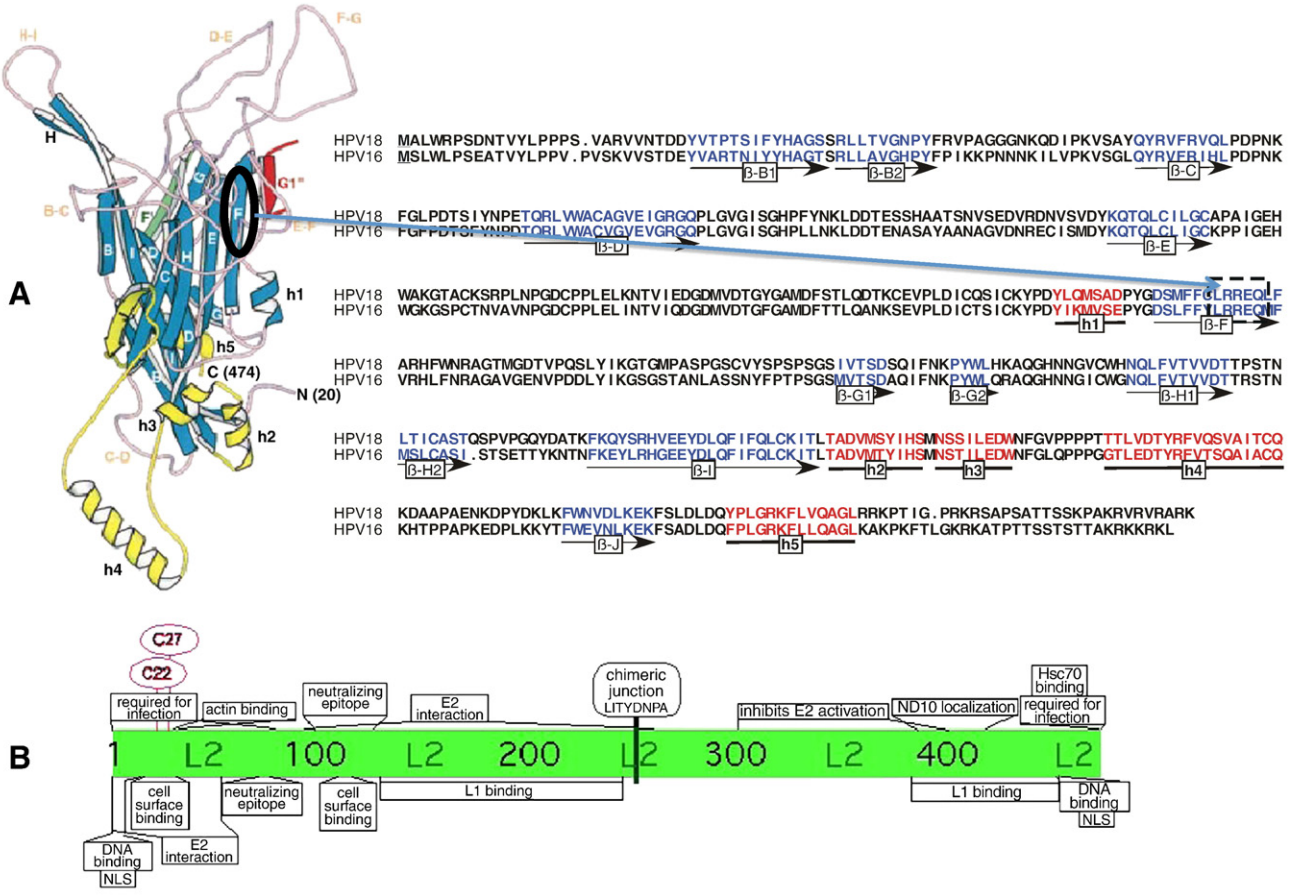

Fig. 5. Capsid proteins L1 and L2 chimeric junctions. Shown are the approximate positions of the cloning junctions used for the L1 and L2 HPV18 and HPV16 chimeras. (A) Shown are the three-dimensional monomeric structure of L1 (left) and the amino acid sequences of HPV16 and HPV18 L1 proteins beginning at the consensus methionine (right) as described by Chen et al. (2000). On the three-dimensional structure, an oval shows the region on the F ß-sheet strand containing the chimeric junction. The position of the chimeric junction can also be seen of the two-dimensional sequence inside of the box. The positions of the ß-sheets (capital letter only for the three-dimensional structure and ß-capital letter for the sequence) and the helixes (h) are shown. (B) Shown are described functional regions of papillomavirus L2 proteins (Bossis et al., 2005; Fay et al., 2004; Florin et al., 2006; Florin et al., 2002; Gornemann et al., 2002; Heino et al., 2000; Holmgren et al., 2005; Kamper et al., 2006; Kawana et al., 1999; Laniosz et al., 2007; Okun et al., 2001; Roden et al., 2001; Roden et al., 1994b; Yang et al., 2003a; Yang et al., 2003b; Zhou et al., 1994). The chimeric junction is shown at its position in the center of the L2 protein.

wild-type HPV18 and HPV16 (Fig. S3) (McLaughlin-Drubin et al., 2004; McLaughlin-Drubin and Meyers, 2004; Meyers et al., 2002; Meyers et al., 1997). Southern blots of whole tissue DNA were first probed with HPV18-specific radiolabeled probes then stripped and reprobed with HPV16-specific radiolabeled probes to demonstrate the presence of both HPV18 and HPV16 DNA (Fig. 6). Undigested DNA showed supercoiled (FI) and nicked (FII) genomic DNAs when probed with either HPV18 or HPV16. Viral genomes digested with *Eco*RI and probed with HPV18 or HPV16 demonstrated a linear (FIII) band consistent with a length of about 8 kb, the size of the HPV genomic DNA. Viral genomes digested with *Bgl*II and probed with HPV18 showed a band consistent with a length of approximately 4900 nt, the size of the URR and early genes (E) of HPV18. Viral genomes digested with *Bgl*II and probed with HPV18 or HPV16 showed a band consistent with a length of around 3 kb, representing the L2 and L1 ORFs (L) of the chimeric HPV (Fig. 6). Southern blots of independently derived chimeric HPV-infected cell lines showed similar results.

Titers of L2 and L1 half and half mutants

We next tested for infectious virus production. Putative viral stocks were prepared as previously described (Alam et al., 2008; Conway et al., 2009a,b; McLaughlin-Drubin et al., 2005; McLaughlin-Drubin et al., 2004; McLaughlin-Drubin and Meyers, 2004; McLaughlin-Drubin et al., 2003; Meyers et al., 2002; Meyers et al., 1997). To measure titers HaCaT cells were infected with 1:20, 1:100, 1:1000, 1:5000, 1:7500, and 1:10,000 dilutions of each viral preparation for 2 days, total RNA was then harvested, and had nested RT–PCR to specifically amplify the HPV18 early spliced viral mRNA E1^E4 transcript and an internal cellular control mRNA β-actin. Nested RT–PCR produces a 269-bp HPV18 E1^E4 and a 429-bp β-actin product. At the lowest dilution of virus, all of the half and half chimeric HPV genomes except HPV18-L2(16)L1(18/16) were able to produce infectious virus in our infectivity assay (Fig. 7 and Table 1). While HPV18-L2(18)L1(18/16) was infectious at a titer of 5000, stocks of HPV18-L2(16)L1(16/18) were somewhat less infectious having a titer of 1000 (Table 2). HPV18-L2(16/18)L1(18), HPV18-L2(16/18)L1(16), HPV18-L2(18/16)L1(18), and HPV18-L2(18/16)L1(16) all had a lower titer of 100 (Table 2). Finally, HPV18-L2(18)L1(16/18) only had a titer of 20 (Table 2). Multiple cell lines and viral stocks prepared from an infected cell line were tested for titers with all giving similar results for each particular mutant. This demonstrates that while the capsid gene chimeras did not appear to affect many aspects of the viral life cycle, there was a significant effect on titers for some of the mutants. At least three independently derived chimeric HPV-infected cell lines were tested for their production of infectious titers after growing in raft cultures. Titers in each case were found to be similar for each cell line infected with the same chimeric virus.

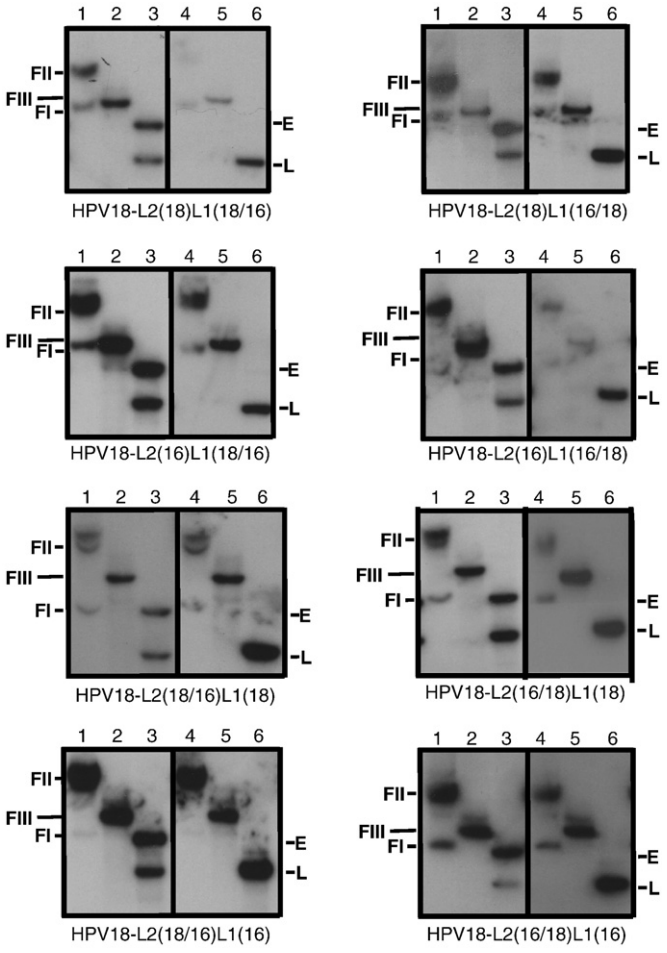

Fig. 6. Southern (DNA) blot hybridization of chimeric HPV DNA-electroporated HFK cell lines grown in monolayer cultures. Cell lines were analyzed for the episomal maintenance, copy number, and integrity of the chimeric genomic DNA. Left panels: The blots were probed with an HPV18-specific probe. Right panels: The blots were stripped and reprobed with an HPV16-specific probe. Samples in lanes 1 and 4 were undigested. Samples in lanes 2 and 5 were digested with *Eco*RI, a single cutter of the chimeric genomes. Samples in lanes 3 and 6 were digested with *Bgl*II to separate the capsid genes (late ORFs) from the rest of the viral genome (early ORFs). Indicated are form I DNA (FI), form II DNA (FII), form II DNA (FIII), early ORFs (E), and late ORFs (L).

Identification of a type-specific domain in the N-terminus of the HPV18 L1 gene

We looked for patterns to try to explain the differences in efficiency of chimeric mutant infectious virus production. One pattern we noticed (although unlikely to be the only pattern) was that chimeric viruses HPV18-L2(18/16)L1(18), HPV18-L2(16)L1(18/16), and HPV18-L2(16)L1(18) were all relatively inefficient for virus production, all had the C-terminus of HPV16 L2 and the N-terminus of HPV18 L1 (Table 3). These results suggested that at least one type-specific domain might reside in these regions affecting virion morphogenesis. We compared the amino acid sequence of the L1s of HPV16 and HPV18 and found that HPV18 L1 has an extra 35 amino acids in its N-terminus compared to HPV16 (Fig. 8). To determine if this extra 35 amino acids of HPV18 L1 affects the interaction of HPV16 L2 and HPV18 L1 during virion morphogenesis or infectivity resulting in poor viral titers, we constructed mutants deleting the first 35 amino acids of HPV18 L1 designated HPV18-L2(18)L1(Δ18), HPV18-L2(16)L1(Δ18), HPV18-L2(18/16)L1(Δ18), and HPV18-L2(16/18)L1(Δ18) (Fig. S4). At least three independently derived cell lines were created for each mutant virus. The loss of the 35 amino acids from the N-terminus had no effect of the stability of the particles.

All Δ18 L1 mutants were able to maintain their genomes episomally. The efficiency of the Δ18 L1 mutants for developing cell lines capable of stable episomal maintenance was 100%, the same as wild-type HPV18 (data not shown). All the tissues harboring HPV18 L1 N-terminal deletions were able to stratify and differentiate, with a similar morphology to that of wild-type HPV18. When we checked HPV mutant genome amplification by Southern blot analyses, all Δ18 L1 mutants, HPV18-L2(18)L1(Δ18), HPV18-L2(16)L1(Δ18), HPV18-

Table 1
Primers used for construction of mutant viruses.

Oligo name	Direction	Sequence 5′→3′
18a5′	5′	GCTAGCAGATCTATGGTATCCCACCGTGCCGC
18L2gHin	3′	GCTAGCAAGCTTCTAGGCCGCCACAAAGCC
18L1hHin	5′	GCTAGAAAGCTTATGTGCCTGTATACACGG
18L1hBgl	3′	GCTAGCAGATCTTTACTTTCCTGGCACGTAC
16c5′	5′	GCTAGCAGATCTATGCGACACAAACGTTCTGC
16L2eHin	3′	GCTAGCAAGCTTCTAGGCAGCCAAGAA
16L1fHin	5′	GCTAGCAAGCTTTTACAGCTTACGTTTTTTGCG
16L1fBgl	3′	GCTAGCAGATCTATGCAGGTGACTTTTATTTAC
CM B1	5′	GCTAGCGCTCTTCATGACAACCCGGCCTTTGAGCCTGTG
CM B2	3′	GCTAGCAAGCTTCTAGGCCGCCACAAAGCC
CM B3	5′	GCTAGCAGATCTATGCGACACAAACGTTCT
CM B4	3′	GCTAGCGCTCTTCATATGTAATAAGTTTAGTGGGAGT
CM C1	5′	GCTAGCAAGCTTATGTGCCTGTATACACGG
CM C2	3′	GCTAGCGCTCTTCCTCACGCCGTAAGCAAAAAAACATGGA
CM C3	5′	GCTAGCGCTCTTCGTGAACAAATGTTTGTTAGACATTTA
CM C4	3′	GCTAGCAGATCTTTACAGCTTACGTTTTTT
CM E1	5′	GCTAGCGCTCTTCATGATAATCCTGCATATGAAGGTATA
CM E2	3′	GCTAGCAAGCTTCTAGGCAGCCAAAGAGAC
CM E3	5′	GCTAGCAGATCTATGGTATCCCACCGTGCC
CM E4	3′	GCTAGCGCTCTTCGTCATATGTAATTAAAGAGGATGGACG
CM F1	5′	GCTAGCAAGCTTATGCAGGTGACTTTTATT
CM F2	3′	GCTAGCGCTCTTCCCTTCGTAAATAAAAAAATAAGCT
CM F3	5′	GCTAGCGCTCTTCGAAGGCAGCTTTTTGCTAGGCATTTT
CM F4	3′	GCTAGCAGATCTTTACTTCCTGGCACGTAC
CM N-35	5′	GCTAGCAAGCTTATGGTACACATTATTATTTGTGGC

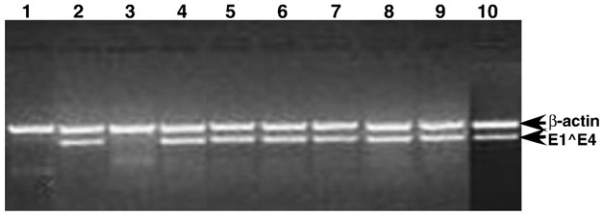

Fig. 7. Infectivity assay of chimeric HPVs. Shown is a 2% agarose gel of nested RT–PCR-amplified HPV18 E1^E4 and ß-actin. All assays were done with use 1:20 dilution of the viral stock. Lane 1: negative control mock-infected; lane 2: positive control wild-type HPV18; lane 3: HPV18-L2(16)L1(18/16); lane 4: HPV18-L2(18)L1(18/16); lane 5: HPV18-L2(18/16)L1(18); lane 6: HPV18-L2(18)L1(16/18); lane 7: HPV18-L2(16)L1(16/18); lane 8: HPV18-L2(16/18)L1(18); lane 9: HPV18-L2(16/18)L1(16); and lane 10: HPV18-L2(18/16)L1(16). Positions of ß-actin and E1^E4 amplified sequences are shown on the right.

Table 2
Virus titers of HPV18/16 L2/L1 chimeras.

Mutant	Titers	Independent measurements[a]	Titer range	Ave/SD[b]
Wt HPV18	7500	8	7500–10,000	8125/1157
HPV18-L2(18)L1(18/16)	5000	3	5000	5000/0
HPV18-L2(18)L1(16/18)	20	3	20	20/0
HPV18-L2(16)L1(18/16)	<20	4	0	0/0
HPV18-L2(16)L1(16/18)	1000	3	1000	1000/0
HPV18-L2(18/16)L1(18)	100	5	0–100	20/44.7
HPV18-L2(18/16)L1(16)	100	3	20–100	73/46.2
HPV18-L2(16/18)L1(18)	100	3	100	100/0
HPV18-L2(16/18)L1(16)	100	3	100	100/0

[a] Number of independent measurements made for each HPV construct.
[b] Titer average/standard deviation.

Table 3
Mutant viruses containing the HPV16 L2 C-terminus and the HPV18 L1 N-terminus.

Virus	Independently immortalized lines (line/attempts[a])	Infectious virus-producing lines (lines producing/total lines[b])	Titers	Independent measurements[c]	Titer range	Ave/SD[d]
HPV18-L2(16)L1(18/16)	4/4	0/4	<20	4	0	0/0
HPV18-L2(18/16)L1(18)	5/5	1/5	100	5	0–100	20/44.7
HPV18-L2(16)L1(18)	20/20	1/20	20	20	0–20	1/4.5

[a] The number of established continuous immortalized cell lines created with the particular HPV chimera over the number of attempts to derive such a cell line.
[b] The number of cell lines able to produce measurable infectious virus over the number of established cell lines.
[c] Number of independent measurements made for each HPV construct.
[d] Titer average/standard deviation.

L2(18/16)L1(Δ18), and HPV18-L2(16/18)L1(Δ18), were able to amplify their genomes when grown in raft cultures (data not shown). All independently derived lines infected with the same mutant virus showed similar results.

Performing our standard infectivity assay we measured titers of the Δ18 L1. Wild-type HPV18 had an infectious titer of 7500, and HPV18-L2(18)L1(Δ18) had an infectious titer of 10,000 (Table 4). This result shows that deletion of the HPV18 L1 35 amino acid N-terminus did not greatly affect virus titers. However, when we compared the Δ18 L1 chimeric mutants to their full-length counterparts, we observed a 125-fold increase in viral titer between HPV18-L2(16)L1(Δ18) (titer = 2500) and HPV18-L2(16)L1(18) (titer = 20) (Table 4). This result demonstrates that the first 35 amino acids in HPV18 L1 N-terminus, through a presently unknown mechanism, affects a necessary interaction of the HPV18 L1 protein and the HPV16 L2 protein resulting in lower viral titers. HPV18-L2(16)L1(18) was very inefficient in virus production, with only 1 out of 20 cell lines able to produce infectious virus. The only cell line able to produce infectious virus did so at the limit of the assay's ability to detect infection. After deleting the first 35 amino acids in HPV18 L1 N-terminus, the virus titer was increased 75-fold when comparing HPV18-L2(18/16)L1(Δ18) (titer = 7500) and HPV18-L2(18/16)L1(18) (titer = 100) (Table 4). Similarly, a 25-fold increase in viral titer was observed between HPV18-L2(16/18)L1(Δ18) (titer = 2500) and HPV18-L2(16/18)L1(18) (titer = 100). Together, these results suggest that the extra 35 amino acids of the HPV18 L1 N-terminus may cause a structural distortion in a HPV16 L2 capsid structure, which was relieved by deleting the HPV18 L1 N-terminus. These studies have identified one type-specific sequence affecting with the interaction between HPV16 L2 and HPV18 L1, resulting in lower levels of infectious virus production. At least three independently derived chimeric HPV-infected cell lines were tested for their production of infectious titers after growing in raft cultures. Titers in each case were found to be similar for each cell line infected with the same chimeric virus. In addition, mutants containing the Δ18 L1 produced benzonase protected genome encapsidated viral particles in numbers similar to wild-type (data not shown). We realize that others have produced VLPs, etc., using the consensus Met and splicing data suggest that these extra 35 amino acids would not contribute to the structure of the virus. However, our genetic analysis suggests that the N-terminal 35 amino acids do play a role in capsid structure.

Neutralization analyses of Δ18 L1 chimeric virus with L1 conformation-dependent antibodies

We hypothesized that changes in viral titers seen with the Δ18 L1 mutants (Table 4) were due to structural changes in the virus evoked by the mutation. To provide preliminary evidence that this may be the case, we used conformation-dependent L1 monoclonal antibodies (mAb) to measure changes in the infectivity neutralization associated with the mutations. To test whether the capsid structure had been affected, HPV18 L1 conformation-dependent antibodies H18.J4 and H18.K2 were tested for their ability to neutralize infection by HPV18-L2(18)L1(Δ18), HPV18-L2(16)L1(Δ18), HPV18-L2(18/16)L1(Δ18), and HPV18-L2(16/18)L1(Δ18). Although the epitopes for these monoclonals have not yet been mapped we know that the deleted N-terminal 35 amino acid sequence cannot contain the epitope for H18.J4 or H18.K2. This is because mAbs H18.J4 and H18.K2 were raised against HPV18 L1 VLPs lacking the first 61 amino acids (Fig. 8; Bishop et al., 2007). Infection by wild-type HPV18, as expected, was completely neutralized by both H18.J4 and H18.K2 (Fig. 9A). Interestingly, all four mutants were neutralized by H18.J4 but not by H18.K2 (Fig. 9A), suggesting that subtle but significant changes were affected by the removal of the N-terminal 35 amino acids from the HPV18 L1 protein. Studies were repeatable with virus from independently derived cell lines.

MAb binding to Δ18 L1 mutants

We reasoned that the lack of neutralization by the mAb H18.K2 was due to an inability to bind to the mutant particle or that the H18.K2 may still be able to bind but unable to block infection due to a change in conformation caused by the loss of the N-terminus 35 amino acids. To test whether the failure of neutralization correlated to the loss of antibody binding by the conformation-dependent antibodies, ELISAs were performed. To perform ELISA analysis, each chimeric HPV was used as antigen with wild-type HPV18 serving as the positive controls. Wells probed with an irrelevant mAb were

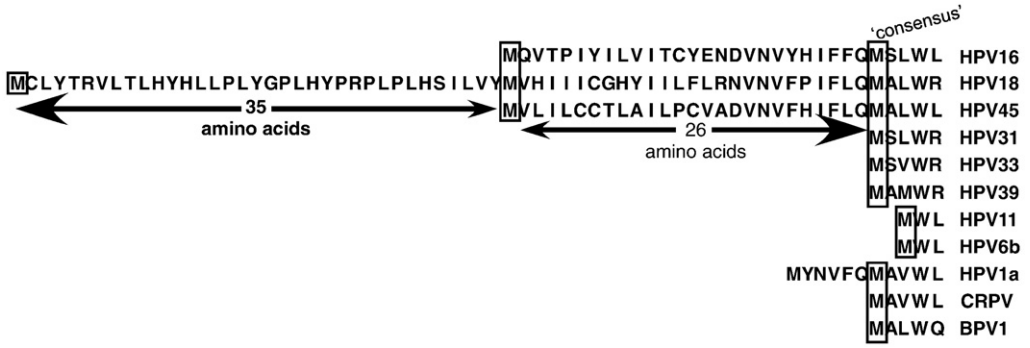

Fig. 8. Papillomavirus L1 N-terminus amino acid alignment. Potential start sites for high-risk HPVs and the 'consensus' methionine are boxed.

Table 4
Virus titers of HPV18 N-terminal 35 aa deletion mutants.

Mutant	Titers	Independent measurements[a]	Titer range	Ave/SD[b]
Wt HPV18	7500	8	7,500–10,000	8125/1157
HPV18-L2(18)L1(Δ18)	10,000	3	10,000	10,000/0
HPV18-L2(16)L1(18)	20	20	0–20	1/4.5
HPV18-L2(16)L1(Δ18)	2500	3	2500	2500/0
HPV18-L2(18/16)L1(18)	100	5	0–100	20/44.7
HPV18-L2(18/16)L1(Δ18)	7500	3	7500	7500/0
HPV18-L2(16/18)L1(18)	100	3	100	100/0
HPV18-L2(16/18)L1(Δ18)	2500	3	2500	2500/0

[a] Number of independent measurements made for each HPV construct.
[b] Titer average/standard deviation.

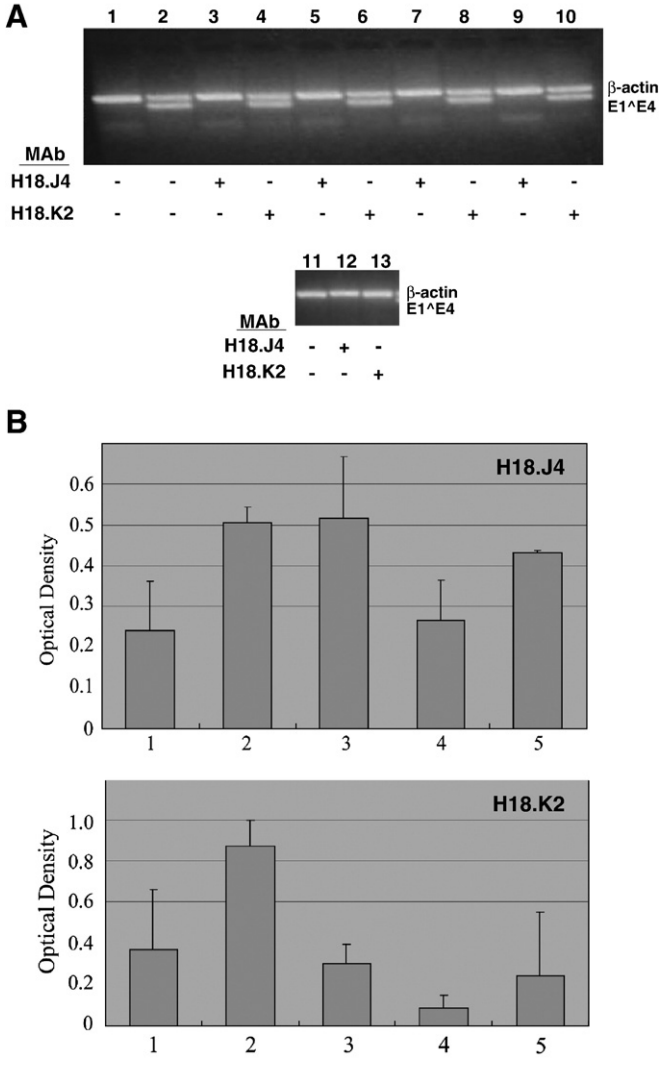

Fig. 9. (A) Neutralization of chimeric HPVs. Shown is a 2% agarose gel of nested RT–PCR-amplified HPV18 E1^E4 and ß-actin. Chimeric viruses were preincubated with the indicated mAb, H18.J4 or H18.K2. Lane 1: negative control, mock-infected; lane 2: positive control, wild-type HPV18; lanes 3 and 4: HPV18-L2(18)L1(Δ18); lanes 5 and 6: HPV18-L2(16)L1(Δ18); lanes 7 and 8: HPV18-L2(18/16)L1(Δ18); lanes 9 and 10: HPV18-L2(16/18)L1(Δ18); lane 11: negative control, mock infected; lanes 12 and 13: positive control, wild-type HPV18. Positions of ß-actin and E1^E4 amplified sequences are shown on the right. (B) Relative binding of conformation-dependent HPV18 L1-specific mAbs H18.J4 and H18.K2 to chimeric HPVs by ELISA assay. Relative binding for each chimeric HPV was defined as the difference between wells probed with (A) H18.J4 or (B) H18.K2 and wells probed with an irrelevant mAb. Values shown are means ± standard errors of the means. Lane 1: wild-type HPV18; lane 2: HPV18-L2(18)L1(Δ18); lane 3: HPV18-L2(16)L1(Δ18); lane 4: HPV18-L2(18/16)L1(Δ18); and lane 5: HPV18-L2(16/18)L1(Δ18).

included and used to subtract background signal. The ELISA measurement for each chimeric HPV was defined as the difference between wells probed with a L1 type-specific and a type-irrelevant antibodies. We first measured binding using the mAb H18.J4, which was capable of neutralizing all of the Δ18 L1 mutants. As seen in Fig. 9B, H18.J4 was able to bind to each of the Δ18 L1 mutants as well or better than wild-type HPV18. We next measured the binding of H18.K2, which was unable to neutralize the Δ18 L1 mutants (Fig. 9A). With the exception of HPV18-L2(18/16)L1(Δ18), H18.K2 was still able to bind to the other three Δ18 L1 mutants similar or better than its binding to wild-type (Fig. 9B). Although the binding of H18.K2 was more variable than what was seen with H18.J4. These results suggest that the change in capsid structure evoked by the deletion of the N-terminus 35 aa of the HPV18 L1 protein blocked the neutralizing capacity of mAb H18.K2 but not necessarily its ability to bind to the capsid. While not quantitative, the ELISA results demonstrated that at least part of the chimeric capsid proteins were correctly folded and could be recognized by conformation-dependent antibodies. These results collectively demonstrate that the extra 35 aa on the HPV18 L1 N-terminus interferes with the ability of the HPV18 L1 and the HPV16 L2 to cooperate and properly assemble into infectious viral particles. Studies were repeatable with virus from independently derived cell lines.

Discussion

For these studies, we used the organotypic raft culture system to test the chimeric mutants instead of coexpressing the viral structural proteins in a simple and less demanding system such as VLP or pseudovirus technology for several reasons. First, we wanted to test the mutants in a system best capable of using the process of naturally differentiating human epithelium by which HPV replicates itself in vivo. Second, we have extensive experience with raft cultures in studying the HPV life cycle and replication of infectious virus. Third, in two recent publications, we have shown that the replication and maturation of native virus in differentiating host tissue differs in significant aspects from particles made using pseudovirus/quasivirus technologies (Conway et al., 2009a,b). When the two conserved L2 cysteines were mutated in pseudo- or quasiviruses, the particles produced were noninfectious (Campos and Ozbun, 2009; Gambhira et al., 2009). When we tested the same mutations in native particles, not only were the mutant viruses infectious but titers were enhanced significantly (Conway et al., 2009a). This showed that viral morphogenesis can differ from particles formed in undifferentiating monolayer culture versus native particles formed in differentiating host epithelium. Finally, we have numerous other studies in the lab underway, including studies on mechanisms of infectivity, neutralization and cross-neutralization epitopes, disinfectant sensitivity, and microbicide sensitivity, all demonstrating differences between particles made in monolayer culture and native virus made in host epithelium.

Our laboratory has previously shown that both structural genes of HPV16 could function with the nonstructural genes of HPV18 for virus

production (Meyers et al., 2002). The present study sought to determine if the structural proteins, L2 and L1, could cooperate to produce infectious viral particles. We replaced either the L2 ORF or the L1 ORF of HPV18 with the counterpart from HPV16. All aspects of the viral life cycle examined including expression of the capsid proteins and particle assembly were found to be similar to wild-type; however, while the HPV18 L2 protein could function with the HPV16 L1 protein to propagate infectious particles, the HPV16 L2 protein was not able to cooperate with the HPV18 L1 protein for infectious particle production.

In an effort to identify the regions of either the HPV18 L1 and/or the HPV16 L2 protein involved in the inability to propagate infectious virus, we made chimeras where only half of either the HPV18 L2 or L1 ORFs were exchanged for the complementary region of HPV16. After testing the eight resulting viral chimeras, it appeared that whenever the N-terminal of the HPV18 L1 was present in the chimera with the C-terminal half of the HPV16 L2 protein, production of infectious virus synthesis was compromised. After comparing the L1 protein N-terminal sequences of HPV18 and HPV16, it was noticed that HPV18 had 35 unique amino acids on its L1 protein N-terminus. In addition, when the L1 protein N-termini of HPV18 and HPV16 were aligned with the N-termini of other papillomaviruses, both had additional in-frame sequences on their N-termini when compared to the other papillomaviruses. Specifically, HPV18 had an additional 61 amino acids and HPV16 had an additional 26 amino acids. In studies where virus-like particles (VLP), pseudovirus, or quasivirus are used, these extra sequences are deleted from the expression vectors used and all L1 expression begins at the 'consensus' ATG (Browne et al., 1988; Carter et al., 1991; Cason et al., 1994; Heino et al., 1995; Kirnbauer et al., 1992; Kirnbauer et al., 1993; Nardelli-Haefliger et al., 1997; Rossi et al., 2000; Xi and Banks, 1991; Zhou et al., 1990; Zhou et al., 1991a,b). While maps of HPV18 late gene mRNAs have yet to be published, splicing studies of the HPV16 capsid ORFs have mapped a splice acceptor at nt 5637 3′ to the first in-frame ATG and 5′ to the second ATG of the L1 ORF (Doorbar et al., 1990; Milligan et al., 2007). Use of this splice acceptor would remove the extra 26 amino acids from the N-terminus of the HPV16 L1 protein. Evidence for the potential expression of L1 proteins initiated from the first ATG comes from a recent publication from our laboratory (Conway et al., 2009b). In this study, we demonstrated that native viral particles consisted of two L1 proteins of different sizes. Importantly, the slower migrating second L1 protein is not present in pseudo/quasiviruses (Conway et al., 2009b). While a posttranslational modification is possible, the sizes of the two proteins are consistent with the larger being 26 amino acids more than the smaller. It is possible that another splice acceptor 5′ of the first ATG was missed in previous studies or it is also possible that the larger L1 protein expressed is translated from one of the several transcripts containing both the L2 and L1 ORFs (Milligan et al., 2007). In eukaryotic cells, initiation of translation can occur by a mechanism of internal initiation where the ribosome is recruited to an internal start site that can be a considerable distance downstream of the 5′ terminus of the RNA (Charnay et al., 2009; Jackson, 2000; Lin et al, 2009; Stoneley and Willis, 2004). Using the Kozak rules for translational initiation, the upstream ATG of HPV18 is either equivalent or superior to that for the downstream ATG (Kozak, 1989).

When the extra 35 amino acids (as compared to the L1 protein from HPV16) in the N-terminus of the HPV18 L1 protein was deleted, chimeric virus containing the L1 protein of HPV18 and the L2 protein of HPV16 produce viral titers equivalent to wild-type. Therefore, these results demonstrate that the first 35 amino acids of HPV18 L1 N-terminus act as a type-specific sequence affecting the proper interactions between HPV16 L2 and HPV18 L1.

HPV11 L1 has been shown to cooperate and form complexes with L2 proteins of HPV5, 6b, 12, 16, 33, and canine oral PV type 1 (COPV1) but not the L2s of either HPV1a or BPV1 (Finnen et al., 2003) demonstrating type specificity in the interaction of L2 and L1. Another study suggested that viral type-restricted domains exist affecting the cooperation between L1 and L2 of different PV types (Okun et al., 2001).

The L1 protein has been shown to be involved in viral DNA binding, cell surface binding, and nuclear localization (Day and Schiller, 2006; Graham, 2006). All these known activities are concentrated in the L1 C-terminus. So far, there are no known activities reported for the L1 N-terminus. In contrast to L1, the minor capsid protein L2 has been shown to be multifunctional. For example, L2 is required to cooperate with L1 and induce the localization of viral L1 proteins to nuclear subdomain ND10 (Becker et al., 2003; Florin et al., 2002; Okun et al., 2001). In addition, the L2 protein consists of interactive sequences for the viral E2 protein (Finnen et al., 2003; Heino et al., 2000; Okun et al., 2001), Hsc70 chaperones (Florin et al., 2004), β-actins (Yang et al., 2003b), t-SNARE (Bossis et al., 2005), and cell surface receptors (Da Silva et al., 2001; Giroglou et al., 2001; McMillan et al., 1999; Roden et al., 1994a). Incorporation of L2 into the viral capsid also enhances the VLP and pseudovirus assembly and infection (Holmgren et al., 2005).

In addition to biological activities, structural analysis of the HPV16 L1 VLP capsid has been characterized (Chen et al., 2000). Because the HPV16 L1 VLP could be crystallized for analysis only after the first 36 amino acids were deleted (the 26 that are all deleted in VLPs plus 9 more), the extreme L1 N-terminal fragment is missing and the structure is unknown. By comparison to the structurally related major capsid protein of SV40, the PV L1 N-terminus is proposed to project into the groove between the capsomeres to help stabilize the structure. The extra piece in HPV18 L1 N-terminus may be located close to the capsid surface and form extra contacts with the minor capsid protein to further stabilize the particle (Chen et al., 2000; Modis et al., 2002). Notably, 19 out of the 35 extra amino acids are hydrophobic. It is likely that this N-terminal sequence is buried inside the capsid or deep in the groove between the capsomeres. HPV18 L1 conformation dependent monoclonal antibodies H18.J4 and H18.K2 were both highly efficient at neutralizing infection of wild-type HPV18 and HPV18-L2(18)L1(18) virus. All four chimeras with the HPV18 L1 N-terminal 35 amino acids deleted were neutralized by H18.J4 but not by H18.K2. The epitope recognized by H18.K2 cannot be contained within the deleted 35 amino acids because this sequence was not part of the VLP used to produce H18.K2. These results suggest that the epitope recognized by H18.K2 is sensitive to structural changes resulting from deletion. In addition, the ELISA results suggest that the structural change does not necessarily inhibit binding of H18.K2 to the viral particle but only prevents its ability to neutralize infection. Using the *in vitro* raft culture system and mutating one methionine at a time may help us to identify the start codon(s) used by the native virus. Although the L1 structure has been studied, the L2 structure is largely unknown.

In this study, we used a genetic approach to study HPV morphogenesis and showed that the N-terminus of HPV18 L1 interfered with the cooperation between HPV18 L1 and HPV16 L2 during virion morphogenesis. After deleting the 35 amino acids in HPV18 L1 N-terminus, infectious virus production was greatly improved. Since HPV18 virus can be efficiently produced with or without the first 35 amino acids, its potential role in viral life cycle is intriguing. In addition, because most available conformation-dependent antibodies were raised against PV L1 VLPs, atomic structure analysis of VLPs combined with immunological studies using our chimeric viruses may also help us to better understand the similarities and differences between VLPs and authentic virions.

Materials and methods

Plasmid construction

HPV18 DNA, a generous gift from Harold zur Hausen, was cloned into pBluescript SK(+) [pBSSK(+)] (Stratagene, La Jolla, CA) at the

unique HPV18 *Eco*RI site within the E1 ORF and named pBSHPV18. pBSHPV18 was used as a template to separately amplify the L2 and L1 ORFs using primers 18a5′, 18L2gHin, 18L1hHin, and 18hBgl (Table 1). The HPV16 L2/L1 ORFs were *Bgl*II digested out of the HPV18/16 chimeric genome (Meyers et al., 2002) and ligated into the *Bgl*II site of the pGL2 Basic Vector (Promega, Madison, WI) to make pGL216L2/L1. The electroporation protocol requires the release of the HPV genomic DNA from the vector DNA sequences. Since the HPV18 genomic DNA was cloned into the vector DNA at the *Eco*RI site, it was necessary to mutate the *Eco*RI site contained within the HPV16 L1 ORF at 6819 (Meyers et al., 2002). Using pGL216L2/L1 as a template the HPV16 L2 and L1 ORFs were separately amplified using primers 16c5′, 16L2cHin, 16L1fHin, and 16L1fBgl (Table 1). Each amplified ORF was cloned individually into the pCR2.1 vector (Invitrogen, Eugene, OR) using the TOPO TA cloning procedure according to the manufacturer's instructions (Invitrogen). Each capsid ORF was then individually cloned into pBSSK + vectors. The plasmid pBSHPV18ΔL2/L1 contains a HPV18 genome with its L2 and L1 ORFs removed and replaced with a *Bgl*II site in a pBSSK(+) vector (Meyers et al., 2002). pBSHPV18ΔL2/L1 was linearized by digesting with *Bgl*II, the HPV18 and HPV16 capsid ORFs were separated from the pBSSK + vector following digestion with *Bgl*II and *Hind*III, and each combination of L2 and L1 ORFs was cloned into the linearized pBSHPV18ΔL2/L1 plasmid as shown in Fig. S1. All mutant genomes were analyzed by restriction digestion and sequenced to ensure they were correct.

Each half (N-terminal and C-terminal) of the HPV18 and HPV16 L2 and L1 ORFs were PCR amplified using primers B1-4, C1-4, E1-4, and F1-4 (Table 1) as shown in Fig. S2. This created N-terminal and C-terminal halves with the N-termini of the L2 ORFs and the C-termini of the L1 ORFs containing a *Bgl*II (BII) restriction site, and the C-termini of the L2 ORFs and the N-termini of the L1 ORFs containing a *Hind*III (HIII) restriction site. The other end of each amplified sequence contained a *Sap*I (SI) restriction site. Each amplified sequence was then digested with *Bgl*II and *Sap*I or *Hind*III and *Sap*I as appropriate and isolated. To create chimeric L2 or L1 ORFs, the N-termini and C-termini of HPV18 L2 and L1 ORFs were ligated to the N-termini and C-termini of HPV16 L2 and L1 ORFs, respectively, and with the pGL2 vector that was previously linearized by double digestion with *Bgl*II and *Hind*III (Fig. S2). Restriction digests and sequencing were performed to verify the chimeric capsid ORFs. Following digestion with *Bgl*II and *Hind*III, the chimeric capsid ORFs were separated and isolated from the pGL2 vector. Wild-type HPV18 and HPV16 L2 and L1 ORFs were separated and isolated from the HPV18-L2(18)L1(18) and HPV18-L2(16)L1(16). A chimeric L2 or L1 ORF was then ligated with a wild-type L1 or L2 ORF along with pHPV18L2/L1Δ that had been previously digested with *Bgl*II and *Hind*III (Fig. S2). This produced eight chimeric virus genomes as shown in Fig. S2. We designated the resulting chimeric plasmids as HPV18-L2(18)L1(16/18), HPV18-L2(18)L1(18/16), HPV18-L2(16)L1(16/18), HPV18-L2(16)L1(18/16), HPV18-L2(16/18)L1(18), HPV18-L2(16/18)L1(16), HPV18-L2(18/16)L1(18), and HPV18-L2(18/16)L1(16) (Fig. S2). All mutant genomes were analyzed by restriction digestion and sequenced to ensure they were correct.

The HPV18/HPV16 L2 chimeras form a junction in the homologous sequence, LITYDNPA, which corresponds to amino acids 249–256 in HPV16 and amino acids 248–255 in HPV18. The HPV18/HPV16 L1 chimeras form a junction in the homologous sequence, LRREQ, which corresponds to amino acids 276–280 in HPV16 and amino acids 311–315 in HPV18. The amino acid difference in numbering for the L1 and L2 proteins is due to gaps inserted when the HPV18 and HPV16 sequences were lined up according to their homologies. The homologous sequences that form the junction site for the L2 and L1 chimeras are approximately in the middle of each ORF.

To create viral genomes containing a HPV18 L1 with the N-terminal first 35 amino acids deleted, primers N-35 and F4 (Table 1) were used to PCR amplify the ORF sequence lacking the first 105 nt (Fig. S3). Primer N-35 maintained the start sequence ATG and added a *BamH*II site on the 5′ end and the F4 primer added a *Bgl*II site on the 3′ end. This amplimer was then digested with *BamH*II and *Bgl*II and was ligated into the pHPV18L1/L2Δ plasmid that was previously digested with *BamH*II and *Bgl*II along with one of the following wild-type or chimeric L2 ORFs: 18 L2, 16 L2, 18/16 L2, or 16/18 L2 (Fig. S3). This create four mutant viral genomes, HPV18-L2(18)L1(Δ18), HPV18-L2(16)L1(Δ18), HPV18-L2(18/16)L1(Δ18), and HPV18-L2(16/18)L1(Δ18) (Fig. S3). All mutant genomes were analyzed by restriction digestion and sequenced to ensure they were correct.

Western blot analysis

One hundred-microliter aliquots of protein extracts were transferred into a 1.5-ml microfuge tube, followed by adding 100 μl of buffer A (50 mM $NaPO_4$ (pH 7.2), 5 mM EDTA, 50 mM NaF, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 20 μg/ml aprotinin, 0.2 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF). To this suspension, 200 μl of buffer B was added (identical to buffer A but supplemented with 2% wt./vol. SDS) and boiled in a water bath for 8 min. While boiling, the samples were vortexed for 2 min. To each sample, 125 μl of 5× sample loading buffer was added (100 mM Tris, pH 8.0, 5 mM EDTA, 10% (wt./vol.) sucrose, 2% (wt./vol.) SDS, 33 mM DTT, and 0.1 mg/ml Pyronin Y). Total protein concentrations were measured using the Peterson protein assay (Lowry method) as previously described (Meyers et al., 2001). To determine capsid protein expression, 90 and 120 μg of the extracts were loaded onto 7.5 % SDS–PAGE gels. Proteins were transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell). Membranes were incubated overnight with primary monoclonal antibodies (kindly provided by Dr. Neil Christensen, Penn State College of Medicine), H18.E20 (L1-reactive) (Rizk et al., 2008), H18.L9 (L1-reactive) (Bishop et al., 2007), and H16.4B4 (L2-reactive) (Embers et al., 2004). All three monoclonals cross-react with their respective capsid proteins from HPV18 and HPV16 but not with CRPV. After being washed, the blots were incubated with anti-mouse horseradish peroxidase linked secondary antibody (Amersham Life Science) as per the manufacturer's instructions. The proteins were detected using the enhanced chemiluminescence method (Perkin-Elmer) as per the manufacturer's instructions.

Electroporation and maintenance of keratinocyte

Primary human foreskin keratinocyte (HFK) cultures were derived from newborn foreskin via trypsin digestion at 37 °C as previously described (Meyers, 1996). HFKs were maintained in monolayer cultures without feeder cells, with 154 medium (Cascade Biologics, Portland, OR), supplemented with antibiotics (Cascade Biologics) and human keratinocyte growth supplement (Cascade Biologics). Multiple batches of primary human foreskin keratinocytes were electroporated with each mutant genome as previously described (Meyers et al., 2002).

Organotypic raft cultures and histochemical analyses

Organotypic raft cultures were grown as previously described (Meyers et al., 2002). Once at the air–liquid interface the raft cultures were fed by diffusion from below every other day with E medium lacking EGF supplemented with 10 μM 1,2-dioctanoyl-*sn*-glycerol (C8; Sigma Chemical Co.). Raft cultures were allowed to stratify and differentiate for 12 days because viral gene expression has been shown to peak between 10 and 12 days in the raft system (Ozbun and Meyers, 1999a; Ozbun and Meyers, 1997; Ozbun and Meyers, 1998a, b; Ozbun and Meyers, 1999b). For histochemical analyses, harvested raft tissues were fixed in 10% neutral-buffered formalin and were embedded in paraffin. Four-micrometer sections were cut and stained with hematoxylin and eosin.

Southern blot hybridization

Viral DNA was isolated as previously described (Meyers et al., 2002). Five micrograms of total cellular DNA from each sample was digested with either *Eco*RI, to linearize the viral genomes, or *Bgl*II, to separate the structural genes from nonstructural genes, or left undigested and then electrophoresed in a 0.8% agarose gel and transferred onto a GeneScreen Plus membrane (New England Nuclear Research Products, Boston, MA) as previously described (Meyers et al., 2002). Southern blot hybridization was performed as previously described (Meyers et al., 2002). The HPV DNA probes were prepared by gel purification of the 8 kb HPV and labeled with the Random Primed DNA labeling kit (Roche Molecular Biochemicals, Indianapolis, IN). Labeled probe was purified with a Quick Spin Column for radiolabeled DNA purification (Roche Molecular Biochemicals). Blots were probed first with a complete HPV18 genomic probe and then were stripped and reprobed with a complete HPV16 genomic probe. Stripping of a membrane was accomplished by placing the blots in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate and boiling for 1 h.

Preparation of virus stocks

Virus stocks were prepared by peeling the epithelial tissue away from the collagen of three organotypic rafts for each HPV chimera. The peeled epithelial layers were homogenized in 0.6 ml of ice-cold 1 M NaCl/0.05 M Na-Phosphate Buffer with a 7.5-ml homogenizer. The homogenizer was washed twice with 200 µl 1 M NaCl/0.05 M Na–phosphate buffer, pH 8. The homogenized viral solution was centrifuged at 10.5 k for 10 min at 4 °C, and the supernatant was transferred to a 1.8-ml Nalgene cryovial. The virus stocks were stored at −20 °C.

HPV infection and neutralization assays

The infectivity measurement of chimeric HPV used the limited dilution RT–PCR titering assay as described previously (Alam et al., 2008; Conway et al., 2009a,b; McLaughlin-Drubin et al., 2004; McLaughlin-Drubin and Meyers, 2004; McLaughlin-Drubin and Meyers, 2005; McLaughlin-Drubin et al., 2003; Meyers et al., 2002; Meyers et al., 1997) using HaCaT cells, an immortalized human keratinocyte cell line (kindly provided by Norbert Fusenig). The virus titer was determined to be the last dilution at which the spliced transcript could be detected.

Infectivity neutralization assays were performed by infecting HaCaT cells with a 1:20 diluted viral sample that had been preincubated with type-specific monoclonal antibodies (mAb) diluted 1:20 in HaCaT culture medium as previously described (Conway et al., 2009a,b; McLaughlin-Drubin et al., 2004; McLaughlin-Drubin and Meyers, 2005; McLaughlin-Drubin et al., 2003). The type-specific antibodies included L1-reactive conformation-dependent monoclonal antibodies, H18.J4 and H18.K2. Both of these antibodies were previously characterized (Bishop et al., 2007). Following preincubation, HaCaT cells were infected with the virus–antibody mixture and incubated for 2 days as described.

ELISA

Five microliters of HPV stocks was bound per well to 96-well plates overnight at 4 °C in neutral PBS buffer, washed, and blocked for 2 h with 5% nonfat milk in PBS. Conformation-dependent HPV18 L1-reactive monoclonal antibodies H18.J4 and H18.K2 Bishop et al., 2007) were used in ELISA studies. Wells containing HPV mutants were incubated with mAbs diluted 1:100 in blocking buffer for 1 h, followed by incubation with rabbit anti-mouse-AP (Pierce) at 1:1000 and development with 1 mg/ml *p*-nitrophenyl phosphate substrate (Sigma). Wells probed with a negative control mAb H11.B2 (a HPV11 L1 conformation-dependent, neutralizing mAb) were read and used as background values for comparison. Absorbance at 405 nm (A_{405}) was measured via a microplate reader (ThermoLabsystems). The readings from wells probed with capsid-specific mAb minus the readings from wells probed with the negative control mAb was defined as the specific binding to each mutant HPV.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.05.019.

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