Virology 409 (2011) 348–359

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The N-terminal region of the human papillomavirus L2 protein contains overlapping binding sites for neutralizing, cross-neutralizing and non-neutralizing antibodies

Ivonne Rubio [a], Hanna Seitz [a], Elena Canali [b], Peter Sehr [c], Angelo Bolchi [b], Massimo Tommasino [d], Simone Ottonello [b], Martin Müller [a,*]

[a] Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, 69120 Heidelberg, Germany
[b] Department of Biochemistry and Molecular Biology, University of Parma, Viale G.P. Usberti 23/A, 43100 Parma, Italy
[c] European Molecular Biology Laboratory, Meyerhofstraße 1, 69117 Heidelberg, Germany
[d] Infections and Cancer Biology Group, International Agency for Research on Cancer, 150 Cours A. Thomas, 693008 Lyon, France

ARTICLE INFO

Article history:
Received 19 July 2010
Returned to author for revision
18 August 2010
Accepted 13 October 2010
Available online 11 November 2010

Keywords:
Immune epitope
Neutralization
Pseudovirion
L2 protein
Monoclonal antibody
Prophylactic vaccine

ABSTRACT

The N-terminal region of the human papillomavirus (HPV) L2 protein has been shown to contain immune epitopes able to induce the production of neutralizing and cross-neutralizing antibodies (Gambhira et al., 2007; Kawana et al., 1999). Using bacterial thioredoxin as a scaffold, we managed to enhance the immunogenicity of putative L2 neutralizing epitopes, but only a minor fraction of the resulting immune responses was found to be neutralizing (Rubio et al., 2009). To determine the recognition patterns for non-neutralizing, neutralizing and cross-neutralizing antibodies, we isolated and characterized a panel of 46 monoclonal antibodies directed against different HPV16 L2 epitopes. Four of such antibodies proved to be neutralizing, and two of them, both targeting the amino acid (aa) 20–38 region of L2, were found to cross-neutralize a broad range of papillomaviruses. The epitopes recognized by neutralizing and cross-neutralizing antibodies were mapped at high resolution and were found to be characterized by distinct recognition patterns. Even in the case of the L2 20–38 epitope, cross-neutralization of HPV31 pseudovirions proved to be extremely inefficient, and this was found to be primarily due to the lack of a proline residue at position 30. HPV16 specific amino acids in this region also appear to be responsible for the lack of cross-neutralizing activity, thus suggesting a potential immune escape mechanism. For the aa 71–80 region, instead, the data indicate that restriction of neutralization to HPV16 is due to sequence (or structural) differences laying outside of the epitope. Besides providing new insights on the molecular bases of L2-mediated immune reactivity, the present data may pave the way to novel vaccination approaches specifically evoking cross-neutralizing antibody responses.

© 2010 Elsevier Inc. All rights reserved.

Introduction

Human papillomaviruses are a heterogeneous group of double stranded DNA viruses, which can be sub-grouped into cutaneous or mucosal types, depending on their ability to infect the skin or the mucosa of the genital or the upper-respiratory tracts. The mucosal HPV types are further subdivided into two groups: (i) low-risk HPVs (e.g. types 6 and 11) mainly associated with benign genital warts and (ii) high-risk HPVs (e.g. types 16 and 18) as the etiological agents of cervical cancer affecting approximately 500,000 women worldwide (Munoz et al., 2003).

In the last two decades efforts from several independent groups have led to the development of efficient prophylactic vaccines against some mucosal HPV types. They rely on the L1 protein in the form of virus-like particles (VLPs) and are either bivalent vaccines containing HPV16 and HPV18 VLPs (Cervarix™) (Harper et al., 2006; Paavonen et al., 2007), or tetravalent vaccines also including VLPs from the low-risk HPV types 6 and 11 (Gardasil™) (Ault, 2007a; Garland et al., 2007). Both vaccines induce high titers of HPV type-restricted neutralizing antibodies. Protection against non-vaccine HPV types (i.e. cross-protection) was observed against closely related HPV types only (Ault, 2007b), although a broader range of protection has been reported recently for the Cervarix vaccine (Paavonen et al., 2009).

Previous studies have shown that immunogens derived from the N-terminal portion (aa 1–120) of the minor capsid protein L2 can induce the generation of cross-protective antibodies against a broad range of papillomavirus infections (Alphs et al., 2008; Gambhira et al., 2007a; Kawana et al., 2001, 2003; Kondo et al., 2007; Pastrana et al., 2005; Schellenbacher et al., 2009). The major challenge, however, is the rather low immunogenicity of L2 and several studies have specifically addressed this point lately (Alphs et al., 2008; Jagu et al., 2009; Kondo et al., 2008; Rubio et al., 2009; Schellenbacher et al.,

* Corresponding author. DKFZ ATV F035, Im Neuenheimer Feld 242, 69120 Heidelberg, Germany. Fax: +49 6221 424932.
E-mail address: Martin.Mueller@dkfz.de (M. Müller).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.10.017

2009). In a previous study, we immunized mice with L2 peptides comprising the putative neutralizing epitopes aa 20–38, 28–42, 56–75, 64–81, 96–115 and 108–120 inserted into bacterial thioredoxin as a macromolecular scaffold (Rubio et al., 2009). Analysis of the resulting sera evidenced strong anti-L2 antibody responses, yet only a few of these responses proved to be neutralizing and the corresponding epitopes were not characterized.

In this study, we isolated a panel of 46 monoclonal antibodies (mAbs) from mice immunized with the aforementioned Trx-L2 peptides and characterized in-depth the targets of non-neutralizing, neutralizing and cross-neutralizing antibodies within the N-terminus of L2. The data provide information about the specific recognition patterns of cross-neutralizing antibodies and also explain why some HPV16 L2-directed neutralizing antibodies fail to neutralize other HPV types. Our results further indicate that L1 apparently has no role in restricting L2-dependent neutralizing activity to HPV16, but that for some epitopes this restriction is due to single amino acid differences lying either within or outside the L2 epitopes of different HPV types. This study thus provides important insights on the molecular bases of (and requirements for) L2-mediated cross-protective immunity and might have an important impact on the development of novel anti-HPV vaccines.

Results

Characterization of monoclonal antibodies directed against different epitopes within the N-terminal region of L2

Previous studies have shown that the aa 1–120 region of the L2 protein (here referred to as the 'L2 N-terminus') contains potential targets for both neutralizing and cross-neutralizing antibodies (Gambhira et al., 2007b; Kondo et al., 2007; Rubio et al., 2009). We observed, however, that neutralizing titers are generally much lower

Table 1
Neutralizing, cross-neutralizing and non-neutralizing anti-HPV 16 L2 antibodies.

Antigen	mAb	Ig isotype	Neutralization capacity (HPV16)	Cross-neutralization capacity	Targeted peptide (aa)	GST-ELISA	IB	IF	Antigen	mAb	Ig isotype	Neutralization capacity (HPV16)	Cross-neutralization capacity	Targeted peptide (aa)	GST-ELISA	IB	IF
Trx-L2 (20–38)	K2L2 (20–38)	IgG1	–	–	30–35	+	+	+	Trx-L2 (64–81)								
	K4L2 (20–38)	IgG2b	+	+	21–30	+	+	+		K1L2 (64–81)	IgG1	+	–	73–79	+	+	+
	K6L2 (20–38)	IgG1	–	–	30–35	+	+	+		K2L2 (64–81)	IgG1/ IgG2b	–	–	73–80	+	+	+
	K7L2 (20–38)	IgG2b	–	–	27–34					K3L2 (64–81)	IgG1	–	–	73–80	+	+	+
	K8L2 (20–38)	IgG2b	–	–	27–35	+	+	+		K4L2 (64–81)	IgG1	–	–	65–72	+	+	+
	K9L2 (20–38)	IgG1	–	–	28–35	+	+	+		K5L2 (64–81)	IgG1	–	–	65–72	+	+	+
	K11L2 (20–38)	IgG2b	–	–	30–35	+	+	+		K6L2 (64–81)	IgG1	–	–	65–72	+	+	+
	K12L2 (20–38)	IgG2b	–	–	27–35	+	+	+		K7L2 (64–81)	IgG1	–	–	65–72	+	+	+
	K14L2 (20–38)	IgG2b	–	–	29–35	+	+	+		K8L2 (64–81)	IgG1	–	–	65–72	+	+	+
	K15L2 (20–38)	IgG1	–	–	22–31	+	+	+		K9L2 (64–81)	IgG1	–	–	65–72	+	+	+
	K18L2 (20–38)	IgG1	+	+	22–30	+	+	+									
Trx-L2 (28–42)	K1L2 (28–42)	IgG1	–	–	30–35	+	+	+	TrxL2 (96–115)								
	K2L2 (28–42)	IgG1	–	–	30–35	+	+	+		K1L2 (96–115)	IgG1	–	–	96–103	+	+	+
	K3L2 (28–42)	IgG1	–	–	30–35	+	+	+		K2L2 (96–115)	IgG1	–	–	96–111	+	+	+
	K4L2 (28–42)	IgG1	–	–	28–35	+	+	+		K3L2 (96–115)	IgG1	–	–	96–107	+	+	+
	K7L2 (28–42)	IgG1	–	–	29–35	+	+	+		K4L2 (96–115)	IgG1	–	–	97–111	+	+	+
	K8L2 (28–42)	IgG1	+	–	32–39	+	+	+		K5L2 (96–115)	IgG1	–	–	96–107	+	+	+
Trx-L2 (56–75)	K1L2 (56–75)	IgG1	–	–	61–75	+	+	+	Trx-L2 (1–120)								
	K2L2 (56–75)	IgG1	–	–	61–75	+	+	+		K1L2 (1–120)	IgM	–	–	85–103	+	+	+
	K4L2 (56–75)	IgG1	–	–	61–75	+	+	+		K2L2 (1–120)	IgG1	–	–	105–120			
	K5L2 (56–75)	IgG1	–	–	61–75	+	+	+		K3L2 (1–120)	IgM	–	–	105–120	+	+	+
	K6L2 (56–75)	IgG1	–	–	61–75	+	+	+		K4L2 (1–120)	IgM	–	–	105–120	+	+	+
	K7L2 (56–75)	IgG1	–	–	61–75	+	+	+		K5L2 (1–120)	IgM	–	–	105–120	+	+	+
	K9L2 (56–75)	IgG1	–	–	61–75	+	+	+		K8L2 (1–120)	IgM	–	–	105–120	+	+	+
	K11L2 (56–75)	IgG1	–	–	61–75	+	+	+		K9L2 (1–120)	IgM	–	–	105–120	+	+	+

For each monoclonal antibody, the isotype, neutralization capacity (+/−), and the GST-ELISA, immunoblot (IB) and immunofluorescence (IF) reactivities (+/−), along with the name of the starting Trx-L2 antigens and the targeted L2 peptide are reported.

Table 2
Neutralizing activities of monoclonal antibodies $K4L2_{20–38}$, $K18L2_{20–38}$, $K15L2_{20–38}$, $K1L2_{64–81}$, and $K8L2_{28–42}$.

PSV	Associated disease	Monoclonal antibodies				
		aa 20–38			aa 28–42	aa 64–81
		K4	K18	K15	K8	K1
		$K_D = 1.7\ 10^{-8}$	$K_D = 3.3\ 10^{-8}$	$K_D = 8.9\ 10^{-7}$		
HPV16	Cervical cancer	3125	625	<5	3125	125
HPV18	Cervical cancer	625	3125	<5	<5	<5
HPV31	Cervical cancer	<5	50	<5	<5	<5
HPV45	Cervical cancer	625	625	<5	<5	<5
HPV58	Cervical cancer	625	3125	<5	<5	<5
HPV57	Common warts	625	625	<5	<5	<5
HPV27	Common warts	625	625	<5	<5	<5
BPV-1	Bovine fibropapilloma	625	125	<5	<5	<5

KD = dissociation constant, units in molar.
The activities of each mAb are expressed as (cross-)neutralization titers, i.e., the reciprocal of the maximum dilution causing ≥70% neutralization of the indicated pseudovirions (PSV); data are the average of duplicates.
Dissociation constant value for K4 (20–38), K18(20–38) and K15(20–38) obtained by Biacore analysis are indicated.

than ELISA titers, suggesting that most antibodies against the L2 N-terminus are non-neutralizing. In the present work we set out to determine the specific targets recognized by three different types of antibodies: (i) antibodies that bind to various regions of the HPV16 L2 N-terminus, yet fail to neutralize; (ii) antibodies that bind and neutralize the parental HPV16 serotype only; and (iii) antibodies capable of cross-neutralizing different HPV types.

Mice previously immunized with Trx-scaffolded L2 peptides (Rubio et al., 2009) were used to isolate a panel of 46 monoclonal antibodies directed against the L2 N-terminal regions comprised of aa 20–38, 28–42, 56–75, 64–81 and 96–115 as well as the entire aa 1–120 polypeptide. The IgG isotypes as well as the neutralization and cross-neutralization capacities of these mAbs are reported in Table 1. All antibodies were reactive in GST-L2 ELISA and bound to the HPV16 L2 protein expressed by transfected 293TT cells in both immunoblot and immunofluorescence assays, thus indicating that L2 structural changes induced by different analytical procedures did not affect antigen/antibody recognition.

Only four of the 46 mAbs were capable of neutralizing HPV16 pseudovirions. Two of them were directed against the aa 20–38 region ($K4L2_{20–38}$ and $K18L2_{20–38}$) and the other two against the aa 28–42 ($K8L2_{28–42}$) and 64–81 ($K1L2_{64–81}$) regions of HPV16 L2 (Table 1). The ability of these antibodies to cross-neutralize the high-risk types HPV58 31, 45, 18 and BPV type 1 as well as the common warts types HPV57 and 27 was determined using the non-neutralizing $K15L2_{20–38}$ mAb (see later) as a control. As shown in Table 2, only antibodies $K4L2_{20–38}$ and $K18L2_{20–38}$ were capable of cross-neutralizing PV types other than HPV16, albeit with somewhat different efficiencies. Antibodies $K8L2_{28–42}$ and $K1L2_{64–81}$ only neutralized homologous HPV16 pseudovirions without any effect on other PV types.

Binding patterns of neutralizing, cross-neutralizing and non-neutralizing antibodies

The aforementioned results indicate that specific L2 regions, previously reported as targets for the generation of neutralizing antibodies (Gambhira et al., 2007b; Kondo et al., 2007), are also recognized by non-neutralizing antibodies. We thus wished to define more precisely the recognition patterns of these different types of antibodies. To this end, all mAbs were tested in ELISAs using 15-mer overlapping peptides as targets, which covered the N-terminal 120 aa of L2 with an offset of 4 aa each, (Fig. 1).

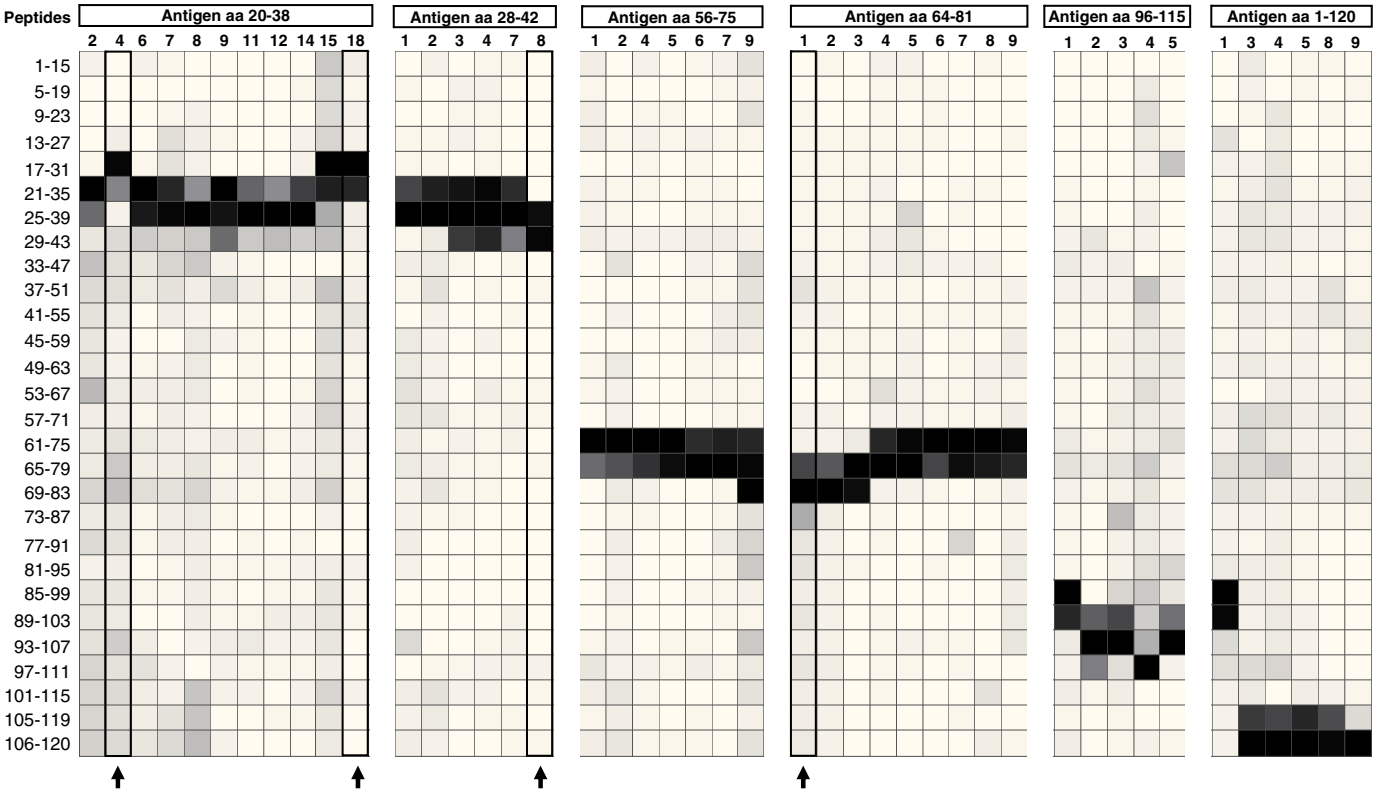

Fig. 1. Peptide epitopes within the HPV16 L2 N-terminus bound by different monoclonal antibodies. Binding of different mAbs, isolated from mice immunized with the indicated Trx-L2 antigens (listed at the top of each panel), to overlapping 15-mer peptides spanning the aa 1–120 region of HPV16 L2 was screened by ELISA. The reactivity of individual peptides with the various mAbs are represented as coloured squares, with black corresponding to the highest reactivity and pale yellow indicating lack of reactivity; the four neutralizing antibodies are boxed.

We observed that antibodies raised against the same L2 region may exhibit different recognition patterns, thereby allowing to distinguish between neutralizing and non-neutralizing responses. Briefly, the cross-neutralizing mAbs $K4L2_{20–38}$ and $K18L2_{20–38}$ recognized a pair of peptides encompassing aa 17–31 and 21–35, whereas the corresponding non-neutralizing mAbs reacted with peptides 21–35 and 25–39 ($K2L2_{20–38}$, $K6L2_{20–38}$, $K7L2_{20–38}$, $K8L2_{20–38}$, $K11L2_{20–38}$, $K12L2_{20–38}$, and $K14L2_{20–38}$) and in one case ($K9L2_{20–38}$) with the further downstream 29–43 peptide, but not with the 17–31 peptide. Interestingly, one antibody ($K15L2_{20–38}$) displayed a similar binding pattern as the two neutralizing antibodies, yet failed to neutralize. As suggested by the results of comparative surface plasmon resonance experiments (K_D in Table 2), this is likely due to the reduced binding affinity of this particular antibody (apparent $K_D = 8.9 \times 10^{-7}$), which is 50- and 30-fold lower than that of $K4L2_{20–38}$ and $K18L2_{20–38}$, respectively.

Antibodies directed against the aa 28–42 region also showed distinct binding patterns. $K8L2_{28–42}$ (HPV16 neutralizing) recognized the aa 25–39 and 29–43 peptides, whereas the other five mAbs (non-neutralizing) additionally reacted with the aa 21–35 peptide (Fig. 1). These data indicate that the aa 20–42 region of L2 contains epitopes capable of inducing all three types of antibody responses.

The fourth neutralizing antibody ($K1L2_{64–81}$) is directed against the aa 64–81 region and recognized peptides spanning aa 65–79, 69–83 and 73–87. By comparison, the non-neutralizing mAbs $K2L2_{64–81}$ and $K3L2_{64–81}$ only recognized peptides spanning aa 65–79 and 69–83, whereas the other six non-neutralizing mAbs targeting this region reacted with peptides 61–75 and 65–79 (Fig. 1). The aa 64–81 region of L2 thus also contains binding sites for neutralizing and non-neutralizing antibodies.

The remaining mAbs, directed against regions 56–75, 96–115 and 1–120, were all non-neutralizing (Fig. 1).

The epitopes recognized by the four neutralizing antibodies were mapped at higher resolution, and compared with those recognized by some of the non-neutralizing antibodies, by using peptides shortened stepwise by one amino acid from the N- or the C-terminus, up to a minimum size of eight amino acids. As shown in Fig. 2A, the neutralizing mAbs $K4L2_{20–38}$ and $K18L2_{20–38}$ reacted with peptides encompassing aa 21–30 and 22–30, respectively, while the corresponding non-neutralizing mAbs recognized a region of L2 comprised between aa 27 and 35. The neutralizing antibody $K8L2_{28–42}$ recognized the aa 32–39 region, whereas the rest of anti-L2(28–42) (non-neutralizing) mAbs recognized immune epitopes located between aa 28 and 35.

For region 64–81, six of the non-neutralizing antibodies recognized an epitope spanning aa 65–71, two non-neutralizing mAbs ($K2L2_{64–81}$ and $K3L2_{64–81}$) recognized the aa 72–79 peptide, while the neutralizing $K1L2_{64–81}$ mAb reacted with a one-residue shorter epitope (aa 72–78) within the same region (Fig. 2B).

Cross-neutralizing antibodies $K4L2_{20–38}$ and $K18L2_{20–38}$ have different minimal amino acid requirements for target recognition

To identify L2 residues critically involved in binding by the cross-neutralizing mAbs $K4L2_{20–38}$ and $K18L2_{20–38}$, we performed an alanine-scanning peptide ELISA. Briefly, replacement of any of the amino acids between positions 21–23 (TCK) and 28–29 (CP) with an alanine resulted in a strong reduction of the binding capacity of mAb $K4L2_{20–38}$ (Fig. 3A). By comparison, loss of $K18L2_{20–38}$ binding was only observed when either one of the two cysteines at positions 22 and 28 was replaced with an alanine (Fig. 3B). These cysteine residues, which were also important for $K4L2_{20–38}$ binding, have recently been shown to exist in an oxidized, disulfide-bonded form in infectious HPV particles (Campos and Ozbun, 2009). As revealed by a comparative immunoblot analysis (Supplementary Fig. 1) conducted on >97% oxidized and >75% reduced $TrxL2(20–38)_3$ (see 'Materials and methods' for details), both mAbs preferentially recognized the oxidized (S–S) form of the antigen (Supplementary Fig. 1).

HPV31 escapes neutralization by mAb $K4L2_{20–38}$ due to a serine/proline replacement at position 30

HPV31 pseudovirions were the least efficiently neutralized by $K18L2_{20–38}$ and were not at all neutralized by $K4L2_{20–38}$. In the L2 protein, amino acids at positions 20 (Q→K), 24 (A→Q) and 30 (S→P) differ between HPV31 and HPV16. By reverting the Ser at position 30

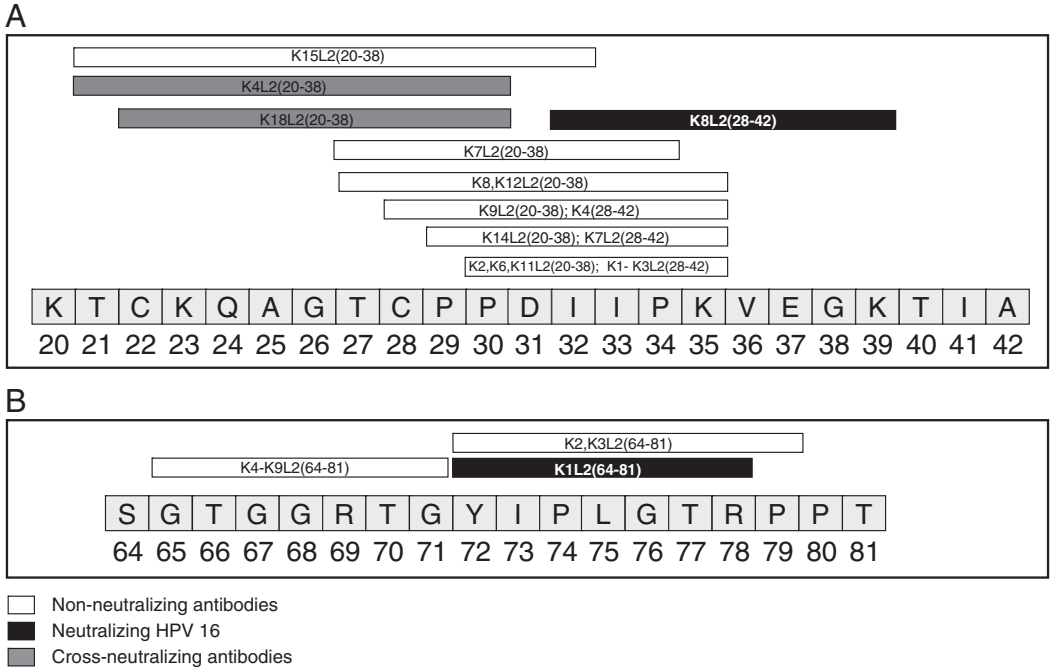

Fig. 2. Binding patterns of neutralizing, cross-neutralizing and non-neutralizing anti-L2 antibodies. Overlapping peptides progressively shortened by one amino acid from either the N- or the C-terminus were used to map the core epitopes recognized by different mAbs in the aa 20–42 (A) and the aa 64–81 (B) regions of L2.

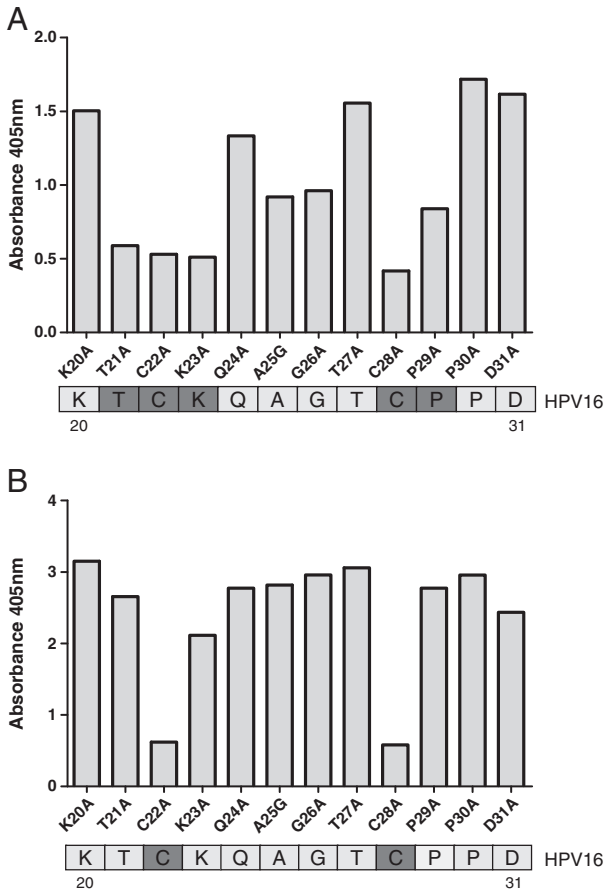

Fig. 3. Recognition profiles of the cross-neutralizing antibodies $K4L2_{20–38}$ and $K18L2_{20–38}$. An alanine scanning analysis of the aa 20–31 L2 epitope was performed to determine the binding patterns of mAbs $K4L2_{20–38}$ (A) and $K18L2_{20–38}$ (B). Amino acid residues that are required for binding by the two antibodies (e.g., the Cys residues at positions 22 and 28) are shown in a dark-grey background.

of the HPV31 L2 peptide to a Pro residue (as in HPV16 L2), $K4L2_{20–38}$ binding to HPV31 L2 increased by about three-fold (Fig. 4). An additional A→Q substitution at position 24 almost completely restored reactivity with mAb $K4L2_{20–38}$. At variance with the S/P substitution at position 30, single changes at position 20 or 24 did not appreciably influence antibody binding even though a somewhat increased $K4L2_{20–38}$ binding was observed upon simultaneous substitution of both residues. In contrast to the stringent sequence requirements of $K4L2_{20–38}$, the $K18L2_{20–38}$ antibody recognized all peptides regardless of modification (Fig. 4). ELISA data further corroborated the notion that Pro30 in HPV16 L2 is indeed crucial for binding by $K4L2_{20–38}$. Thus, the inability of this HPV16 L2 monoclonal antibody to neutralize HPV31 pseudovirions appears to be due to the different primary structure of HPV31 L2. Alternatively, it is possible that this phenomenon may be indirectly caused by the HPV31 L1/L2 interaction, which might negatively influence immune reactivity by limiting access of the antibody to L2 or by changing the conformation of the L2 epitope. To distinguish between these possibilities, we examined the accessibility of this particular epitope in hybrid particles containing HPV16L1/31 L2 or HPV31L1/16 L2. We also examined hybrid particles containing various mutated forms of HPV31 L2, bearing single, double or triple amino acid substitutions in the aa 20–31 region, the latter of which fully converts the HPV31 L2 epitope to the corresponding HPV16 epitope. The results of neutralization assays carried out with these pseudovirions are shown in Fig. 5. In summary, both antibodies were able to neutralize infection by the hybrid particles HPV31L1/16 L2. Consistent with its ability to neutralize HPV31 pseudovirions, mAb $K18L2_{20–38}$ also neutralized HPV16L1/31 L2 particles, albeit with a lower efficiency. In addition, the S30P substitution in HPV31 L2 restored the neutralizing activity of mAb $K4L2_{20–38}$ to about 70% of the level observed with HPV31L1/16 L2 hybrid particles. Both mAbs were able to neutralize pseudovirions bearing an HPV31 L2 with all three amino acids shifted to those of HPV16 L2, although with a slightly decreased efficiency compared to homologous HPV16 or hybrid HPV31L1/16 L2 pseudovirions.

Type-specificity of the HPV 16 L2(28–42) and (64–81) epitopes

$K8L2_{28–42}$ and $K1L2_{64–81}$ are HPV16 type-restricted, neutralizing antibodies which bind the aa 32–39 and 72–78 epitopes, respectively. To find out why these antibodies are only able to neutralize HPV16, critical amino acid residues required for binding of $K8L2_{28–42}$ and $K1L2_{64–81}$ were determined.

We found that the aa 31–40 HPV16 peptide was bound strongly by $K8L2_{28–42}$, while the corresponding peptide from HPV52/58 (HPV types 52 and 58 are identical in this region) was not (Fig. 6A). A V→I substitution at position 32 of HPV52/58 L2 partially restored $K8L2_{28–42}$ binding, which was completely recovered following a T→K substitution at position 39. The aa 31–40 peptide from HPV18 was not strongly bound by $K8L2_{28–42}$ either, probably because, similar to HPV52/58, it also lacks the critical Lys residue at position 39 (as well as an Ile residue at position 32). HPV18 has one additional change compared to HPV16 — a Val instead of an Ile residue at position 33. When only this valine residue was replaced with an isoleucine, full binding by $K8L2_{28–42}$ was recovered. The HPV45 peptide was the most weakly bound by $K8L2_{28–42}$. In addition to the absence of the critical Lys residue at position 39 and Ile at position 32, it bears an Asn at position 34 compared to a Pro residue in HPV16. The fact that a single substitution of this Pro residue in HPV16 with an Asn residue as in HPV45 drastically reduced $K8L2_{28–42}$ binding indicates that this residue is also quite critical for antibody recognition. Thus Lys39 and Pro34, and to a lesser extent Ile33, appear to be key

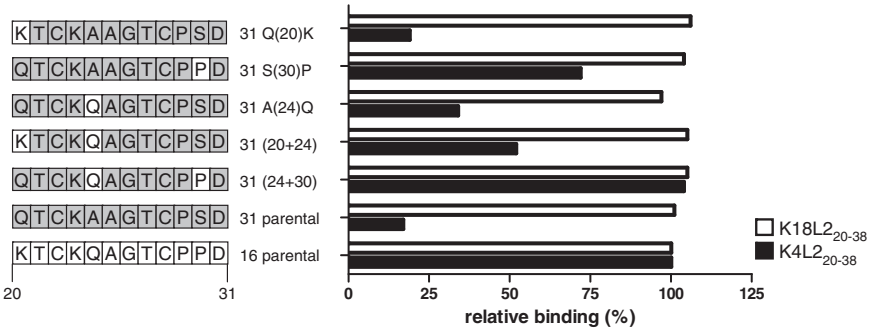

Fig. 4. Reactivity of mAbs $K4L2_{20–38}$ and $K18L2_{20–38}$ with hybrid (HPV31/16) aa 20–31 peptides. HPV31 peptides bearing one or two amino acids derived from the corresponding positions of HPV16, along with the unmodified parental peptides, were used to identify key amino acid residues responsible for the lack of HPV31 cross-neutralization by mAb $K4L2_{20–38}$. Antibody reactivity with each L2 aa 20–31 peptide was determined by peptide ELISA (see 'Materials and methods' for details).

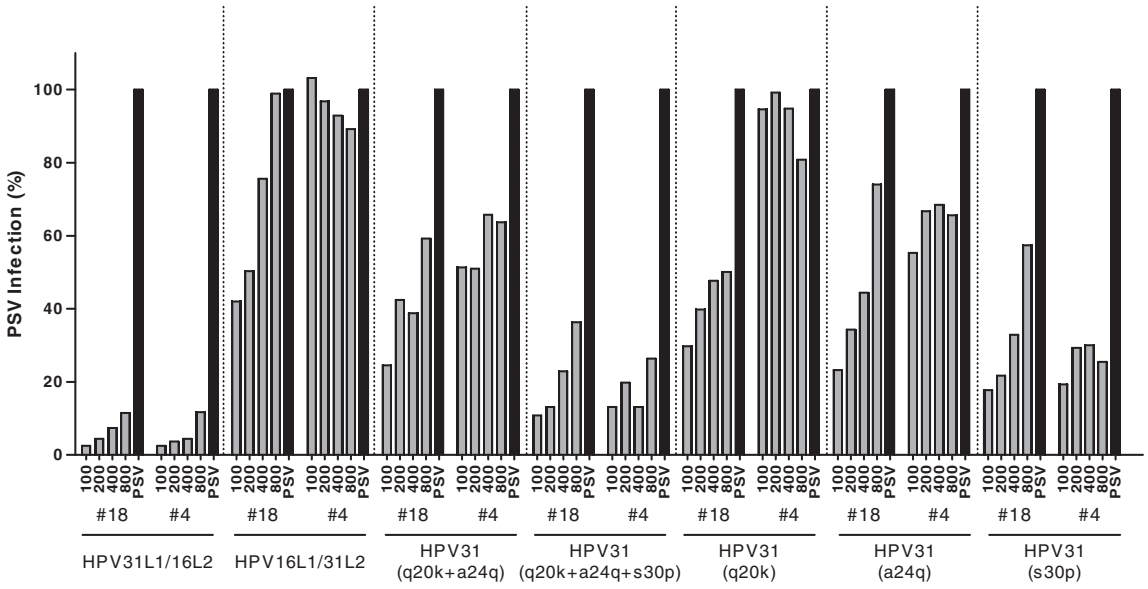

Fig. 5. Neutralization of hybrid HPV31 L1/L2-HPV16 L1/L2 pseudovirions by mAbs K4L2$_{20-38}$ and K18L2$_{20-38}$. Hybrid particles containing the unmodified HPV31 L1/HPV16 L2 proteins (and vice versa) as well as hybrid particles containing HPV16 L1 and various HPV16-adapted variants of the aa 20–31 peptide from HPV31 L2 (see also Fig. 4) were used to analyze the (cross-)neutralization capacity of mAb K4L2$_{20-38}$ (#4); mAb K18L2$_{20-38}$ (#18) served as a reference for these experiments. Different dilutions of each antibody were incubated with the various pseudovirions prior to cell infection (see 'Materials and methods' for details); untreated pseudovirions (PSV) were used as a full infection (100%) control in each assay.

determinants of the HPV16-restricted binding/neutralization capacity of mAb K8L2$_{28-42}$.

For the aa 72–78 L2 epitope bound by K1L2$_{64-81}$, the primary HPV58 sequence differs at three positions from that of HPV16. While the HPV16 peptide was recognized by mAb K1L2$_{64-81}$, the HPV58 peptide was not (Fig. 6B). An I→V substitution at position 73 of the HPV58 peptide as well as a T→S substitution at position 77 only partially restored K1L2$_{64-81}$ binding. By contrast, a full recovery of K1L2$_{64-81}$ binding was obtained upon substitution of the Thr residue at position 78 of HPV58 with an Arg residue as in HPV16.

The L1 protein is not responsible for HPV type-restricted neutralization by K1L2$_{64-81}$ and K8L2$_{28-42}$

The results of peptide ELISAs suggested that one or two amino acid residues are apparently sufficient to determine the observed HPV

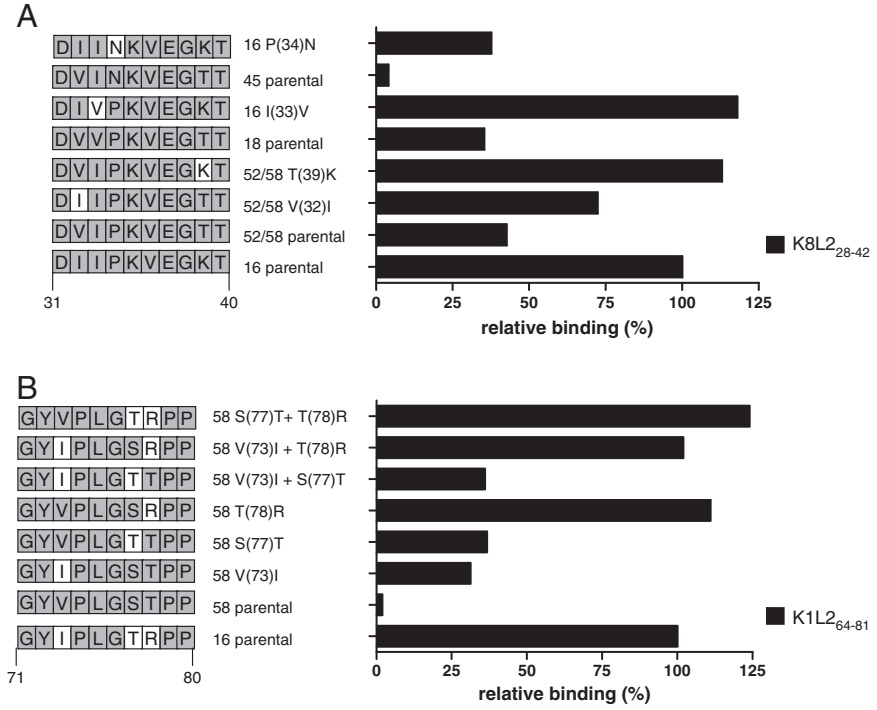

Fig. 6. Reactivity of antibodies K8L2$_{28-42}$, and K1L2$_{64-81}$ with hybrid peptides derived from HPV types 16, 18, 45, 52, and 58. L2 peptides covering the core epitopes recognized by mAbs K8L2$_{28-42}$ (aa 31–40; panel A) and K1L2$_{64-81}$ (aa 71–79; panel B) were used to investigate the amino acid sequence determinants of the HPV type-restricted neutralizing activity of these antibodies. The reactivities of each antibody with unmodified L2 peptides from the indicated HPV types as well as with different variants bearing single or double amino acid substitutions were determined by peptide ELISA (see 'Materials and methods' for details).

type-specificity of mAbs K1L2$_{64–81}$ and K8L2$_{28–42}$. To find out whether the L1 protein influences HPV16-restricted neutralization by these antibodies, e.g. by imposing structural constraints on the L2 protein, we studied the effect of L1 on K1L2$_{64–81}$- and K8L2$_{28–42}$-mediated neutralization with the use of hybrid L1/L2 pseudovirions. In particular, HPV16 L2 was analyzed in combination with HPV31 L1, HPV18 L1 and HPV45 L1. In all cases, as long as HPV16 L2 was present, equal levels of neutralization by K1L2$_{64–81}$ and K8L2$_{28–42}$ were observed. Conversely, HPV31 L2 was studied in combination with HPV16 L1. In this case, despite the presence of HPV16 L1, neutralization by K1L2$_{64–81}$ and K8L2$_{28–42}$ was not observed, while all pseudovirions were efficiently neutralized by the cross-neutralizing antibody K18L2$_{20–38}$ (data not shown).

Neutralization of HPV31 pseudovirions bearing altered L2 epitopes by K1L2$_{64–81}$ and K8L2$_{28–42}$

To identify key amino acid residues within the aa 32–39 and 72–78 L2 epitopes that are required for neutralization by the type-restricted K8L2$_{28–42}$ and K1L2$_{64–81}$ antibodies, the corresponding regions of HPV31 were chosen as models of an HPV type that cannot be neutralized by such antibodies. For K8L2$_{28–42}$, the aa 31–40 region of HPV31 L2 was mutagenized systematically in order to introduce amino acid residues found in HPV16 (see Fig. 7A). The HPV31 pseudovirion construct comprising all four HPV16 L2 amino acid substitutions (Ile 32, Val 36, Gly 38 and Lys 39) was efficiently neutralized by K8L2$_{28–42}$ (Fig. 7A; for transducing activities of the different pseudovirions see Supplementary Table 1). Pseudovirions lacking either Val 36 or Lys 39 escaped neutralization, while Ile 32 and Gly 38 did not appreciably influence neutralization efficiency. This finding further confirms the requirement of a lysine residue at position 39 for neutralization, as observed in peptide ELISAs.

A similar strategy was applied to the aa 71–80 region of HPV31 L2 (Fig. 7B). In this sequence, three amino acids differ between HPV31 and HPV16 at positions 73, 76 and 80. Surprisingly, even modified HPV31 pseudovirion constructs bearing all three HPV16 substitutions failed to be neutralized by K1L2$_{64–81}$, as were constructs bearing only single or double substitutions. Hybrid particles consisting of HPV31L1/16 L2 were neutralized effectively by K1L2$_{64–81}$. Altered pseudovirions and the HPV31L1/16 L2 showed comparable transducing activity in this assay (Supplementary Table 1). We then performed a peptide ELISA in which HPV 31 L2 (aa 71–80) peptides bearing single as well as double amino acid changes at positions 73, 76 and 80 were compared with the corresponding HPV16 peptide with respect to K1L2$_{64–81}$ binding. As revealed by these assays (not shown), the authentic HPV31 peptide was bound by K1L2$_{64–81}$, albeit to a lower extent compared to the corresponding HPV16 peptide, and binding was improved by introduction of a glycine at position 76 or a proline at position 80. Taken together, these findings indicate that the HPV31 peptide is recognized, albeit rather weakly, by K1L2$_{64–81}$ and suggest that the linear, aa 71–80 sequence of HPV31 L2 is not primarily responsible for the lack of neutralization.

From neutralization assays using mixed (L1/L2) pseudovirions we concluded that neutralization by K1L2$_{64–81}$ is not influenced by the L1 protein. To find out whether L1 influences HPV type-specificity, we investigated the combined effects of the L1 type and of the primary L2 epitope sequence on neutralization by this antibody. To this end, the HPV31 L2 variant V73I, S76G, S80P was used in combination with HPV16 L1 to assemble hybrid pseudovirions that were analyzed for neutralization by K1L2$_{64–81}$. We found that despite the presence of HPV 16 L1, K1L2$_{64–81}$ was not able to neutralize any of the aforementioned hybrid pseudovirions, which were neutralized instead by the positive control antibodies K18L2$_{20–38}$ and L1-1.3.5.15 (data not shown). Thus, neither the L1 protein nor the sequence of the aa 71–80 epitope embedded in a heterologous (HPV 31) L2 protein restrict neutralization by mAb K1L2$_{64–81}$ to HPV16.

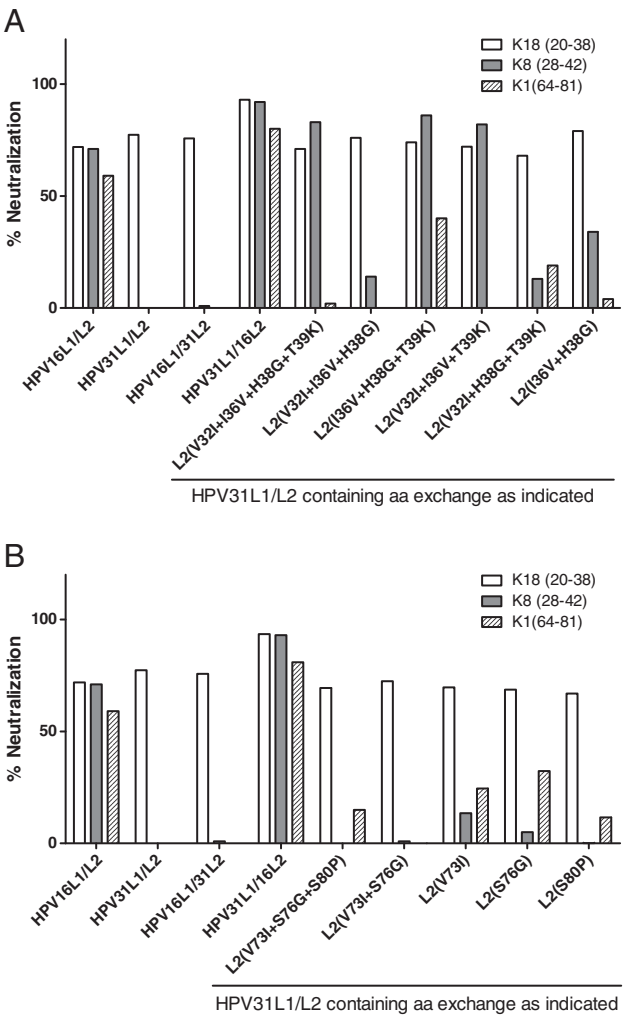

Fig. 7. Neutralization of HPV31 pseudovirions containing sequence-modified variants of the L2 protein by antibodies K8L2$_{28–42}$, and K1L2$_{64–81}$. Pseudovirions bearing the indicated HPV16-adapted variants of HPV31 L2 in combination with HPV31 L1 were used to investigate the molecular bases of the type-restricted neutralizing activity of mAbs K8L2$_{28–42}$ (A) and K1L2$_{64–81}$ (B). Single or multiple (2–4) amino acid substitutions were inserted in the core peptide epitopes (aa 31–40 and 71–79) recognized by the two antibodies. Unmodified HPV16 and HPV31 pseudovirions, mixed pseudovirions containing HPV16L1/31 L2 and HPV31L1/16 L2 were used as controls. The cross-neutralizing K18L2$_{20–38}$ and the K1L2$_{64–81}$ antibodies served as controls for the experiment shown in Fig. 7A, and the K18L2$_{20–38}$ and the K8L2$_{28–42}$ were used as controls for the experiment shown in Fig. 7B.

Discussion

This study focused on the fine mapping of immune epitopes within the N-terminus of the minor capsid protein L2, a promising candidate for the development of novel broad range anti-HPV vaccines. Forty-six monoclonal antibodies directed against regions of the L2 protein potentially capable of eliciting virus neutralizing immune responses were used as probes. From the reactivity patterns of these mAbs we learned that all but four antibodies reacting with different L2 epitopes are in fact non-neutralizing. This is quite surprising, as each of the examined epitopes comprises only a short region of the L2 protein. Although a particular subset of monoclonal antibodies might not necessarily reflect total serum reactivity, the data are in line with our previous finding that only a fraction of antibodies directed against selected L2 epitopes are indeed neutralizing (Rubio et al., 2009). The observation that antibodies directed against HPV16 L2 neutralizing epitopes can be either non-

neutralizing, neutralizing but HPV16 type-restricted, or cross-neutralizing, raises a question as to the determinants of these three different immune responses. Failure to neutralize could be explained by the inability of the antibodies to bind to the L2 target when present in the context of virus particles. Alternatively, non-neutralizing antibodies might be unable to interfere with key infection processes such as virion uptake, transport or uncoating, despite epitope binding. Further experiments aimed to analyze virus neutralization mechanisms are under way. In particular, it will be interesting to determine whether non-neutralizing antibodies are able to compete with neutralizing antibodies directed against the same region of L2. It will also be interesting to find out whether some non-neutralizing epitopes can act as decoys for the neutralizing ones.

Another important issue is what distinguishes antibodies only capable of neutralizing HPV16 from broadly cross-neutralizing antibodies. The simplest possibility would be that type-restricted antibodies fail to neutralize different HPV types because of their inability to react with different variants of the same L2 target sequence. It is equally conceivable, however, that not the L2 protein itself, but rather the structural constraints imposed by the L1 scaffold are limiting access of certain types of antibodies to their target antigens. In the case of HPV31 pseudovirions, which partially (or totally) escaped neutralization by two otherwise cross-neutralizing antibodies, the data obtained with hybrid pseudovirions indicate that escape is not due to the L1 protein, but rather to subtle sequence differences between the HPV31 and HPV16 L2 proteins. These results, however, also indicate that at least the N-terminal portion of the L2 proteins from different HPV types can adopt a very similar structural organization within viral capsids.

The mechanisms of L2-directed neutralization are not yet fully understood.

For mAb RG1 it has been proposed that exposure of the aa 17–36 epitope in HPV16 only occurs after (pseudo) virion binding to the cell surface and L2 cleavage by the cellular furin protease (Day et al., 2008a,b; Richards et al., 2006). In the presence of RG1 virions are released from the cells and reattach to the extracellular matrix because the antibody prevents interaction with a secondary receptor (Day et al., 2008a,b). The two cross-neutralizing antibodies characterized in this study ($K4L2_{20–38}$ and $K18L2_{20–38}$) both overlap the RG1 epitope and require the two cysteine residues present in this region (C22 and C28) for binding. These cysteines are conserved among different papillomaviruses and have recently been shown to be disulfide-bonded in infectious virus particles (Campos and Ozbun, 2009). The latter finding is in line with our observation that both mAbs ($K4L2_{20–38}$ and $K18L2_{20–38}$) have a strong preference for the oxidized (S–S) rather than the reduced (SH) form of the aa 20–38 L2 peptide. As suggested by the behaviour of mAb $K15L2_{20–38}$, which shares a recognition pattern very similar to that of $K4L2_{20–38}$ and $K18L2_{20–38}$, yet failed to neutralize, binding affinity represents another important requirement for neutralization. In addition, eight antibodies recognize the C-terminal portion of the RG1 epitope (aa 27–35), yet lack neutralizing activity. This suggests that this region may remain inaccessible even after furin cleavage. It should be noted, however, that a sequence immediately downstream to the epitope recognized by these non-neutralizing mAbs has been shown to interact with syntaxin 18, albeit at a later stage of the infection cycle (Bossis et al., 2005). In fact, we found another neutralizing antibody ($K8L2_{28–42}$) which binds to an epitope located just upstream of the putative syntaxin 18 interaction site. This is consistent with results showing that residues 36–49 of BPV 1 also become exposed during virus entry (Laniosz et al., 2007). In contrast to $K4L2_{20–38}$ and $K18L2_{20–38}$, the $K8L2_{28–42}$ antibody specifically neutralizes HPV16 pseudovirions only. Therefore the aa 21–39 region of L2, which includes the most upstream portion of the epitope recognized by the RG1 mAb, contains the target epitopes for two different types of neutralizing antibodies (HPV16-restricted and cross-neutralizing), separated by a short stretch of amino acids recognized by non-neutralizing antibodies.

We isolated monoclonal antibodies targeting three other regions of L2 (aa 56–75; aa 64–81; aa 96–115). Although the sera of mice from which these antibodies were derived were all able to neutralize HPV16 pseudovirions, only one of the corresponding mAbs, directed against the aa 64–81 region, was endowed with neutralizing activity. The epitope recognized by this mAb overlaps the binding site of a previously isolated anti-HPV16 neutralizing antibody (mAb6; Kawana et al., 1999). Virus neutralization mechanisms based on binding to this particular region of L2 have not been described yet and it remains to be determined at what stage in the infection cycle this epitope becomes exposed.

Two of the four neutralizing antibodies ($K8L2_{28–42}$ and $K1L2_{64–81}$) failed to neutralize papillomavirus types other than HPV16. This lack of cross-neutralization can be ascribed to the relatively poor amino acid sequence conservation of the corresponding epitopes (see Fig. 8). In fact, binding of $K8L2_{28–42}$ to the HPV52/58 aa 31–40 peptide could be restored by replacing Thr39 with a lysine, the amino acid residue present at this position in HPV16 L2. The same T39K replacement, together with two additional amino acid substitutions, allowed for neutralization of hybrid pseudovirions bearing HPV16-adapted variants of HPV31 L2 by $K8L2_{28–42}$. This resembles the behaviour of the epitope recognized by $K4L2_{20–38}$ and $K18L2_{20–38}$, where single amino acid substitutions reduced (or abolished) neutralization of HPV31 compared to HPV16 pseudovirions. Although monoclonal antibodies represent a somewhat artificial situation, our findings raise a question as to whether there are natural escape mechanisms for L2-directed neutralizing antibodies analogous to the hypervariable loops present in L1 VLPs. Unfortunately, there is no information on naturally occurring anti-L2 neutralizing antibodies, and by using hybrid particles we only detected neutralizing activity directed against the L1 protein in a screening conducted on 10 human sera (M.M., unpublished observations).

At variance with the other three epitopes recognized by neutralizing antibodies, type-restricted neutralization of HPV16 pseudovirions by $K1L2_{64–81}$ was not affected by the introduction of HPV16-specific amino acids in the heterologous L2 protein from HPV31 (aa 71–80), despite improved binding of the $K1L2_{64–81}$ mAb to the corresponding isolated peptides. This indicates the involvement of higher-order constraints in the recognition of, and neutralization mediated by, this particular epitope. One possibility is that this epitope has an intrinsically reduced accessibility within a HPV31 L1/L2 particle. Alternatively, HPV31 L2 might allow for alternative virus entry pathways, which are not blocked by antibody binding. In either case, a vaccination approach relying on this particular HPV31 L2 peptide as antigen is expected not to elicit a neutralizing immune response.

Because of its ability to induce broadly protective antibody responses, the L2 protein is an attractive candidate for the development of a novel prophylactic anti-HPV vaccine. Several attempts have been made to increase the immunogenicity of L2 (Alphs et al., 2008; Rubio et al., 2009; Schellenbacher et al., 2009; Slupetzky et al., 2007). Our data indicates that the immunogenicity of the L2 N-terminus is not so poor *per se*, but that only a minor fraction of the immune responses induced by peptide epitopes from this region leads to effective neutralization. This is in stark contrast to VLP-based vaccines, where antibodies directed against intact viral capsids are almost always neutralizing. Therefore, in the case of L2 the challenge is to re-direct the B-cell response so to produce mainly cross-neutralizing antibodies with a reactivity pattern similar to that of mAb $K18L2_{20–38}$.

What defines a cross-neutralizing L2 epitope? As suggested by this study, the most important requirements are a strong sequence conservation, a high accessibility, and spatial proximity to functionally critical regions of L2, antibody binding to which hinders interaction

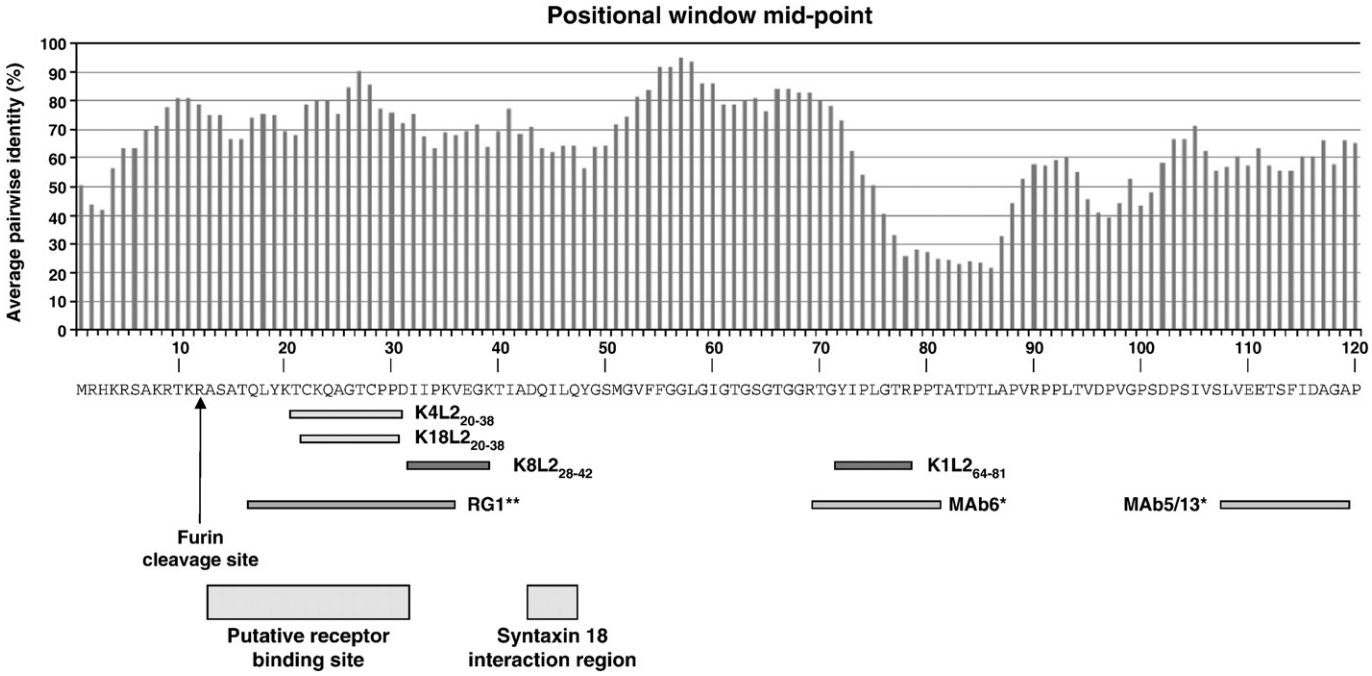

Fig. 8. Positional conservation profile of N-terminal HPV L2 polypeptides. Aligned N-terminal (aa 1–120) L2 polypeptide sequences from 21 HPV serotypes were analyzed for sequence conservation (see 'Materials and methods' for details). Numbers on the x-axis are referred to the HPV16 L2 sequence and indicate the mid-point position of the sliding window utilized for analysis (7 amino acids, moved by incremental steps of 1 residue positions). For each window position, average pairwise percent identity values derived from multiple sequence alignment are represented as histogram bars. The reference sequence shown below the graph is from HPV16 L2. Also shown are the epitopes recognized by the four neutralizing antibodies identified in this study as well as the binding sites of previously characterized neutralizing mAbs (MAb6 and MAb5/13, Kawana et al, 1999; RG1, Gambhira et al., 2007b), and various functional regions potentially involved in HPV internalization.

with cellular components involved in virus internalization. The aa 20–31 epitope seems to fulfill the aforementioned criteria, as it is the second best conserved sequence element within the L2 N-terminus, and by being located between the furin cleavage site and the syntaxin 18 binding site it likely overlaps the putative L2 receptor (see Fig. 8).

So far, all attempts to induce anti-L2 neutralizing antibody responses have targeted linear L2 epitopes. The reason for this has to do with the inability to present L2 to the immune system in the context of L1 VLPs providing for an authentic L2 conformation. Notably, anti-L1 neutralizing antibodies are almost exclusively directed against conformational epitopes.

Besides important insights on the molecular bases of L2-mediated cross-protective immunity and new antibody reagents for studying the HPV infection cycle, the results of the present high resolution analysis provide a useful framework for the development of novel L2-based vaccines. The anticipated major challenge will be to effectively re-direct the immune response from irrelevant epitopes to functionally important sites of the L2 protein.

Materials and methods

Isolation of monoclonal antibodies

HPV type 16 L2 peptides covering aa regions 20–38, 28–42, 56–75, 64–81, 96–115 and 1–120 were internally fused to bacterial thioredoxin as described before and used to vaccinate 6–8 month-old female Balb/c mice (Charles River Laboratories; Sulzfeld, Germany) (Rubio et al, 2009). After analysis of immune sera by GST-L2 ELISA and neutralization assays, mice with the strongest serum reactivities were selected for mAb production. Briefly, splenocytes were fused with SP2/0 myeloma cells and maintained in selection medium containing Hypoxanthine Aminopterin Thymidine (HAT) supplemented with Hybridoma Fusion and Cloning Supplement (HFCS; Roche). Positive clones, selected by GST-L2 ELISA, were sub-cloned four times and the immune reactivity of the corresponding hybridoma supernatants was evaluated by immunofluorescence (IF), immunoblot (IB) and the ability to neutralize PV pseudovirions.

Detection of anti-HPV16 L2 antibodies

GST-capture ELISA was used for the detection of HPV16 L2 antibodies. To this end, microtiter plates were coated overnight at 4 °C with 50 µl of coating buffer containing glutathione-casein, blocked with 0.2% casein in PBS, pre-warmed at 37 °C, followed by the addition of 50 µl of GST-L2 (aa 1–120) and incubation for 1 h at 37 °C (Sehr et al., 2001). Plates were washed with PBS-0.3% Tween 20 prior to the addition of 50 µl aliquots of hybridoma supernatants and incubation for 1 h at 37 °C. Plates were then washed again and incubated for 1 h at 37 °C with 50 µl/well of a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG polyclonal antibody (Sigma-Aldrich) diluted 1:3000 in PBS-0.3% Tween 20 plus 0.2% casein. After a further washing with PBS-Tween 20, 100 µl of 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS; 1 mg/ml in 100 mM sodium acetate-phosphate buffer, pH 4.2, containing 0.015% H_2O_2) were added to each well and absorbance at 405 nm was measured after 10–20 min with an automated plate reader (Titertek).

Immunoglobulin isotype identification

Monoclonal antibody producing cell lines were isotyped using a Hybridoma Sub-isotyping Kit (Calbiochem Novagen). Briefly, 96-well plates were coated with 100 µl of goat anti-mouse IgG diluted 1:1000 in coating buffer. Plates were incubated overnight at 4 °C and, after washing three times with PBS-0.1% Tween 20, were blocked with 200 µl of blocking serum diluted 1:4 in PBS-0.1% Tween 20 and incubated at room temperature (RT) for 1 h. Fifty µl of hybridoma supernatants were added to each well and incubated at RT for 1 h. Individual supernatants were tested with 100 µl of rabbit anti-IgM, anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3 and anti-IgGA, using PBS

as a negative control. Following incubation for 1 h at RT, reactions were developed by adding 100 µl of HRP-conjugated sheep anti-rabbit IgG polyclonal antibody (Sigma-Aldrich) diluted 1:3000 with PBS-0.1% Tween 20 in blocking serum. After a further washing with PBS-Tween 20, plates were stained as described earlier.

Indirect immunofluorescence

1.5×10^5 293TT cells/ml were seeded in 12-well plates with glass cover slides for 24 h before transfection with a plasmid encoding HPV16 L2. Cells were washed twice with PBS and fixed with 1 ml of absolute ethanol at −20 °C for 10 min; cells were then re-hydrated by washing twice with PBS and blocked with a solution of 5% PBS-milk overnight at 4 °C. Wells were incubated with hybridoma supernatants for 1 h at RT and washed three times with PBS. After washing, wells were incubated for 1 h at RT in the darkness with fluorochrome-conjugated anti-mouse rabbit antibody diluted 1:400. Glasses were washed three times with PBS-0.3% Tween 20, embedded in Fluoprep (BioMérieux) and analyzed with a Leica-DMRD microscope.

Immunoblotting

293TT cells transfected as described earlier for immunofluorescence were washed with PBS, collected in 200 µl of PBS-lysis buffer containing SDS and β-mercaptoethanol, and boiled at 100 °C for 10 min. Samples were fractionated on 12.5% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes, which were blocked overnight at 4 °C in PBS–0.1% Tween 20 containing 5% skim milk powder (blocking solution) and incubated for 1 h at RT with mAb-containing supernatants. Membranes were washed three times with PBS–0.3% Tween 20 for 5 min and then incubated with a HRP-conjugated goat anti-mouse IgG polyclonal antibody (Sigma-Aldrich) diluted 1:3000 in PBS-0.3% Tween 20 plus 5% milk for 1 h at RT. After washing, the L2 protein was detected with an enhanced chemiluminescence detection kit (Applichem).

A similar immunoblotting procedure was used to determine the immune reactivity of mAbs $K18L2_{20-38}$ and $K4L2_{20-38}$ with the oxidized (S–S) and the reduced (SH) form of the $TrxL2(20–38)_3$ antigen. As revealed by 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB) analysis using N-acetyl cysteine as standard, >97% of the 8 SH groups/molecule harboured by $TrxL2(20–38)_3$ are in a disulfide-bonded form in the recombinant polypeptide purified from bacterial lysates. A reduced form of the polypeptide, bearing on average 6 ± 1 DTNB-reactive SH groups/molecule, was prepared by treatment of $TrxL2(20–38)_3$ (1 mg/ml) with 0.3 M β-mercaptoethanol at 25 °C for 30 min and checked by DTNB analysis after removal of excess β-mercaptoethanol on size-exclusion spin columns (Micro BioSpin 6, BioRad).

IgG quantification

Antibody concentrations in the supernatants of hybridoma cells were determined by ELISA. 96 well plates (BD-Falcon) were incubated overnight at 4 °C with 50 µl of sheep anti-mouse antibody IgG diluted in PBS (1.6 µg/µl). Plates were washed three times with PBS-0.3% Tween 20 and blocked with 3% milk in PBS for 1 h at 37 °C. Ten serial dilutions of a commercial mouse IgG (1 mg/ml), starting from 1:1000, were used as standards. Fifty µl of calibrators and supernatants diluted 1:100, 1:200 and 1:400 was seeded in the wells and incubated at 37 °C for 1 h. After three more washings with PBS-0.3% Tween 20, plates were incubated with 50 µl of HRP-conjugated goat anti-mouse IgG polyclonal antibody diluted 1:3000 in PBS-0.3% Tween 20 plus 3% milk. Following a further washing with PBS-Tween 20 plates were stained as described earlier. Calibration curves were used to calculate IgG concentration.

Biacore analysis

Surface plasmon resonance (SPR) measurements were performed on a Biacore T100 machine equipped with CM5 sensor chips (Biacore, Uppsala, Sweden) using 10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% Tween20 and 1mM DTT as running buffer at 25°C. Polyclonal immunosorbent purified rabbit anti-mouse Ig (Biacore, BR-1005-14) was immobilized to the sensor surface of two flow cells using standard amine-coupling chemistry according to the manufacturers' instruction. Monoclonal antibodies ($K4L2_{20-38}$, $K18L2_{20-38}$, and $K15L2_{20-38}$) were consecutively captured from undiluted hybridoma supernatants to one flow cell, the other flow cell coated with capture antibody served as reference. Twofold serial dilutions of Trx-L2 (1-120) ranging from 0.98-1000 nM were injected over the captured mAb and reference surface. Regeneration of the capture surface between different mAbs (supernatants) was done with 10 mM glycine pH 2.0 at a flow rate of 30 µl/min for 30 s. Sensorgrams were processed using double referencing and the equilibrium dissociation constants were determined by steady state affinity evaluation with the Biacore T100 Evaluation Software.

Pseudovirion preparation

Pseudovirions were prepared as previously described (Buck et al., 2005) by co-transfection of the 293TT cell line derived by transformation of human embryonic kidney cells, with a plasmid bearing the humanized HPV16 L1 and L2 genes along with an SV40 replication origin (pCDNA4-HPV16L1h-L2h/SV40ori) and a second plasmid harbouring the reporter enzyme "secreted form of placental alkaline phosphatase" (SEAP) under the control of the CMV promoter (pCMVSEAP). Co-transfection was routinely performed on 7×10^6 293TT cells previously seeded on 10 cm culture dishes in Dulbecco modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS; Gibco-BRL), 1% penicillin/streptomycin (Life Technologies), and 125 µg/ml hygromycin (Roche), using TurboFect (Fermentas) according to the manufacturer's instructions with minor modifications. Briefly, 8 µg of each plasmid was mixed with 80 µl of PolyFectR in 800 µl of culture medium, incubated for 10 min and added to the cells, which were then incubated at 37 °C for 3–4 days. For pseudovirion extraction, 5×10^7 cells were harvested by trypsinization, washed once with PBS, and resuspended in 1 ml of PBS containing 1 mM $CaCl_2$ and 5.6 mM $MgCl_2$. After the addition of 4 µl of benzonase (250 U/ml; Sigma-Aldrich) and 50 µl of Brij 58, cells were incubated at 37 °C overnight to induce pseudovirion maturation. They were then supplemented with NaCl to a final concentration of 710 mM, followed by a 10 min incubation at 4 °C. The resulting lysate was cleared by centrifugation (10 min at $3500\times g$), pseudovirions were collected, divided in aliquots and immediately stored at −70 °C.

Neutralization assays

Prior to infection, 293TT target cells were seeded for 24 h at a density of 15,000 cells per well in 96-well plates filled with DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and 125 µg/ml hygromycin. The following day, pseudovirions were diluted in DMEM depending on their transducing activity to obtain a value between 2 10^4 and 3 10^5 relative units of luminiscense (RUL) and mixed with different dilutions of the anti-L2 antibodies. After 15 min at room temperature, the culture medium was replaced with 200 µl of the aforementioned pseudovirion solution. SEAP activity in cell culture supernatants was determined five days post-infection using the chemiluminescent SEAP Reporter Gene Assay (Roche) as per manufacturer's instructions. All anti-L2 mAbs were tested in duplicate. In each neutralization (and cross-neutralization) assay, the following controls were included: untreated cells, cells treated with the pseudovirions alone (i.e., without any added antiserum), cells treated with pseudovirions preincubated (as described for Trx-L2 peptide antisera; Rubio et al., 2009) with established

neutralizing mAbs specific for HPV31 (H31.A6 diluted 1:1000; (Christensen et al., 2001, 1996)), HPV45 (H45.L10 diluted 1:1000; (Combita et al., 2002)) and HPV16 (Ritti01 diluted 1:1000; (Rizk et al., 2008)), or with polyclonal antibodies against HPV18 (rat #2; diluted 1:2000) and HPV58 (human 109; diluted 1:1000) as required by each neutralization experiment.

Peptides

Biotinylated 15-mer peptides covering the N-terminal 120 aa of L2 with an offset of 4 amino acids each were produced by chemical synthesis. Other peptides covering the aa 20–38 region were generated by stepwise (one amino acid) shortening from the N- and the C-terminus until an 8-mer peptide size was reached. For alanine-scanning, variants of the aa 20–31 region of HPV16 L2 were produced by replacing each amino acid residue with alanine. All peptides were synthesized by Mimotopes (Australia). Biotinylated peptides covering the aa 20–31 regions of HPV31 and HPV16 L2 as well as variant peptides with amino acid residues of the original HPV16 sequence replaced by glycine residues were synthesized at the DKFZ core facility.

Peptide ELISA

Supernatants were tested for peptide binding using biotinylated peptides. Microtiter plates were coated with 50 µl of streptavidin (Sigma S4762; 1 mg/ml) diluted 1:400 with distilled water. Plates were incubated overnight at 37 °C; blocked with PBS-Casein 0.2% and further incubated for 1 h at RT. After each step plates were washed three times with PBS-0.3% Tween 20. Peptides previously resuspended in 50% acetonitrile and 5% acetic acid (or DMSO) were diluted to a concentration of 1 µg/µl in PBS; 50 µl of each peptide dilution were then added to the wells and incubated for 1 h at RT. Fifty µl of each supernatant diluted 1:10 were added to ELISA plates and incubated for 1 h at RT. Plates were then washed, incubated for 1 h at RT with 50 µl/well of a HRP-conjugated goat anti-mouse IgG polyclonal antibody (Sigma-Aldrich) diluted 1:3000 in PBS-0.3% Tween 20 plus 0.2% casein, and stained as described earlier.

Site-directed mutagenesis

Three mutations were introduced into the HPV 31 L2 gene (at once or stepwise) in order to adapt the HPV31 L2 sequence at specific positions to that of HPV16; the mutated genes are referred to as Q20K, A24K and S30P. Mutants were generated with the Quick Change Mutagenesis Kit (Stratagene). Four additional mutations (referred to as V32I, I36V, H38G and T39K) were introduced (at once or stepwise) in the aa 31–40 epitope and three mutations (V73I, S76G and S80P) in the aa 71–80 epitope of the HPV31 L2 gene in order to revert the HPV31 sequence to that of HPV16 at these positions; these mutants were generated with the QuikChange II-E and QuikChange II-XL Site-Directed Mutagenesis Kits (Stratagene) (oligonucleotide sequences are available on request).

Sequence analysis

N-terminal (aa 1–120) L2 sequences from 21 HPV serotypes were retrieved from GenBank (NP_040308.1, AAY86489.1, AAY86491.1, P06419.1, AAF00067.1, ACL12349.1, AAD33258.1, ABP99806.1, AAY86493.1, AAA46955.1, ACL12332.1, CAA52589.1, ACL12324.1, AAA46971.1, ACL12340.1, ABO76829.1, AAA47055.1, P26539.1, gb| ACL12357.1, CAA63886.1, BAA90741.1), aligned with ClustalW, and percent identity values were calculated using the AlgnIO module of Bio-Pearl. Average pairwise percent identities were determined using sliding windows of 7 amino acids with an incremental step of 1 residue positions.

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2010.10.017.

Acknowledgments

E.C. is a recipient of a PhD student fellowship from the University of Parma.

References

Alphs, H.H., Gambhira, R., Karanam, B., Roberts, J.N., Jagu, S., Schiller, J.T., Zeng, W., Jackson, D.C., Roden, R.B., 2008. Protection against heterologous human papillomavirus challenge by a synthetic lipopeptide vaccine containing a broadly cross-neutralizing epitope of L2. Proc. Natl Acad. Sci. USA 105 (15), 5850–5855.

Ault, K.A., 2007a. Effect of prophylactic human papillomavirus L1 virus-like-particle vaccine on risk of cervical intraepithelial neoplasia grade 2, grade 3, and adenocarcinoma in situ: a combined analysis of four randomised clinical trials. Lancet 369 (9576), 1861–1868.

Ault, K.A., 2007b. Human papillomavirus vaccines and the potential for cross-protection between related HPV types. Gynecol. Oncol. 107 (2 Suppl 1), S31–S33.

Bossis, I., Roden, R.B., Gambhira, R., Yang, R., Tagaya, M., Howley, P.M., Meneses, P.I., 2005. Interaction of tSNARE syntaxin 18 with the papillomavirus minor capsid protein mediates infection. J. Virol. 79 (11), 6723–6731.

Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T., 2005. Generation of HPV pseudovirions using transfection and their use in neutralization assays. Meth. Mol. Med. 119, 445–462.

Campos, S.K., Ozbun, M.A., 2009. Two highly conserved cysteine residues in HPV16 L2 form an intramolecular disulfide bond and are critical for infectivity in human keratinocytes. PLoS ONE 4 (2), e4463.

Christensen, N.D., Reed, C.A., Cladel, N.M., Hall, K., Leiserowitz, G.S., 1996. Monoclonal antibodies to HPV-6 L1 virus-like particles identify conformational and linear neutralizing epitopes on HPV-11 in addition to type-specific epitopes on HPV-6. Virology 224 (2), 477–486.

Christensen, N.D., Cladel, N.M., Reed, C.A., Budgeon, L.R., Embers, M.E., Skulsky, D.M., McClements, W.L., Ludmerer, S.W., Jansen, K.U., 2001. Hybrid papillomavirus L1 molecules assemble into virus-like particles that reconstitute conformational epitopes and induce neutralizing antibodies to distinct HPV types. Virology 291 (2), 324–334.

Combita, A.L., Touze, A., Bousarghin, L., Christensen, N.D., Coursaget, P., 2002. Identification of two cross-neutralizing linear epitopes within the L1 major capsid protein of human papillomaviruses. J. Virol. 76 (13), 6480–6486.

Day, P.M., Gambhira, R., Roden, R.B., Lowy, D.R., Schiller, J.T., 2008a. Mechanisms of human papillomavirus type 16 neutralization by l2 cross-neutralizing and l1 type-specific antibodies. J. Virol. 82 (9), 4638–4646.

Day, P.M., Lowy, D.R., Schiller, J.T., 2008b. Heparan sulfate-independent cell binding and infection with furin-precleaved papillomavirus capsids. J. Virol. 82 (24), 12565–12568.

Gambhira, R., Jagu, S., Karanam, B., Gravitt, P.E., Culp, T.D., Christensen, N.D., Roden, R.B., 2007a. Protection of rabbits against challenge with rabbit papillomaviruses by immunization with the N terminus of human papillomavirus type 16 minor capsid antigen L2. J. Virol. 81 (21), 11585–11592.

Gambhira, R., Karanam, B., Jagu, S., Roberts, J.N., Buck, C.B., Bossis, I., Alphs, H., Culp, T., Christensen, N.D., Roden, R.B., 2007b. A protective and broadly cross-neutralizing epitope of human papillomavirus L2. J. Virol. 81 (24), 13927–13931.

Garland, S.M., Hernandez-Avila, M., Wheeler, C.M., Perez, G., Harper, D.M., Leodolter, S., Tang, G.W., Ferris, D.G., Steben, M., Bryan, J., Taddeo, F.J., Railkar, R., Esser, M.T., Sings, H.L., Nelson, M., Boslego, J., Sattler, C., Barr, E., Koutsky, L.A., 2007. Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. N Engl J. Med. 356 (19), 1928–1943.

Harper, D.M., Franco, E.L., Wheeler, C.M., Moscicki, A.B., Romanowski, B., Roteli-Martins, C.M., Jenkins, D., Schuind, A., Costa Clemens, S.A., Dubin, G., 2006. Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. Lancet 367 (9518), 1247–1255.

Jagu, S., Karanam, B., Gambhira, R., Chivukula, S.V., Chaganti, R.J., Lowy, D.R., Schiller, J.T., Roden, R.B., 2009. Concatenated multitype L2 fusion proteins as candidate prophylactic pan-human papillomavirus vaccines. J. Natl Cancer Inst. 101 (11), 782–792.

Kawana, K., Yoshikawa, H., Taketani, Y., Yoshiike, K., Kanda, T., 1999. Common neutralization epitope in minor capsid protein L2 of human papillomavirus types 16 and 6. J. Virol. 73 (7), 6188–6190.

Kawana, K., Kawana, Y., Yoshikawa, H., Taketani, Y., Yoshiike, K., Kanda, T., 2001. Nasal immunization of mice with peptide having a cross-neutralization epitope on minor capsid protein L2 of human papillomavirus type 16 elicit systemic and mucosal antibodies. Vaccine 19 (11–12), 1496–1502.

Kawana, K., Yasugi, T., Kanda, T., Kino, N., Oda, K., Okada, S., Kawana, Y., Nei, T., Takada, T., Toyoshima, S., Tsuchiya, A., Kondo, K., Yoshikawa, H., Tsutsumi, O., Taketani, Y., 2003. Safety and immunogenicity of a peptide containing the cross-neutralization epitope of HPV16 L2 administered nasally in healthy volunteers. Vaccine 21 (27–30), 4256–4260.

Kondo, K., Ishii, Y., Ochi, H., Matsumoto, T., Yoshikawa, H., Kanda, T., 2007. Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. Virology 358 (2), 266–272.

Kondo, K., Ochi, H., Matsumoto, T., Yoshikawa, H., Kanda, T., 2008. Modification of human papillomavirus-like particle vaccine by insertion of the cross-reactive L2-epitopes. J. Med. Virol. 80 (5), 841–846.

Laniosz, V., Nguyen, K.C., Meneses, P.I., 2007. Bovine papillomavirus type 1 infection is mediated by SNARE syntaxin 18. J. Virol. 81 (14), 7435–7448.

Munoz, N., Bosch, F.X., de Sanjose, S., Herrero, R., Castellsague, X., Shah, K.V., Snijders, P.J., Meijer, C.J., 2003. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J. Med. 348 (6), 518–527.

Paavonen, J., Jenkins, D., Bosch, F.X., Naud, P., Salmeron, J., Wheeler, C.M., Chow, S.N., Apter, D.L., Kitchener, H.C., Castellsague, X., de Carvalho, N.S., Skinner, S.R., Harper, D.M., Hedrick, J.A., Jaisamrarn, U., Limson, G.A., Dionne, M., Quint, W., Spiessens, B., Peeters, P., Struyf, F., Wieting, S.L., Lehtinen, M.O., Dubin, G., 2007. Efficacy of a prophylactic adjuvanted bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a phase III double-blind, randomised controlled trial. Lancet 369 (9580), 2161–2170.

Paavonen, J., Naud, P., Salmeron, J., Wheeler, C.M., Chow, S.N., Apter, D., Kitchener, H., Castellsague, X., Teixeira, J.C., Skinner, S.R., Hedrick, J., Jaisamrarn, U., Limson, G., Garland, S., Szarewski, A., Romanowski, B., Aoki, F.Y., Schwarz, T.F., Poppe, W.A., Bosch, F.X., Jenkins, D., Hardt, K., Zahaf, T., Descamps, D., Struyf, F., Lehtinen, M., Dubin, G., Greenacre, M., 2009. Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women. Lancet 374 (9686), 301–314.

Pastrana, D.V., Gambhira, R., Buck, C.B., Pang, Y.Y., Thompson, C.D., Culp, T.D., Christensen, N.D., Lowy, D.R., Schiller, J.T., Roden, R.B., 2005. Cross-neutralization of cutaneous and mucosal Papillomavirus types with anti-sera to the amino terminus of L2. Virology 337 (2), 365–372.

Richards, R.M., Lowy, D.R., Schiller, J.T., Day, P.M., 2006. Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. Proc. Natl Acad. Sci. USA 103 (5), 1522–1527.

Rizk, R.Z., Christensen, N.D., Michael, K.M., Muller, M., Sehr, P., Waterboer, T., Pawlita, M., 2008. Reactivity pattern of 92 monoclonal antibodies with 15 human papillomavirus types. J. Gen. Virol. 89 (Pt 1), 117–129.

Rubio, I., Bolchi, A., Moretto, N., Canali, E., Gissmann, L., Tommasino, M., Muller, M., Ottonello, S., 2009. Potent anti-HPV immune responses induced by tandem repeats of the HPV16 L2 (20–38) peptide displayed on bacterial thioredoxin. Vaccine 27 (13), 1949–1956.

Schellenbacher, C., Roden, R., Kirnbauer, R., 2009. Chimeric L1–L2 virus-like particles as potential broad-spectrum human papillomavirus vaccines. J. Virol.

Sehr, P., Zumbach, K., Pawlita, M., 2001. A generic capture ELISA for recombinant proteins fused to glutathione S-transferase: validation for HPV serology. J. Immunol. Meth. 253 (1–2), 153–162.

Slupetzky, K., Gambhira, R., Culp, T.D., Shafti-Keramat, S., Schellenbacher, C., Christensen, N.D., Roden, R.B., Kirnbauer, R., 2007. A papillomavirus-like particle (VLP) vaccine displaying HPV16 L2 epitopes induces cross-neutralizing antibodies to HPV11. Vaccine 25 (11), 2001–2010.