Conformational and linear epitopes on virus-like particles of human papillomavirus type 33 identified by monoclonal antibodies to the minor capsid protein L2

Christoph Volpers, Martin Sapp, Peter J. F. Snijders, Jan M. M. Walboomers and Rolf E. Streeck

Institut für Medizinische Mikrobiologie, Johannes-Gutenberg-Universität Mainz, Hochhaus am Augustusplatz, D-55101 Mainz, Germany and Department of Pathology, Free University Hospital, 1081 HV Amsterdam, The Netherlands

The organization of epitopes on the minor capsid protein L2 of human papillomavirus (HPV) type 33 has been analysed using three monoclonal antibodies (MAbs) generated against a large fragment of the L2 protein (amino acids 82-259) expressed as a glutathione S-transferase fusion protein. The topology of the L2 epitopes has been investigated with respect to the structure of HPV-33 virus-like particles (VLPs). Two of the MAbs reacted with linear epitopes which were mapped to amino acids 153-160 and 163-170, respectively. These epitopes were accessible in denatured but not in native VLPs consisting of L1 and L2, suggesting an internal location. The third antibody was unable to detect denatured L2 protein but reacted with native VLPs. This is the first demonstration of an apparent conformational epitope of the HPV L2 protein. A model for the putative orientation of L2 in the papillomavirus capsid is deduced from the location of these and other antigenic sites.

Introduction

Human papillomaviruses (HPV) are non-enveloped double-stranded DNA viruses infecting epithelial cells of skin and mucosa. Some of the more than 70 different HPV types identified cause benign skin papillomas and genital condylomas; others, especially HPV-16, -18, -31 and -33, are regularly associated with cervical intraepithelial neoplasias and impose a high risk for malignant progression to invasive carcinomas of the genital tract (de Villiers, 1989). HPVs have a capsid of 55 nm diameter with icosahedral symmetry (T=7) composed of 72 pentameric capsomers consisting of the major capsid protein, L1, and a minor structural component, L2 (Baker et al., 1991). Since viral particles of genital HPV types can neither be isolated from infected tissue in preparative amounts nor efficiently produced in cell culture, several groups have prepared virus-like particles (VLPs) using eukaryotic expression systems (Hagensee et al., 1993; Kirnbauer et al., 1992, 1993; Rose et al., 1993; Zhou et al., 1991). We have recently shown that HPV-33-encoded L1 and L2 proteins synthesized in insect cells self-assemble into empty virus-like particles (Volpers et al., 1994). VLPs have been shown to be structurally indistinguishable from virions (Hagensee et al., 1994), to induce neutralizing antibodies (Kirnbauer et al., 1992; Rose et al., 1994; Volpers et al., 1995), and to be useful for screening human sera for the presence of conformation-dependent antibodies (Kirnbauer et al., 1994; Carter et al., 1993).

To verify the incorporation of the L2 protein and to study its topographical location in HPV-33 VLPs, we generated L2-specific monoclonal antibodies (MAbs) which have now been characterized in detail. They may also be useful for the detection of L2 expression in genital precancerous lesions infected with viruses of the HPV-16/33 group.

Methods

Cloning, expression, and purification of fusion proteins. The cloning, expression in Escherichia coli, and affinity purification of glutathione S-transferase (GST) fusion proteins have previously been described (Volpers et al., 1993).

Immunization of mice and isolation of MAbs. BALB/c mice were immunized by five injections of 20 µg each of G33L2 protein at 4-week intervals. Three days after the final boost a mouse was sacrificed and the spleen removed. A single cell suspension was obtained by pressing the spleen through a 60 mesh sieve. The spleen cells were fused to myeloma cell line X63Ag8.653 maintained in Iscove's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and 50 µg/ml-mercaptoethanol. The fusion was performed in PEG 1500 (Boehringer Mannheim) using standard protocols (Galfre et al., 1977).
Cells were distributed into 1000 wells and selected with hypoxanthine and azaserine (Sigma). After 6-8 days the cell culture supernatants were screened for secretion of antibodies directed against the L2 portion of the fusion protein by ELISA using G33L2 and GST, respectively, as antigens. Immunoreactions were visualized by use of horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch) and 1,2-phenyldiamine dihydrochloride. Positive clones were subcloned twice by limited dilution.

Peptide synthesis. Peptides were synthesized by Fmoc amino acid chemistry coupled to a derivatized cellulose membrane according to the vendor's instructions (SPOT kit, Cambridge Research Biochemicals).

Preparation of virus-like particles. Virus-like particles were solubilized from isolated nuclei of bac33L1 and bac33L2 infected Sf9 cells and purified by two subsequent CsCl density gradients exactly as described previously (Volpers et al., 1994).

Biopsy specimen, DNA isolation, HPV detection and typing. Biopsy specimens were taken, DNA was isolated, and the HPV DNA was detected and typed exactly as described previously (Sapp et al., 1994).

Immunohistochemistry. Paraffin-embedded sections on coated slides (0-1% poly-L-lysine) were deparaffinized with xylene, rehydrated, and treated for 3 min each for 95 °C in citrate buffer, pH 6.0. Endogenous peroxidase was blocked by incubating for 30 min with methanol containing 0.3% H2O2. After washing repeatedly in PBS, sections were pre-incubated with normal rabbit serum (1:50) for 10 min. Subsequently, incubation was performed with the MAb at room temperature for 60 min at the indicated dilution. MABs were detected with biotinylated rabbit anti-mouse Fab antibody fragment (1:500) (Vector Lab). Detection was performed by incubation with horseradish peroxidase coupled to avidin-biotin complex (1:500) (Vector Lab) for 60 min. The complex was visualized using diaminobenzidine (DAB) and H2O2. Slides were counterstained with haematoxylin, dehydrated and mounted in Depex.

Immunofluorescence. Sf9 cells were seeded onto microscope slides, infected with bac33L2 and grown for 48 h at 27 °C in supplemented Grace's insect medium containing 10% fetal calf serum. The cells were fixed with PBS-2% formaldehyde (20 min), treated with PBS-1% Nonindet P-40 for 20 min, incubated with MABs (5 to 10 μg/ml) for 1 h at 37 °C, given several washes with PBS, and subsequently incubated with goat anti-mouse IgG-dilhioaminofluorescein conjugate (1:20; Jackson Immunochemicals) for 1 h at 37 °C. Unbound secondary antibodies were removed by washing with PBS and the cells were covered with Histogel (Camon) and a cover slide. Photographs were taken with a Photomicroscope 3 (Zeiss).

Results

Isolation and specificity of MABs to the L2 minor capsid protein

To generate MABs to the minor capsid protein of HPV-33 a fragment of the L2 ORF of the HPV-33 genome encoding amino acids 82-259 was expressed in Escherichia coli as fusion protein with GST (G33L2). The fusion protein was used to immunize mice. Hybridomas obtained using standard procedures were screened with G33L2. Three stable hybridoma cell lines secreting L2-specific monoclonal antibodies, designated 33L2-1, 33L2-2 and 33L2-3, respectively, were obtained. We identified MABs 33L2-1 and 33L2-2 as IgG1 antibodies and MAb 33L2-3 as an IgM antibody.

All three MABs strongly reacted with native, purified G33L2 protein in ELISA, but only MABs 33L2-1 and 33L2-2 reacted equally strongly with SDS-denatured antigen (Table 1) and stained the L2 fusion protein and numerous degradation products in Western blots (Fig. 1b, c). G33L2 migrates aberrantly in SDS-PAGE (52 kDa vs the expected 46 kDa) as was previously observed for the full-length L2 protein (Volpers et al., 1994). MAb 33L2-3 was unreactive with denatured antigen in ELISA and weakly stained the upper two bands of the G33L2 protein but not smaller degradation products in Western blots (Fig. 1d), suggesting that almost the full-length L2 moiety of the fusion protein is required to retain the binding site of this antibody. We conclude from these data that MAb 33L2-3 is directed against a conformational antigenic determinant and that the fusion protein was partially renatured during the transfer to nitrocellulose, as has previously been shown for other proteins (Christensen et al., 1990b).

The linear epitopes of MABs 33L2-1 and 33L2-2 were mapped using GST-fusion proteins with truncated L2 moieties (Fig. 1). Both MABs reacted with fusion protein G33F2 carrying L2 amino acids 135-175 in Western blots (Fig. 1b, c), whereas MAB 33L2-3 showed no reactivity with either of the shortened L2 proteins (Fig. 1d) nor with two synthetic peptides comprising the overlap regions between them (amino acids 130-139 and 170-179, respectively) (not shown), confirming the conformation-dependent nature of the corresponding antigenic site. To further localize the linear epitopes of MABs 33L2-1 and 33L2-2, we synthesized 16 10-amino-acid peptides overlapping by eight amino acids each and spanning the L2 amino acid sequence 135-174. They were synthesized by Fmoc amino acid chemistry, and coupled to a derivatized cellulose membrane. The membrane was saturated with blocking buffer, incubated with the respective hybridoma supernatant (diluted 1:100) and developed with an anti-mouse IgG phosphatase conjugate. MAb 33L2-1 reacted with two peptides containing the overlapping sequence FTEPSVLH (HPV-33 L2 aa 163–170) (Fig. 2a). The epitope of MAB 33L2-2 was similarly mapped to the sequence QTISTHNLN (HPV-33 L2 aa 153–160) (Fig. 2b); since a peptide lacking the first two amino acids Q and T of the epitope was also weakly reactive, ISTHNLN seems to be the minimum binding site of this antibody.

Using affinity-purified GST/L2 fusion proteins of HPV-1,-8,-11,-16 and -18 (Volpers et al., 1993) in Western blotting, MAb 33L2-1 was shown to be cross-reactive with HPV-16 (Fig. 3b), whereas MAb 33L2-2 was type-specific for HPV-33 (Fig. 3c). Identical results were obtained by ELISA (not shown). MAB 33L2-3 was also found to be specific for HPV-33 in an ELISA with the purified L2 fusion proteins (not shown), but it cannot be excluded that the lack of reactivity with the other
Table 1. ELISA reactivities of monoclonal antibodies and polyclonal rabbit antisera against fusion protein G33L2 and virus-like particles*

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
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<tr>
<td>33L2-1</td>
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<tr>
<td>33L2-3</td>
<td>33L1-4</td>
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<td>33L1-7</td>
<td>K28</td>
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* Antibodies were used at the following dilutions: MAbs 33L2-1, 33L2-2, 1:100; MAb 33L2-3, undiluted; MAb 33L1-4, 1:1000; MAb 33L1-7, 1:3000; antisera K18, 1:200; K28, 1:100. Secondary reagents conjugated with horseradish peroxidase were used at the following dilutions: anti-mouse IgG, 1:5000; anti-mouse Ig, 1:1000; Protein A, 1:3000. Absorbance values were measured at 492 nm; NT, not tested.

Fig. 1. Epitope mapping of the MAbs using fusion proteins. (a) Coomassie-blue-stained SDS-polyacrylamide gel of affinity purified GST/L2 fusion proteins containing HPV-33 L2 amino acid sequence 82–259 (G33L2), 82–134 (G33F1), 135–175 (G33F2) and 174–259 (G33F3), respectively. (b–d) Western blots of the L2 fusion proteins reacted with MAb 33L2-1 (b), MAb 33L2-2 (c) and MAb 33L2-3 (d).

HPV types is due to an incomplete presentation of the corresponding putative conformational epitopes in the fusion proteins.

Reactivity with eukaryotically expressed L2 protein

The reactivity of the MAbs raised against a bacterially expressed fusion protein with eukaryotically expressed, possibly modified L2 protein was demonstrated by immunofluorescence and immunohistochemistry. All three MAbs stained the nuclei of Sf9 insect cells infected with an HPV-33 L2 recombinant baculovirus (Volpers et al., 1994), as shown for MAb 33L2-2 in Fig. 4(a), but not of uninfected or baculovirus wild-type-infected cells. The reactivity of MAb 33L2-1 with HPV-16 capsid antigen was demonstrated by the strong immunohistochemical nuclear staining of the superficial cell layers of CIN II lesions containing HPV-16 DNA (Fig. 4b). Using samples containing HPV-11 and HPV-18 capsid protein, respectively, no staining with MAb 33L2-1 was obtained (not shown), in agreement with the Western blot data. These results prove the capacity of the MAbs to react with full-length minor capsid protein in infected cells and suggest that they may be useful as HPV-16/33 subgroup-specific probes for diagnostic immunocytochemical detection of capsid protein in genital precancerous lesions and for epidemiological studies.

Characterization of epitopes on virus-like particles

To investigate the topology of L2 epitopes on HPV-33 VLPs, the reactivity of intact and disrupted VLPs with
Fig. 2. Epitope mapping of the MAbs using synthetic peptides. A cellulose membrane carrying 16 synthetic 10-amino-acid peptides spanning HPV-33 L2 amino acid sequence 135-174 was reacted with MAb 33L2-1 (a), MAb 33L2-2 (b) and developed with anti-mouse IgG–phosphatase conjugate and 5-bromo-4-chloro-3-indolyl phosphate/thiazolyl blue as substrate. (c) Amino acid sequences of the overlapping peptides; reactive peptides are in bold type.

Fig. 3. Cross-reactivity of MAbs with GST/L2 fusion proteins of different HPV types. (a) Coomassie-blue-stained SDS–polyacrylamide gel of fusion proteins of HPV-33 (G33L2), HPV-1 (G1L2, containing HPV-1 L2 amino acid sequence 42-211), HPV-8 (G8L2, with amino acids 55-278), HPV-11 (G11L2, with amino acids 48-209), HPV-16 (G16L2, with amino acids 79-226) and HPV-18 (G18L2, with amino acids 81-254). (b, c) Western blots of L2 fusion proteins reacted with MAb 33L2-1 (b) and MAb 33L2-2 (c).
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Fig. 4. Reactivity of MAbs with eukaryotically expressed L2 protein.
(a) Immunofluorescence staining of HPV-33 L2-recombinant baculovirus-infected insect cells (Sf9) with MAb 33L2-2. Fluorescein isothiocyanate-conjugated anti-mouse IgG was used as secondary antibody. (b) Immunohistochemical staining of a formalin-fixed, paraffin-embedded tissue section of a cervical intraepithelial neoplasia grade II, containing HPV-16 DNA, with MAb 33L2-1. Bound antibody was visualized using diaminobenzidine. Bars: (a) 10 μm (1500 x); (b) 20 μm (500 x).

A polyclonal rabbit serum (K18) raised against a Protein-A/HPV-33 L2 fusion protein carrying L2 amino acids 82–134 has recently been characterized as containing antibody specificities for several binding sites; the immunodominant epitope has been mapped to amino acids 117–130 (Volpers et al., 1993). This polyclonal antiserum, which had a slightly elevated background reactivity with L1-VLPs, was preferentially reactive with L2 protein after denaturation of L1/L2-VLPs (Table 1). Similar results were obtained with a monospecific antiserum (K28) generated by immunization with a synthetic peptide corresponding to L2 amino acids 117–130 (Volpers et al., 1993), indicating that amino acid sequence 117–130 is not accessible at the capsid surface.

We conclude from these data and the lack of reactivity of MAbs 33L2-1 and 33L2-2 with native L1/L2-VLPs that most or all of amino acid sequence 117–170 is buried in the major capsid protein or masked by other parts of the L2 protein.

Discussion

This communication describes the isolation and characterization of MAbs to the HPV-33 L2 minor capsid protein defining linear as well as conformational epitopes on the L2 protein. The minor capsid protein of papillomaviruses has previously been demonstrated to induce type-specific antibodies in animals immunized with fusion proteins and virus particles (Christensen et
in the capsid is largely unknown. Recent biochemical analyses suggest that some regions of the L2 protein interact with monomeric capsomers whereas other parts interact with intercapsomer regions stabilizing oligomeric capsomers (Sap et al., 1995). Yaegashi et al. (1991) obtained a MAb directed against amino acids 102–108 of the HPV-1 L2 protein by immunization with intact virions, suggesting that this region is accessible at the virion surface. Christensen et al. (1991) identified external, linear neutralizing epitopes on the C-terminal half of the cottontail rabbit papillomavirus (CRPV) L2 protein using polyclonal rabbit sera. Recently, Roden et al. (1994) showed that the N-terminal section of the bovine papillomavirus type 1 (BPV-1) L2 protein (amino acids 45–173) induces neutralizing antibodies in rabbits. Vaccination experiments with CRPV (Lin et al., 1992) and BPV-4 (Campo et al., 1993) demonstrated a protective effect of neutralizing L2-specific antibodies indicating an external presentation of the corresponding epitopes on the virus particle. In contrast, MAbs (Jin et al., 1989) and polyclonal antisera (Christensen et al., 1990b) raised against bacterially expressed fusion proteins containing the complete L2 sequence of BPV-1 and HPV-6, respectively, reacted only with denatured, not with intact virions in immunoassays, indicating an internal location of their antigenic determinants, as has been shown in this work for MAbs 33L2-1 and 33L2-2. Taken together with the recent finding of Zhou et al. (1994) that the N terminus of the HPV-16 L2 protein seems to be responsible for the interaction of the capsid with the viral genome, these results suggest the following model for the orientation of L2 in the papillomavirus particle: the N terminus of the L2 protein is located inside the capsid, possibly interacting with DNA; the region around amino acid 100 adjacent to the strongly conserved N-terminal 80-amino-acid stretch (Danos & Yaniv, 1983) and an as yet undefined segment of the C-terminal half are positioned at the outside of the capsid; the in-between polypeptide chain, including amino acid sequences 120–130 and 150–170, is internal and probably in close contact with the major capsid protein. An external localization of conformational epitopes on L2, like the one defined by MAb 33L2-3, is consistent with this concept.

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References

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