Identification of seroreactive regions of the human papillomavirus type 16 proteins E4, E6, E7 and L1

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Small fragments of the DNA of human papillomavirus type 16 (HPV-16) were randomly cloned into the bacteriophage fd which expresses the resulting peptides as part of its capsid. Antisera raised against different HPV-16 fusion proteins were used for screening of the phage clones and the reacting peptides were determined by sequencing the inserted HPV-16 DNA fragments of the positive recombinants. Seroreactive regions of the proteins derived from the E4, E6, E7 (two regions) and L1 (three regions) open reading frames could be found by this approach. Of these seven regions, four were defined by at least two overlapping inserts, thus limiting the domains to between 10 and 15 amino acids. In the case of the E4 open reading frame, the same region identified by immunoscreening was also found when synthetic overlapping octapeptides were tested by ELISA with the anti-E4 antiserum. Using an approach to predict 'receptor-like' regions within the respective proteins, five of the seven regions were also identified. From the data on these regions, synthetic peptides were produced and used for the detection of antibodies against HPV-16 proteins in human sera by ELISA.

Introduction

Human papillomavirus (HPV) type 16 is implicated in not only benign lesions of the anogenital tract but also malignant cancer of the uterine cervix, penis and vulva (for review see zur Hausen, 1989). In addition, HPV-16 can also be found in genital scrapes from clinically asymptomatic individuals. Such information became available by analysing tissue samples for the presence of viral genomes using molecularly cloned HPV-16 DNA as a probe in different hybridization assays (Schneider et al., 1987; de Villiers et al., 1987). On the other hand, little is known about the immune response to infections by HPV-16 and papillomaviruses in general. Only recently, human sera obtained from patients with papillomavirus-associated genital lesions, from patients suffering from cervical cancer, as well as from healthy individuals, were tested for the presence of antibodies directed against viral proteins (Jochmus-Kudielka et al., 1989). These proteins were expressed as fusion proteins, with different prokaryotic sequences linked to their N terminus, and used as antigens in Western blot experiments. This test, however, is relatively tedious, thus preventing the quantitative analysis of large serum collections. Moreover, due to the relatedness of the different papillomavirus types, cross-reactivity of antibodies cannot be excluded. Therefore, we decided to identify seroreactive regions of the HPV-16 proteins. The open reading frames (ORF) E4, E6, E7 and L1 were selected for this as they are involved either in virus replication or virus-related transformation (Schwarz et al., 1985; Doorbar et al., 1986; Münger et al., 1989). Knowledge of such domains is the prerequisite for the establishment of a peptide-based ELISA which may subsequently be used for large-scale screening of human sera.

Methods

Bacterial strains and phages. The filamentous phage derivative fuse 1 (fd-tet-J6; Zacher et al., 1980; Smith, 1985; Parmley & Smith, 1988) was used as the expression system for HPV-16 genomic DNA fragments cloned into its unique PvuII site. Escherichia coli strain K802 (F' galK2 galT22 metB1 supE44 kdr) (Wood, 1966) was transformed with the fuse 1 vector, by the method of Hanahan (1983). The tetracycline-resistant colonies produce bacteriophages which are not infectious for this strain because of its F' phenotype. Recombinant phages were plated onto E. coli strain K91 (F', derivative of K38; Lyons & Zinder, 1972). Insertion of a DNA fragment of the size 3n + 2 nucleotides, without internal stop codons for translation, restores a
frameshift mutation within gene III of bacteriophage fd which does not otherwise permit production of infectious progeny.

**Antisera.** Polyclonal rabbit antisera prepared against an MS2 polymerase—(Seedorf et al., 1985) or CII—HPV-16 E4 fusion protein (Jochmus-Kudielka & Gissmann, 1990), or raised against an MS2 polymerase—HPV-16 E7 fusion protein (Seedorf et al., 1985) were used. The E7 part of the fusion protein includes the amino acids between nucleotide positions 3399 and 3617 of the HPV-16 genome. Two different anti-E7 monoclonal antibodies (MAbs) raised against the same fusion protein were also used (E7 II and E7 IV; Oltersdorf et al., 1987). The anti-HPV-16 E6 antiserum was prepared against an MS2 polymerase—E6 fusion protein containing the main part of the E6 ORF (nucleotide positions 110 to 556; Seedorf et al., 1985). Two different polyclonal rabbit antisera prepared against the N-terminal (L1/I; nucleotide positions 5692 to 6819) and the C-terminal part (nucleotide positions 6819 to 7152) of HPV-16 L1 fused to the N-terminus of MS2 polymerase were also used (Seedorf et al., 1985).

**Preparing the HPV-16 fuse 1 expression library.** HPV-16 plasmid DNA (5 μg) was sonicated for 120 s to yield a fragment size of approximately 1 kb and further digested to about 300 bp with 0.2 to 2 units of DNase I for 10 min at 15 °C in the presence of 10 mM-MnCl₂. The DNase I had been diluted and preincubated for 1 h in a buffer containing 50 mM-Tris-HCl pH 8.0, 10 mM-MnCl₂ and 0.1 mg/ml bovine serum albumin (BSA).

To obtain blunt ends the DNA fragments were treated for 60 min at 15 °C with 15 units of T4 polymerase and 10 units of E. coli DNA ligase and 100 μM of each of the four deoxyribonucleotides. The DNA was then ligated into the unique PstI site of fuse 1. Competent E. coli K802 (F⁻) were transformed with the ligated DNA and plated on LB plates (Sambrook et al., 1989) containing 15 μg/ml tetracycline. A total of approximately 3 × 10⁶ tetracycline-resistant phage-producing colonies were obtained from eight different experiments. To obtain recombinant phages, the colonies were rinsed with LB. This amplification yielded eight different libraries with a total number of approximately 5 × 10¹² infectious particles but also resulted in under-representation of certain recombinants (see results). The phage suspensions were centrifuged and heated for 10 min at 65 °C to remove the remaining bacteria. Unless otherwise indicated, these amplified libraries were kept separately and used for further experiments.

**Immunoscreening.** Between 2000 and 6000 phages were plated with 0.2 ml of exponentially growing K91 cells in 3:5 ml 0.5% agar plates. Nitrocellulose replicas were taken and incubated further on fresh minimal medium plates for 6 h at 37 °C to enhance the signals. Afterwards the filters were centrifuged for 60 min in 100 μl non-fat milk in phosphate-buffered saline (PBS) and then incubated overnight in 5% milk in PBS containing a 1:100 to 1:1000 dilution of HPV-specific antisera (preabsorbed with sonicated K91 cells) or MAbs. The filters were then washed three times with 5 min in PBS/0.1% Tween-20 and incubated for 3 h at room temperature with goat anti-rabbit (or anti-mouse) peroxidase antibodies (1:1000) in 5% non-fat milk. After washing, the filters were stained in 0.2 ml PBS containing 30 mg diamino benzidine, 30 μl H₂O₂ and 1.5 ml NiSO₄. Finally the filters were washed in H₂O for 30 min and dried on paper.

**Preparation of single-stranded DNA of fuse 1 recombinants.** A protocol similar to previously described procedures was used (Sanger et al., 1977). LB (50 ml) was inoculated with tetracycline-resistant E. coli K91 harbouring the fuse 1 plasmid, incubated for 16 h at 37 °C and the bacteria were then pelleted at 6000 r.p.m. for 30 min. After adding 2 ml of 40% polyethylene glycol 6000 and 2 ml of 5 M-sodium acetate pH 6.5 to the supernatant, the phages were precipitated for 60 min at 0 °C and then centrifuged at 6000 r.p.m. for 60 min. The pellet was resuspended in 0.3 ml TE buffer and, after two extractions with phenol, the DNA was precipitated. Approximately 25% of the material obtained from such preparations was used for one sequencing reaction.

**Sequencing.** For DNA sequencing the standard United States Biochemicals protocol was used with the universal primer replaced by a 20-mer oligonucleotide (5'-TCCAGACGTTAGTAAAAATGAA-3'). An example of a sequencing reaction is given in Fig. 1.

**Peptide synthesis.** A set of overlapping peptides corresponding to the HPV-16 E4 ORF was synthesized on the tips of polyethylene rods derivatized with beta-alanine (Cambridge Research Biochemicals), essentially following the strategy described by Geyser et al. (1984, 1985). Deviating from the protocol recommended by Geyser et al., the protein sequence was divided into octamer peptides overlapping by six residues and the synthesis was carried out using Fmoc chemistry and in situ activation by benzotriazolyl-N-oxytris(dimethylamino)- phosphonium hexafluorophosphate (BOP; Castro's reagent) (Castro et al., 1975). Fmoc amino acid derivatives (6 μmol), BOP and N-methyl morpholine solutions were distributed into polyethylene reaction trays
(Cambridge Research Biochemicals) according to the respective peptide sequences being synthesized. All other reactions were carried out according to the manufacturers' instructions. As a positive control, the peptide RPDYLDFA, derived from human tumour necrosis factor alpha (amino acid positions 138 to 145; Cerami & Beutler, 1986), was synthesized together with the HPV-16-specific peptides and tested with an appropriate antiserum by ELISA.

**ELISA with synthetic peptides.** For epitope mapping all tests were made on peptides covalently bound to the polyethylene pins on which they had originally been synthesized. Racks with 96 pins fixed in a configuration that allowed insertion into the wells of a microtitre tray were used and incubations for ELISA were performed by sticking the pins into the wells. The rods were washed with methanol and PBS and then blocked with 0.25% gelatine, 0.1% Tween-20 in PBS for 2 h at 37°C, followed by incubation with sera diluted 1:200 to 1:4000 in 0.125% gelatine, 0.05% Tween-20 for 1 h at 37°C. After washing with PBS/0.1% Tween-20 the pins were incubated for 1 h at 37°C with Protein A-peroxidase (1:4000) followed by a further washing and staining with tetramethylbenzidine (Holland et al., 1974) for 15 min. Staining was terminated by lifting the rods out of the dye and adding 100 μl 0.5 M-H₂SO₄. The absorbance was measured in an automatic ELISA reader.

To remove the antibody–enzyme complex after ELISA, the pins were sonicated for 1 h (water bath, 30 W, 48 kHz) at 60°C in PBS/1% SDS/0.1% 2-mercaptoethanol and finally washed with methanol. The efficiency of the disruption procedure was tested by ELISA using Protein A-peroxidase without any primary serum. The same peptides were used more than 40 times in subsequent ELISAs.

For screening of human sera, microtitre plates were coated with 0.5 to 20 μg of synthetic peptides in 0.5 mM-sodium carbonate buffer pH 9.6 per well overnight at 4°C. After three washings with H₂O the plates were blocked with 5% BSA in PBS for 3 h at 37°C, followed by three washings with PBS containing 0.1% Tween-20. The plates were incubated with human sera diluted 1:100 to 1:500 in 2-5 μg BSA in PBS, 0.05% Tween-20 for 1 h. The plates were again washed four times and then incubated with a goat anti-human–peroxidase antibody for 1 h. Staining was performed as described above.

**Computer analysis of potential protein structure.** The mean α-helical hydrophobic moment (αH) and the mean hydrophobicity (H) were calculated according to the procedure of Eisenberg et al. (1982, 1984). A segment of seven amino acids was moved through the protein sequences of the HPV-16 E4, E6, E7 and L1 ORFs (Brasseur, 1988; Brasseur et al., 1988). The (H) and the αH per segment were calculated. These two parameters were plotted as a function of the midpoint of the segment along the sequence. The normalized consensus hydrophobicity scale (Eisenberg, 1984) was used because it is especially suitable for membrane-associated proteins. Protein fragments with low (H) and high (αH) may have receptor-like properties (de Loof et al., 1986). The secondary structure of the identified antibody binding sites was predicted using the Garnier method (Garnier et al., 1987). All calculations were performed on an Olivetti XPS with an Intel 80387 arithmetic coprocessor with the PC-Prot+ procedure.

**Results**

The identification of seroreactive peptides was performed by two different experimental methods and the data were compared with the sequences obtained using a theoretical approach to define 'receptor-like' peptides within a protein.

**Identification of epitopes using the fd expression library**

Approximately 25000 recombinant bacteriophages obtained from separately constructed HPV-16–fd expression libraries were plated onto *E. coli* K91. Replicas were taken and incubated with a polyclonal rabbit antiserum against HPV-16 E7 followed by incubation with goat anti-rabbit-peroxidase complexes and staining as described in Methods.

![Fig. 2. Identification of recombinants expressing HPV-16 E7 epitopes.](Image)

Approximately 3000 bacteriophage particles obtained from an initially identified plaque were plated on *E. coli* K91. Replicas were taken and incubated with a polyclonal rabbit antiserum against HPV-16 E7 followed by incubation with goat anti-rabbit-peroxidase complexes and staining as described in Methods.

Region E7-221 is represented by 22 clones which fall into four groups of different sizes (221, 209, 212, 108; see Table 1). Group 221 consists of only one isolate of 68 nucleotides (bp 576 to bp 643). The other groups are represented by three, nine and nine clones, respectively, all of which are shorter than clone 221 and overlap between nucleotide positions 589 and 618, corresponding to the amino acid sequence EYMLDLQPET.

Region E7-107 is represented by nine clones of two different groups overlapping by 41 nucleotides with the
common sequence GPAGQAEPDRAHY (bp 679 to bp 717; Table 1).

Unlike E7, it was not possible to identify E6-positive bacteriophages by screening an equivalent number of recombinants with a polyclonal rabbit antiserum prepared against the MS2 polymerase–HPV-16 E6 fusion protein. This may be explained by an under-representation of such clones in the phage library obtained from the original colonies. To enrich E6-reacting phages, the immunoglobulin fraction of the anti-E6 rabbit serum was bound to Protein A-Sepharose. Approximately 1010 bacteriophages, obtained as a pool of seven different fd expression libraries constructed by individual ligation of sheared and DNase I-digested HPV-16 DNA, were incubated with the Protein A–Sepharose–immunoglobulin complexes. The specifically bound bacteriophages were eluted and plated on E. coli K91 and approximately 3000 plaques were obtained. For amplification, the phages were suspended in medium and plated again. In order to test the efficiency of the enrichment step, identical numbers of recombinants eluted from the Protein A column were plated and hybridized with HPV-16 DNA probes specific for the E6 (nucleotides 24 to 654) or the E4 (nucleotides 2714 to 3693) ORF. By comparison with the hybridization of the original recombinants, an approximately 20-fold enrichment of E6 DNA sequence-containing phages was calculated (data not shown). As the immunoreactive recombinants express only a part of the E6 protein (see below), it was assumed that such recombinants were even less prevalent in the original library and thus the factor of enrichment is much higher. In fact, approximately 300 positive signals were obtained when the phages were screened with the same E6 antiserum used for the binding to the Protein A column. Of the positive recombinants, 14 were re-screened, nine of which were finally analysed by DNA sequencing and shown to fall into two different groups representing one region of the HPV-16 E6 ORF. The overlapping sequence (bp 101 to 145) encodes the peptide AMFQDPQERPRKLPQ (Table 1).

Three different antigenic regions were identified within the HPV-16 L1 protein. Recombinant bacteriophages (20000) of one HPV-16 library were screened

Table 1. Antigenic regions on the HPV-16 proteins E4, E6, E7 and L1

<table>
<thead>
<tr>
<th>fd clone (HPV-16 ORF)</th>
<th>Antigenic region*</th>
<th>Number of identical clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4-815</td>
<td>bp 3423–1 PKSPWAPKK–bp 3455</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>KPSWAPKPKHRLS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WAPKPKHRRLSSDQD</td>
<td></td>
</tr>
<tr>
<td>E6-670</td>
<td></td>
<td></td>
</tr>
<tr>
<td>670 (E6)</td>
<td>bp 68–LSRHFMQKRTAMFQDPQERPRKLPQ–bp 145</td>
<td>4</td>
</tr>
<tr>
<td>676 (E6)</td>
<td>bp 101–AMFQDPQERPRKLPQLCTELQTTIDIDELC–bp 193</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E7-221</td>
<td></td>
<td></td>
</tr>
<tr>
<td>221 (E7)</td>
<td>bp 577–PTLHEYMLDLQPETTDLYCEQ–bp 642</td>
<td>1</td>
</tr>
<tr>
<td>209 (E7)</td>
<td>bp 586–HHEYMLDLQPET–bp 618</td>
<td>3</td>
</tr>
<tr>
<td>212 (E7)</td>
<td>bp 580–TLHHEYMLDLQPETTD–bp 624</td>
<td>9</td>
</tr>
<tr>
<td>108 (E7)</td>
<td>bp 589–EYMLDLQPETTDLY–bp 630</td>
<td>9</td>
</tr>
<tr>
<td>107 (E7)</td>
<td>bp 667–DEIDGPAGQAEPDRAHY–bp 717</td>
<td>6</td>
</tr>
<tr>
<td>710 (E7)</td>
<td>bp 679–GPAGQAEPDRAHYN1–bp 723</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>L1-809</td>
<td></td>
<td></td>
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<tr>
<td>809 (L1)</td>
<td>bp 5998–PLLNLKDDENTENASAYAANAAGVDN–bp 6066</td>
<td>3</td>
</tr>
<tr>
<td>830 (L1)</td>
<td>bp 6307–ICTSICKYPD–SDAQIFNKPY–bp 6570</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>bp 6379–FYLREREQM–bp 6402</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1-842</td>
<td></td>
<td></td>
</tr>
<tr>
<td>842 (L1)</td>
<td>bp 6922–KHTPPAPKEDPLKK–bp 6966</td>
<td>2</td>
</tr>
<tr>
<td>905 (L1)</td>
<td>bp 6907–AACQKTTTPAPKEDPLKKYTFWEVNLKEFKFSADLD–bp 7017</td>
<td>4</td>
</tr>
<tr>
<td>877 (L1)</td>
<td>bp 6958–LKKYTTFWEVNLKEFKFSADLDQF–bp 7023</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| * Designated by first or single fd clone.  
† Epitope identified by overlapping octapeptides.  
‡ Predicted sites matching experimentally defined epitopes.  
§ L1-830 encodes 88 amino acids and is shown in part.
with a rabbit antiserum raised against the N-terminal part of the HPV-16 L1 ORF (bp 5695 to 6818). Of 80 positive plaques, 25 were rescreened and 11 of these were sequenced. Three clones were shown to contain an identical fragment of the HPV-16 L1 ORF, i.e. nucleotide positions 5998 to 6066, encoding the peptide PLLNKLDDTENSASAYAANAGVDN (L1-809; Table 1). The other eight clones contained an insert (nucleotide positions 6307 to 6570) encoding a peptide 88 amino acids long (ICTSICKYPD-----SDAQIFNKPY; L1-830).

Screening of 8000 recombinants of the same HPV-16 library with a polyclonal rabbit antiserum raised against the C-terminal part of HPV-16 L1 (bp 6818 to 7152) resulted in 52 positive recombinants. Of these, seven were confirmed by repeated re-screening and their inserts were sequenced. All clones were shown to belong to the same immunogenic region (L1-842; see Table 1). The insert of two clones (842, nucleotides 6922 to 6966) encodes the peptide KHTPPAPKEDDPLKK which overlaps the insert of clone 877 (nucleotides 6958 to 7023), encoding the peptide LKKYTFWEVNLKEKF-SADLDQF, by three amino acids. A third group of recombinants (905; nucleotides 6907 to 7017), represented by four clones, overlaps with clones 842 and 877 and encodes a 37-mer peptide (AIACQKHTPPAPKEDDPLKKTWFWEVNLKEKFSA). Since a sequence of only three amino acids (LKK) is common to all three groups of clones there are probably two independent binding sites within the region L1-842 (nucleotides 6907 to 7023), although the existence of such a short epitope cannot be excluded.

To identify epitopes of the E4 protein, the same library was used as for the L1 protein. By screening with an anti-E4 serum prepared against an MS2 fusion protein, 28 recombinants were identified. Three of these were analysed by sequencing and were shown to contain the HPV-16 E4 ORF-specific insert (nucleotide positions 3422 to 3456) corresponding to the peptide IPKPSPWAPKK.

Identification of the binding site of an anti-E7 mouse MAb

As described for the identification of E6-specific epitopes, $10^{10}$ phages obtained from seven different HPV-16–fd libraries were bound to Protein A–Sepharose–IgG (anti-HPV-16 E7 MAb II; Oltersdorf et al., 1987) complexes. After elution, approximately 300 recombinants were obtained, plated and the resulting plaques rinsed with medium. Five-hundred recombinants of this amplified sub-library were screened with the MAb and two of the clones were sequenced after three steps of re-screening. Both clones were shown to contain an insert identical to that of clone 108 of the immunogenic region 221 of the HPV-16 E7 ORF (Table 1).

Further mapping of immunoglobulin binding sites of the antigenic region E7-221

By screening of the expression library clone 108 was identified as binding the anti-E7 MAb. In order to determine whether all different clones of antigenic region 221 bind HPV-16 anti-E7 MAbs, competition assays were performed. MS2 polymerase–E7 fusion protein on Western blot strips was stained with MAb II against HPV-16 E7 (Oltersdorf et al., 1987). The antibody was preabsorbed with preparations of different phage clones and incubated with the lanes containing the HPV-16 E7–MS2 fusion protein. Only preparations of phage particles of clone 221 (lanes 2 and 4; different concentrations of phage particles used for absorption; for clone numbers see Table 1) or clone 108 (lane 5) can react with the MAbs, therefore preventing the reaction with the fusion protein on the lanes. Clones 209 (lanes 3 and 6, different concentrations of phage particles) or 212 (lane 7) did not react with the MAb. In lane 1 the antibodies were preabsorbed without phage particles; in lane 8 they were incubated with particles containing an insert not related to E7 sequences.

Fig. 3. Inhibition of immunostaining of the MS2 polymerase–HPV-16 E7 fusion protein with MAbs by fd bacteriophages expressing the antigenic region E7-221 (see Table 1). Western blot strips were stained with MAb II against HPV-16 E7 (Oltersdorf et al., 1987). The antibody was preabsorbed with preparations of different phage clones and incubated with the lanes containing the HPV-16 E7–MS2 fusion protein. Only preparations of phage particles of clone 221 (lanes 2 and 4; different concentrations of phage particles used for absorption; for clone numbers see Table 1) or clone 108 (lane 5) can react with the MAbs, therefore preventing the reaction with the fusion protein on the lanes. Clones 209 (lanes 3 and 6, different concentrations of phage particles) or 212 (lane 7) did not react with the MAb. In lane 1 the antibodies were preabsorbed without phage particles; in lane 8 they were incubated with particles containing an insert not related to E7 sequences.
was observed when MAb E7 IV was used for staining. Therefore it was concluded that there is at least one additional binding site for antibodies adjacent to the peptide EYMLDLQPET of the antigenic region E7-221 described above. Similar experiments demonstrate that the rabbit polyclonal anti-E7 antiserum also recognizes two epitopes of the clones 221 and 108.

Identification of antigenic regions of the HPV-16 E4 protein using overlapping peptides

A total of 45 octapeptides representing the HPV-16 E4 ORF were synthesized on polyethylene pins as described in Methods. The peptides overlapped by six amino acids, thus peptide number one represents amino acids 1 to 8 of the HPV-16 E4 ORF, peptide number two encompasses amino acids 3 to 10 and so on. The peptides were incubated with the polyclonal rabbit antiserum raised against the MS2-HPV-16 E4 fusion protein mentioned above. Rabbit immunoglobulins were detected with Protein A-peroxidase conjugates as described in

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**Fig. 4.** ELISA of overlapping octapeptides representing the HPV-16 E4 ORF. The 45 peptides were incubated first with a polyclonal rabbit antiserum against HPV-16 E4 followed by incubation with the Protein A-peroxidase conjugate. The peptides were stained as described in Methods and the extinction was measured. A very similar pattern was obtained in four different experiments using two different rabbit antisera.

**Fig. 5.** Plot of hydrophobicity (H) against the hydrophobic moment (\(\mu_H\)) for all seven-residue-long segments of HPV-16 E4. Characteristic regions occupied by protein domains are designated as G (globular), M (membrane), T (transmembrane), S (surface) and R (‘receptor-like’). The cluster of residues representing a receptor-like region of the E4 protein is boxed. The numbers indicate the position of the corresponding amino acid in the HPV-16 E4 ORF. The enclosed area contains amino acids 37 (K) to 44 (S). Because a seven residue segment was used for the calculation and each amino acid shown is the centre of such a segment, three additional amino acids for the first and the last residue in the ‘receptor-like’ area also belong to the predicted epitope. The resulting sequence is therefore WAPKKHRRLSSDQD, corresponding to numbers 34 to 47.
Table 2. Prediction of antigenic epitopes on the HPV-16 proteins E4, E6, E7 and L1

<table>
<thead>
<tr>
<th>ORF</th>
<th>Epitope</th>
<th>Sequence</th>
<th>Nucleotide positions*</th>
<th>Structure†</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7</td>
<td>1</td>
<td>NDSSEEDEID</td>
<td>685 to 720</td>
<td>Helix/β-turn</td>
</tr>
<tr>
<td>E7</td>
<td>2</td>
<td>AGQAEPDRAHY</td>
<td>645 to 675</td>
<td>Coil/β-turn</td>
</tr>
<tr>
<td>E6</td>
<td>1</td>
<td>MFQDPQERPRKLPQL</td>
<td>104 to 148</td>
<td>Coil/β-turn</td>
</tr>
<tr>
<td>E6</td>
<td>2</td>
<td>CKQQLRREV</td>
<td>200 to 229</td>
<td>Helix/β-turn</td>
</tr>
<tr>
<td>E6</td>
<td>3</td>
<td>PEEKQRHLKDKQRFHNIR</td>
<td>437 to 490</td>
<td>Coil/β-turn</td>
</tr>
<tr>
<td>E6</td>
<td>4</td>
<td>SCCRSTRTRETLQ</td>
<td>515 to 556</td>
<td>β-turn</td>
</tr>
<tr>
<td>E4</td>
<td>1</td>
<td>WAPKKHRRLSSLQD</td>
<td>3441 to 3482</td>
<td>Coil/β-turn</td>
</tr>
<tr>
<td>L1</td>
<td>1</td>
<td>PIKKPNNNKI</td>
<td>5788 to 5817</td>
<td>β-turn</td>
</tr>
<tr>
<td>L1</td>
<td>2</td>
<td>YNPDTORL</td>
<td>5908 to 5931</td>
<td>Coil/β-turn</td>
</tr>
<tr>
<td>L1</td>
<td>3</td>
<td>FYLRREQM</td>
<td>6379 to 6402</td>
<td>β-turn</td>
</tr>
<tr>
<td>L1</td>
<td>4</td>
<td>CQKHTPPAPKEDDDPKYY</td>
<td>6916 to 6969</td>
<td>Coil/β-turn</td>
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<tr>
<td>L1</td>
<td>5</td>
<td>GKKKATPTT</td>
<td>7084 to 7110</td>
<td>β-turn</td>
</tr>
<tr>
<td>L1</td>
<td>6</td>
<td>TSTAKKKKRL</td>
<td>7117 to 7152</td>
<td>Helix/coil</td>
</tr>
</tbody>
</table>

* First base pair of first coding triplet to last base pair of last coding triplet.
† Calculated by the method of Garnier et al. (1987).

Methods. As shown in Fig. 4, a major cluster of antibody-binding peptides was obtained with the rabbit antiserum. It includes the peptide KPSPWAPKKHRRLS of the HPV-16 E4 ORF. This sequence is partially included in the reactive region identified by immunoscreening (Table 1). Testing of the peptides with an independently produced rabbit antiserum prepared against a CII-HPV-16 E4 fusion protein resulted in a very similar pattern compared to that obtained with the anti-MS2 E4 antiserum (data not shown). Incubation with rabbit control sera or with Protein A–peroxidase alone gave no significant signals.

The significance of signals obtained with single peptides (e.g. peptides 5/6; see Fig. 4) is not clear since, in contrast to the peptide KPSPWAPKKHRRLS, a synthetic oligopeptide derived from this region fails to react with the antiserum when bound to microtitre plates and tested by ELISA (data not shown).

Prediction of immunogenic sites on HPV-16 proteins

Together with the experimental systems described above, a theoretical approach was used for the identification of antibody-binding regions. The \(<H>\) and \(<\mu H>\) were calculated for the HPV-16 E7, E4, E6 and L1 ORFs according to the procedure of Eisenberg et al. (1982, 1984). Protein fragments with low \(<H>\) and high \(<\mu H>\) may have ‘receptor-like’ properties (de Loof et al., 1986). The plot for the HPV-16 E4 protein is shown in Fig. 5. All antibody-binding domains identified by this procedure are listed in Table 2.

By comparison with the data described above it was shown that two of six regions in L1, one of the two regions in E7, one of four regions in E6 and the epitope in E4 coincide with the seroreactive areas identified by the experimental approaches (see Table 2). For all but one of the experimentally identified epitopes, a coil/β-turn secondary structure was predicted (Garnier et al., 1987). Thus, four of the seven regions with a ‘receptor-like’, coil/β-turn structure were identified as seroreactive epitopes.

Use of synthetic peptides in an ELISA

As described in previous sections, immunogenic regions of HPV-16 proteins were identified by the use of specific antisera prepared in rabbits or mice. In order to test whether these regions react with human sera too, the synthetic oligopeptides KPSPWAPKKHRRLS and DEIDGPAGQAEPDRAHY, corresponding to the regions E4-815 and E7-107 (see Table 1), were used to screen by ELISA a total of 36 sera obtained from immunosuppressed patients for the presence of antibodies. Of the sera, 42.8% and 30.5% were shown to react with the E4- and E7-specific peptide, respectively. Similar numbers have been obtained when the sera were tested in Western blot experiments using fusion proteins expressed in E. coli (Jochmus-Kudielka et al., 1989; I. Jochmus-Kudielka & L. Gissmann, unpublished data).

Discussion

In order to identify seroreactive epitopes within proteins E4, E6, E7 and L1 of HPV-16, the experimental approach described by Smith (1985) was applied. Short fragments of the viral genome were randomly cloned into gene III of the ssDNA bacteriophage fd. Recombinants
were screened by immunostaining with the appropriate antisera prepared against bacterial fusion proteins (Seedorf et al., 1987; Jochmus-Kudielka & Gissmann, 1990) and the epitopes were determined by sequencing of the inserted DNA. Owing to some background reactions probably depending upon the quality of the antiserum used, only a proportion of the initially identified recombinants could be verified in subsequent tests after isolation and re-plating. In the cases of repeatedly positive recombinants, however, this method proved to be highly specific because the antisera reacted only with bacteriophages expressing peptides from those ORFs against which they were originally prepared and, in most instances, the respective sequences were identified repeatedly (see Table 1). Except for the E4 ORF and regions 809 and 830 of the L1 ORF, independent overlapping clones were found, further underlining the reliability of the expression library method. Additional support was provided by the fact that identical results were obtained with different antisera prepared in individual rabbits (in the case of E4) or even with sera derived from different species (rabbit and mouse, in the case of E7-221). Epitope 221 of the E7 protein (Table 1) has also been found by Tindle et al. (1990), and Cason et al. (1989) described an epitope within the L1-830 region. Moreover, synthetic peptides derived from the E7 epitope 107 and from the E4 epitope were found to react with a number of human sera and experiments by Dillner et al. (1990) demonstrated the presence of human antibodies directed against L1 peptides overlapping with antigenic regions L1-809 (three amino acids overlap), L1-830 (12 amino acids overlap) and L1-842 (complete overlap; see Table 1) (Dillner et al., 1990).

In the case of the E4 ORF, only one type of positive recombinant bacteriophage could be found. The epitope was confirmed, however, by an independent approach. Overlapping octapeptides spanning the entire ORF were synthesized and used to test an anti-E4 antiserum by ELISA. Of these peptides four, covering a total of 14 amino acids (positions 17 to 20), were shown to bind the immunoglobulins of the rabbit sera (see Fig. 4). Peptide 17 is contained completely within the fd-derived epitope (see Table 1) but overlaps with peptide 20 by only two amino acids. Therefore it is not clear whether there are actually two adjacent epitopes within the E4 protein, the second of which was not detected within the pool of recombinant fd phages tested so far. This has no practical consequence as this study was initiated mainly to identify seroreactive epitopes for testing human sera and we would include as many epitopes as possible in the test kit. In fact in our first experiments the peptide KPSPWAPKKHRRLS, when bound to a microtitre plate, was shown to detect human antibodies by ELISA in 43% of the sera. On the other hand, a synthetic oligopeptide derived from the region at peptide positions 5/6 (Fig. 4) failed to react although it had been identified as positive after coupling to the polyethylene pins. This divergent behaviour may be explained by the different presentation of the peptide to the antibodies in both assays.

The methods used in our investigation permit the identification only of sequential but not conformational epitopes. Therefore it is an open question whether these domains within the native proteins are actually exposed to the immune system. In the case of the E7 epitopes this appears to be rather likely because they completely overlap with or are immediately adjacent to the putative E7-binding sites of the retinoblastoma protein (Dyson et al., 1989) and (as mentioned above) the respective oligopeptide was shown to react with human sera. For the other epitopes such direct proof of their immunogenicity in vivo is still missing but the prediction that all but one of the experimentally identified regions are 'receptor-like' structures (Table 2) is supportive of this notion.

As mentioned before, the purpose of this work was to identify epitopes to be used as synthetic peptide antigens for the screening of human sera for the presence of anti-HPV-16 antibodies. Probably due to low antibody titres and the relatively small number of epitope-containing phage proteins (approximately $5 \times 10^9$ molecules per plaque), immunoscreening of the fd libraries with human sera has not so far resulted in the identification of seroreactive recombinants, but synthetic peptides derived from the regions E4 and E7-107 can be used to detect antibodies by ELISA in human sera which have been tested in parallel by Western blotting using bacterial fusion proteins (Jochmus-Kudielka et al., 1989). Owing to the low number of sera tested so far the significance of the data is not clear but it did become apparent that by Western blot experiments identical results were obtained in about two-thirds of the sera (Müller et al., 1990). Similar experiments using synthetic peptides for the other regions are in progress. An important question in this context is whether all the epitopes within a given protein have been identified. The binding pattern obtained with the individual octapeptides of the E4 protein (see Fig. 4) suggests that the major seroreactive region is represented by the peptide KPSPWAPKKHRRLS. In the case of E7, preincubation of the animal sera with bacteriophage particles obtained from clones E7-221 and E7-107 prior to reaction with the respective fusion protein in Western blot experiments resulted in an almost complete loss of the signal (data not shown; see also Fig. 3), indicating that no major epitope has actually been missed. Similar experiments using human sera are presently in progress in our laboratory.
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