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Development of Type-Specific and Cross-Reactive Serological Probes for the Minor Capsid Protein of Human Papillomavirus Type 33

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Human papillomavirus type 33 (HPV33) is associated with malignant tumors of the cervix. In an attempt to develop immunological probes for HPV33 infections, antisera against various bacterial fusion proteins carrying sequences of the minor capsid protein encoded by L2 were raised in animals. Antigenic determinants on the HPV33 L2 protein were identified by using truncated fusion proteins and were classified as type specific or cross-reactive with respect to HPV1, -8, -11, -16, and -18. Cross-reactive epitopes map to amino acids 98 to 107 or to amino acids 102 to 112 and 107 to 117, respectively, depending on the fusion protein used for immunization. Antibodies directed toward these epitopes detect L2 proteins of HPV11, -16, and -18, but not of HPV1 and -8, in Western immunoblots and enzyme-linked immunosorbent assays. HPV33 L2 amino acids 82 to 94 and 117 to 130 induce type-specific antibodies, with the major response directed to amino acids 117 to 130. By using a synthetic peptide corresponding to L2 amino acids 117 to 130, high-titered, type-specific antisera were obtained. These antisera should be useful as immunological probes for HPV33 infection.

Papillomaviruses (PVs) comprise a heterogeneous group of double-stranded DNA viruses infecting the skin and mucosa. More than 60 types of human PV (HPV) have been identified (for a review, see reference 10). They infect epithelial cells in a highly tissue specific manner and are thought to induce abnormal growth of the affected cells (26, 42, 49, 61). On the basis of their tissue specificity and the associated pathobiology, they can be classified into different groups. The HPVs infecting cells in the urogenital tract are subdivided into viruses that are mostly associated with benign lesions such as common genital warts (condyloma acuminata [HPV6, -11, and -42) and those associated with cervical intraepithelial neoplasias, which can progress to invasive cervical carcinoma (HPV16, -18, -31, -33, -35, -39, and -52) (10).

The confirmation of HPV infection currently relies either on the detection of viral DNA by using hybridization techniques (2, 11, 32, 35, 57) and more recently polymerase chain reaction (PCR) technology (52, 62) or on the use of a commercially available polyclonal antiserum directed against disrupted bovine PV particles (Dako, Copenhagen, Denmark). The bovine PV antiserum is used to detect viral antigen in tissues (24, 30, 38, 59). These antibodies are broadly cross-reactive and recognize most animal and human PVs (6, 14, 19, 22–24, 50).

To enable clinicians to distinguish between the HPVs associated with benign and malignant lesions, reagents that are type or group specific and easy to use in routine screening must be developed.

Great emphasis has been placed on the development of serological tests to detect HPV infection. To this end, regions of proteins encoded by the more common genital HPV types, which give rise to a humoral immune response in humans, have been mapped by use of either overlapping peptides (12, 13, 33) or bacterially expressed fusion proteins (18–20, 28, 60). Thus far, the best-characterized proteins are the major and minor capsid proteins L1 and L2, respectively, and the early protein E7. It was shown that the most commonly observed antibodies against HPV16 and HPV18 proteins are directed against L2 and that they are mostly type specific (20). Even though many but not all studies find an increased prevalence for anti-HPV antibodies in patients with low- and high-grade lesions, a significant percentage of healthy individuals are seropositive for HPV proteins (21, 27). The best correlation between the grade of the lesion and seroconversion seems to exist for antibodies directed against the transforming proteins E6 and E7 (25). Recently, however, it was pointed out that the antibody prevalence to these proteins increases only after the development of malignant lesions (36).

A different approach is to screen for viral antigens in tissue of possibly infected individuals. For this purpose, well-defined antibodies of polyclonal or monoclonal origin are needed. Whereas the late proteins L1 and L2 are easily detectable in cervical intraepithelial neoplasias (7, 15, 16, 34, 54), early proteins with the exception of E4 are expressed at levels that are difficult to detect. Thus, antibodies directed against the capsid proteins seem to be best suited to detect HPV infection in early stages. Since no viral particles can be isolated in large enough quantities in most cases (40, 43, 51), recombinant proteins have been used for the immunization of animals (15, 41, 53, 54). Several groups have shown that the L2 protein expressed as a fusion protein in bacteria induces a type-restricted immune response in animals (29, 53). In contrast, L1 is highly conserved among PV types and gives rise to cross-reactive antisera (24, 34, 54). We have thus initiated a study to identify type- and group-specific antigenic determinants on the L2 protein by using sera from animals immunized with bacterially expressed protein. For

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this work, we have chosen HPV33, which was found in 10% of cervical carcinomas (1). Different parts of the L2 protein were expressed in *Escherichia coli* in the context of different fusion moieties and used for immunizing guinea pigs or rabbits. Here we report our results on the identification of cross-reactive and type-specific antigenic regions of the L2 protein recognized by the animal sera.

MATERIALS AND METHODS

Construction of expression plasmids. We used pRIT2T and pGEX3X (Pharmacia LKB Biotechnology) for the synthesis of HPV DNA-encoded polypeptides as fusion proteins in *E. coli*. pRIT2T carries the coding sequence of the staphylococcal protein A under the control of the lambda p_R promoter followed by a polylinker (39). pGEX3X contains the inducible *tac* promoter followed by the glutathione *S*-transferase (GST) gene and a polylinker sequence at its carboxy terminus (47). All manipulations with DNA were carried out according to Maniatis et al. (37).

To construct pRI33F1, a *Pvu*II-*Pst*I fragment (nucleotides [nt] 4451 to 4614) of the L2 open reading frame of HPV33 (5) was fused in frame to the protein A gene by cloning it into *Sma*I-*Pst*I-cut pRIT2T. A *Pvu*II-*Hin*dIII fragment of HPV33 (nt 4451 to 4983) was ligated to *Eco*RI-cut pGEX3X DNA after Klenow polymerase treatment of both DNAs, generating plasmid pG33L2. Corresponding fragments of the L2 open reading frames of HPV1 (nt 4018 to 4529), HPV8 (nt 4442 to 5111), HPV11 (nt 4558 to 5043), HPV16 (nt 4469 to 4906), and HPV18 (nt 4484 to 5003) were cloned in frame to the GST gene of pGEX3X to generate plasmids pG1L2, pG8L2, pG11L2, pG16L2, and pG18L2, respectively. The L2 sequences of the various HPV types present in the fusion proteins correspond to amino acids 42 to 211 (HPV1), 55 to 278 (HPV8), 48 to 209 (HPV11), 79 to 226 (HPV16), and 81 to 254 (HPV18). pBR322-based bacterial plasmids with the complete DNA sequences of HPV1 (8), HPV8 (17), HPV11 (9), and HPV16 (46) were kindly provided by E. M. de Villiers (Deutsches Krebsforschungszentrum, Heidelberg, Germany). pHPV18 (4) was a gift from G. Orth (Institut Pasteur, Paris, France). For the fusion of part of L2 to *lacZ*, a *Pvu*II-*Hin*dIII fragment corresponding to nt 4451 to 4983 of the HPV33 genome was inserted into pSKS105 (3) cleaved by *Sma*I and *Hin*dIII, yielding plasmid pHPL2.33βG.

Restriction enzymes, the large fragment of DNA polymerase I, T4 DNA ligase, and T4 DNA polymerase were purchased from Boehringer Mannheim.

Construction of deletion mutants. Deletion mutants of the HPV33 L2 fragment were obtained either by use of naturally occurring restriction sites or by introduction of additional sites via PCR with synthetic oligonucleotide primers (44).

The 5′ primer for obtaining carboxy-terminal deletions hybridized to pGEX3X vector DNA at its unique *Csp*45I site and had the sequence 5′-GCTGAAAATGTTCGAAGATC-3′. All 3′ primers were complementary to successive parts of the L2 fragment present in pG33F1b and contained a nonhybridizing tail carrying an *Eco*RI restriction sequence. Deletions from the amino terminus were introduced by PCRs using pG33F1 DNA as the template, a series of 5′ primers, and a 3′ primer hybridizing to pGEX3X DNA at its unique *Aat*II site (5′-GCCACCTGACGTCTAAGAAACCA-3′). Each of the 5′ primers possessed a *Bam*HI restriction sequence at its 5′ end which did not hybridize with the L2 sequence. The oligonucleotides were synthesized on a Milligen/Biosearch Cyclone Plus DNA synthesizer.

PCRs were usually run at an annealing temperature of 56 to 65°C with *Thermus aquaticus* DNA polymerase (AmpliTaq; Perkin-Elmer/Cetus) in a Perkin-Elmer Thermocycler. PCR products were double-digested with *Csp*45I-*Eco*RI or *Bam*HI-*Aat*II, respectively, purified by agarose gel electrophoresis, isolated from the gel, and ligated with pGEX3X which had been cleaved correspondingly and purified from agarose gels. All recombinant expression plasmids were sequenced to confirm the in-frame orientation of the insert (45).

Expression and purification of fusion proteins. The protein A/L2 fusion gene was expressed in *E. coli* N4830-1 carrying the temperature-sensitive lambda *c*I857 repressor. Fusion proteins were purified by column chromatography on immunoglobulin G-Sepharose 6 FF (Pharmacia LKB Biotechnology) (39).

The L2/β-galactosidase (β-Gal) fusion protein was purified from an overnight culture of *E. coli* MC1060 carrying plasmid pHPL2.33βG according to the affinity chromatography procedure described by Ullmann (56).

Expression plasmids derived from the GST fusion vector pGEX3X were transformed into *E. coli* MC1061. Fusion proteins were purified from the supernatants of bacterial lysates by affinity chromatography on glutathione-Sepharose 4B (Pharmacia LKB Biotechnology) (47). For further purification, the GST fusion proteins (approximately 50 to 100 μg) were subjected to preparative sodium dodecyl sulfate—12.5% polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized as potassium dodecyl sulfate-protein precipitates and cut out of the gel. The protein was eluted by diffusion.

Immunization of animals. Polyclonal antisera of three guinea pigs were raised against a purified L2/β-Gal fusion protein containing amino acids 82 to 259 of the HPV33 L2 protein; two guinea pigs and two rabbits were immunized with the A/L2 (amino acids 82 to 134) fusion protein. The animals were subcutaneously immunized with approximately 200 μg of affinity-purified fusion protein in complete Freund's adjuvant (Sigma) followed by two injections of the same dose of antigen in incomplete Freund's adjuvant at intervals of 3 to 4 weeks. Sera were collected 10 days after the third injection. Monospecific antisera directed toward the epitope encompassing amino acids 117 to 130 were obtained by immunization of two rabbits with the peptide YGAPAPSIPTPSGFD, conjugated to ovalbumin via bisdiazobenzidine as described previously (57). Sera of the same animals collected before immunization were used as controls.

Western immunoblotting. Purified fusion proteins or whole bacterial lysates were subjected to SDS-PAGE (31) and blotted onto nitrocellulose membranes (Hybond C; Amersham) as described before (55). Guinea pig antisera were diluted 1/500, and rabbit antisera were diluted 1/200 to 1/400 in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA); the antisera were preincubated with untransformed *E. coli* lysates for 1 h at room temperature. To selectively abolish the immunoreactivity of rabbit antiserum against distinct epitopes, undiluted serum was incubated with an equal volume of an unpurified preparation of the corresponding fusion protein overnight at 4°C and for 1 h at room temperature.

Enzyme-linked immunosorbent assay (ELISA). L2 fusion proteins of different HPV types which had been purified by affinity chromatography and gel electrophoresis were diluted to about 2 μg/ml in 50 mM sodium carbonate (pH 9.5) and were bound to microtiter plates (Falcon Microtest plates). All washing steps were carried out with PBS (pH 7.5)

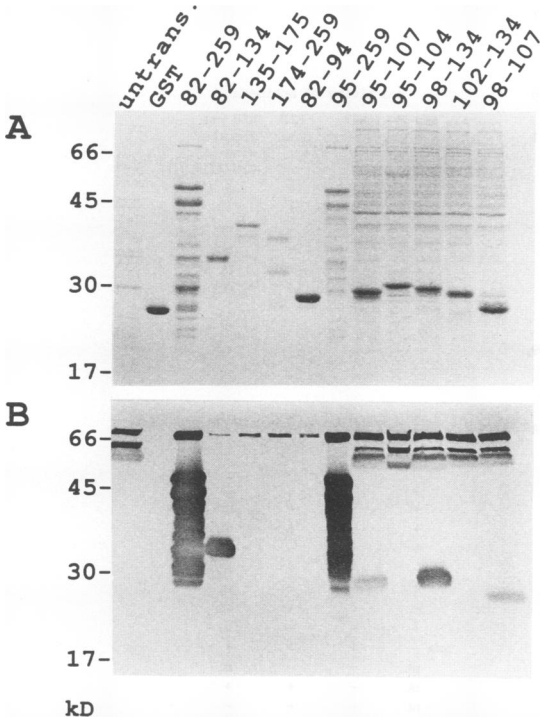

FIG. 1. Mapping of the immunoreactive region of HPV33 L2 protein with guinea pig anti-L2/β-Gal antiserum ME1. (A) Coomassie blue-stained SDS-polyacrylamide gel of GST/L2 fusion proteins. Numbers above the lanes indicate the amino acid boundaries of the L2 fragment expressed in the respective fusion protein (see Fig. 3 for names of the clones). untrans., lysate of untransformed *E. coli* MC1061 cells. (B) Western blot of the same proteins reacted with antiserum ME1 (diluted 1:400).

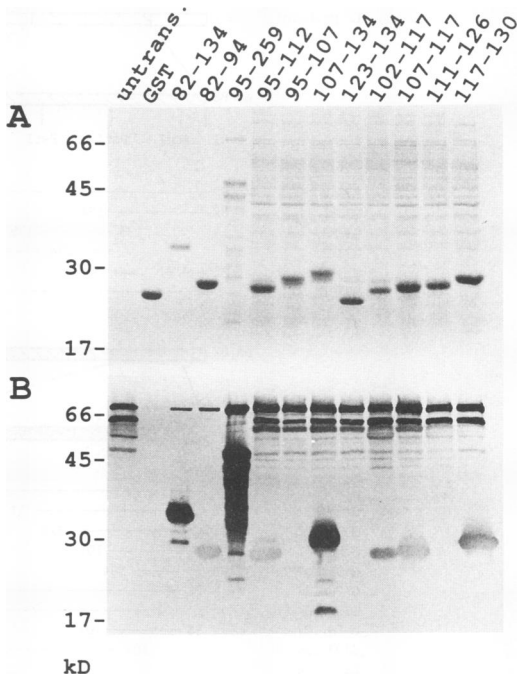

FIG. 2. Mapping of immunoreactive regions of HPV33 L2 protein with rabbit anti-protein A/L2 antiserum K18. (A) Coomassie blue-stained SDS-polyacrylamide gel of GST/L2 fusion proteins. Numbers above the lanes indicate the L2 fragments expressed in the respective fusion proteins (for names of the clones, see Fig. 3). untrans., lysate of untransformed *E. coli* MC1061 cells. (B) Western blot of the same proteins reacted with antiserum K18 (diluted 1:400).

containing 0.1% Tween 20. After blocking by 2% BSA in PBS-Tween 20, the plates were incubated with various antiserum dilutions for 90 min at 37°C. Immunoreactions were visualized by use of horseradish peroxidase-conjugated protein A (dilution of 1:300; Bio-Rad) and 1,2-phenyldiamine dihydrochloride (Sigma) as the substrate. BSA and GST were used as negative controls.

RESULTS

Immune response in guinea pigs to a L2/β-Gal fusion protein. To identify antigenic determinants on the minor capsid protein of HPV33, three guinea pigs were immunized with the L2/β-Gal fusion protein carrying L2 amino acids 82 to 259 at the amino terminus. The immune sera obtained were tested in Western blots with truncated GST/L2 fusion proteins synthesized in *E. coli*. Either total lysates of induced bacterial cells or purified fusion proteins were used.

None of the sera tested contained antibodies directed against GST alone (Fig. 1). However, the animal sera reacted with two bacterial proteins of slightly more and less than 66 kDa, respectively, present in lysates of both untransformed and transformed *E. coli* cells (Fig. 1B and 2B). The larger of these two *E. coli* proteins also appeared in Western blots of affinity-purified preparations of the GST/L2 fusion proteins, suggesting a copurification (Fig. 1B and 2B; see also Fig. 4).

All three guinea pig antisera reacted with fusion protein G33L2, which contained L2 amino acids 82 to 259 and was used for immunization. This fusion protein was partially degraded in *E. coli* (Fig. 1). When the L2 region was subcloned, reactivity was exclusively displayed by L2 amino acids 82 to 134 (fusion protein G33F1), whereas no reaction was observed with clones expressing amino acids 135 to 175 (pG33F2) and 174 to 259 (pG33F3) (Fig. 1B and 3). In addition, protein G33F1a carrying L2 sequence 82 to 94 was not recognized by the immune sera.

To more precisely locate the L2 epitope(s), a detailed deletion mutagenesis of the region from 95 to 134 was performed by using PCR amplification of overlapping fragments, which were subsequently expressed as GST fusion proteins. The corresponding Western blot is shown in Fig. 1, and the sizes, names, and reactivities of all deletion mutants constructed are summarized in Fig. 3.

Step-by-step deletion starting from the carboxy terminus of the sequence from amino acids 95 to 134 did not abolish reactivity with any of the guinea pig antisera unless the fragment was shortened beyond amino acid 107. A fusion protein containing amino acids 95 to 104 (G95/104) did not react. Using deletions starting from the amino terminus, only three residues could be deleted without loss of reactivity (G98/134). The removal of four additional amino acids (G102/134) destroyed the antibody binding site. The common epitope reactive with all three guinea pig antisera (ME1 to ME3) thus maps to the segment between amino acids 98 and 107 (GPLDSSIVSL). This is the only epitope on the L2 fragment used for immunization, since preincubation of the antisera (ME1 to ME3) with the G98/107 fusion protein completely abolished the reactivity of all antisera to the whole fragment (G33L2; not shown).

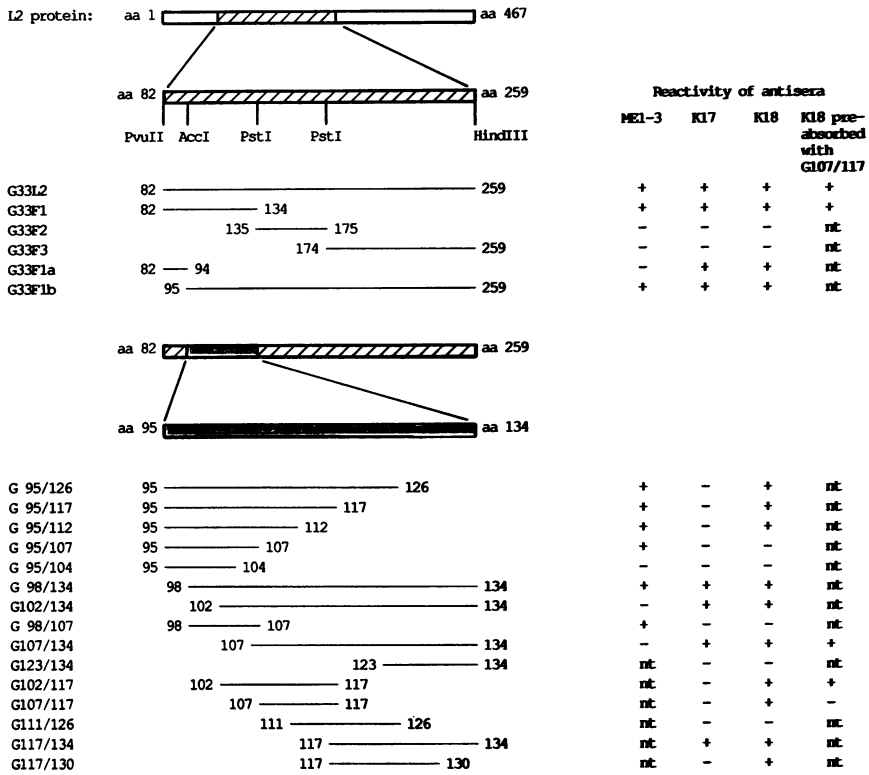

FIG. 3. Epitope mapping of the HPV33 L2 protein with guinea pig antisera (ME1 to ME3) and rabbit antisera (K17 and K18). Positions and sizes of L2 deletion mutants of a GST/L2 fusion protein (G33L2) are indicated by horizontal bars. Names of the various proteins are listed on the left. Reactivity of the antisera with the deletion mutants in Western blotting is indicated by + (positive) or − (negative). aa, amino acid; nt, not tested.

Immune response in rabbits and guinea pigs to a protein A/L2 fusion protein. To examine the immunogenicity of the same segment of the HPV33 minor capsid protein in the context of another fusion protein and in another animal species, two rabbits and two guinea pigs were immunized with a protein A/L2 fusion protein carrying L2 amino acids 82 to 134 at the carboxy terminus. Both rabbit antisera obtained strongly reacted with the GST fusion protein G33F1 carrying the same L2 sequence (Fig. 2 and 3). However, a more complex pattern of antibody binding sites on L2 was found for the rabbit antisera (K17 and K18) than for the guinea pig antisera ME1 to ME3 previously characterized. The rabbit antisera also differed from each other in reactivity. Both antisera reacted with a determinant located between amino acids 82 and 94 (AIPLQPIRPPVTV). When the L2 fragment 95 to 134 was deleted from the carboxy terminus, reactivity of the K18 antiserum was still observed with fusion protein G95/112 (Fig. 2 and 3), although to a lesser extent than with deletion mutants G95/117 and G102/117, which retained the L2 sequence up to amino acid 117. This result indicated that an antigenic site extending to amino acid 117 was destroyed in deletion mutant G95/112. We therefore analyzed the antiserum with fusion protein G107/117 (amino acids 107 to 117) and found a strong signal in Western blots (Fig. 2). When the K18 antiserum was preabsorbed with G107/117, eliminating all reactivity with the epitope 107 to 117, fusion protein G102/117 was still reactive (Fig. 3), albeit more weakly than with untreated antiserum. We conclude that G102/117 contains two independent but overlapping epitopes extending from amino acids 102 to 112 (SSIVSLIEETS) and 107 to 117 (LIEETS FIEAG), respectively.

Using fusion protein G102/117 to preabsorb the K18 antiserum, we identified an additional antigenic determinant; preabsorption did not abolish reactivity to fusion protein G107/135. However, fusion proteins G123/134 and G111/126 were unreactive. This result indicated that an epitope might be centered around amino acid residues 123 to 125. This observation was confirmed by the strong positive reaction of the G117/130 deletion mutant carrying the L2 sequence GAPAPSIPTPSGFD. Compared with reactivities of other fusion proteins carrying a single epitope, the reactivity of G117/130 was particularly strong (Fig. 2). Although not obvious from the photographic reproduction, this result was clearly seen and repeatedly found with the original blotting membranes. This finding prompted us to call the epitope a major antigenic site.

Thus, one major and three minor antigenic sites reactive with the rabbit antiserum K18 have been located on the L2 protein: amino acids 82 to 94, 102 to 112, 107 to 117, and 117 to 130; i.e., the epitopes identified are spread over nearly the entire L2 fragment used for immunization.

Apart from the antigenic determinant localized between amino acids 82 and 94, which was common to both rabbit antisera, the reactivity of antiserum K17 was confined to one additional epitope located within L2 amino acids 117 to 135 (Fig. 3). Since the K18-reactive fusion protein G117/130 was not detected by antiserum K17, the binding site of the corresponding antibodies present in K17 should include

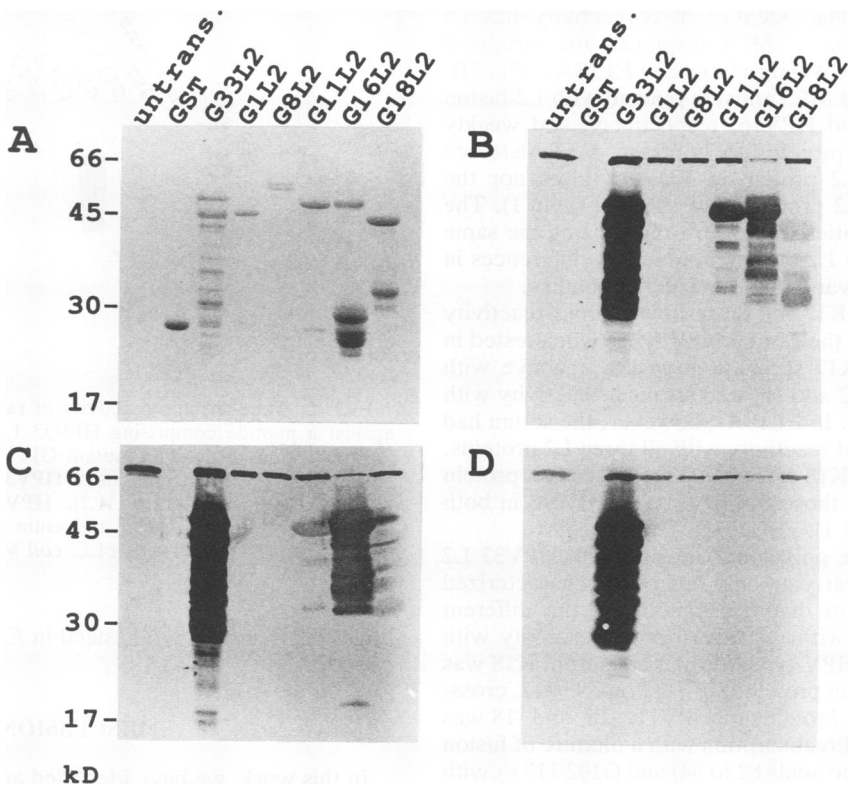

FIG. 4. Cross-reactivity of polyclonal antisera with GST/L2 fusion proteins of different HPV types. (A) Coomassie blue-stained SDS-polyacrylamide gel of fusion proteins of HPV33 (G33L2), HPV1 (G1L2), HPV8 (G8L2), HPV11 (G11L2), HPV16 (G16L2), and HPV18 (G18L2). (B to D) Western blots of L2 fusion proteins reacted with guinea pig antiserum ME1 (B), rabbit antiserum K18 (C), and rabbit antiserum K18 after preabsorption with protein G102/117 (D).

amino acid residues located carboxy terminal of the major antigenic site of K18.

From two guinea pigs (ME4 and ME5) immunized with the same protein A/L2 fusion protein used for the immunization of the rabbits, we collected antisera which were reactive with the epitope common to K17 and K18 (amino acids 82 to 94) and with further antigenic sites, yielding a pattern of antibody specificities similar to those of rabbit sera K17 and K18 (data not shown). However, no reactivity was found toward the only epitope recognized by guinea pig sera ME1 to ME3 (amino acids 98 to 107). Since the immune responses of the guinea pigs to the L2/β-Gal fusion were virtually the same and the immune reactions of the guinea pigs and the rabbits to different batches of the protein A/L2 fusion protein were also similar but different from the reaction to L2/β-Gal, we feel that the different fusion protein constructs used rather than differences between the animal species were responsible for the different immune responses observed.

The antisera contain cross-reactive and type-specific antibodies. To examine the type specificity of the antisera obtained, fragments of the L2 open reading frames of various HPV types were expressed as fusion proteins with GST. The following HPV types were selected for comparison: HPV1 and HPV8 as representatives of cutaneous and epidermodysplasia verruciformis-associated types, respectively; HPV11, which induces benign genital warts; and HPV16 and HPV18, which are associated with various malignancies of the genital tract and are most closely related to HPV33 (10). The reactivities of the different L2 fusion proteins were tested in Western blots and ELISAs. For ELISAs, the affinity-purified L2 fusion proteins were further purified by SDS-PAGE and subsequent elution from the gel, to avoid an unspecific background signal due to the cross-reactivity with contaminating *E. coli* proteins (Fig. 1, 2, and 4). The corresponding Western blots are shown in Fig. 4; the results of the ELISAs are shown in Table 1.

None of the animal sera assayed stained the L2 fusion proteins of HPV1 and HPV8 (Fig. 4). However, the guinea pig antisera ME1 to ME3 reacted with L2 fusion proteins of

TABLE 1. ELISA reactivities of guinea pig antisera and rabbit antisera against GST/L2 fusion proteins of HPV33, -1, -8, -11, -16, and -18[a]

Virus	Optical density at 492 nm								
	Guinea pig antisera			Rabbit antisera					
	ME1	ME2	ME3	K17	K18	K18[b]	K18[c]	K28	K29
HPV33	1.66	1.80	1.74	1.58	1.82	1.62	1.72	1.95	1.80
HPV1	0.0	0.02	0.0	0.02	0.01	0.0	0.0	0.0	0.0
HPV8	0.01	0.05	0.03	0.06	0.05	0.0	0.01	0.0	0.0
HPV11	0.87	0.31	0.61	0.17	0.28	0.0	0.0	0.0	0.0
HPV16	0.50	0.05	0.27	0.11	0.48	0.0	0.0	0.0	0.0
HPV18	0.20	0.03	0.12	0.12	0.24	0.0	0.0	0.0	0.0

[a] Antisera were used at following dilutions (end-point titers are indicated in parentheses): ME1, 1:400 (1:6,400); ME2, 1:100 (1:3,200); ME3, 1:800 (1:12,800); K17, 1:100 (1:1,600); K18, 1:200 (1:6,400); K28, 1:800 (1:51,200); K29, 1:800 (1:25,600).

[b] Preabsorbed with proteins G33F1a and G102/117.

[c] Preabsorbed with protein G33F1b.

HPV11, -16, and -18. The extent of cross-reactivity differed among the three antisera. ME1 displayed the strongest cross-reactivity in both Western blots and ELISAs (Fig. 4B; Table 1). The ME1 and ME3 antisera reacted with L2 fusion proteins of HPV11 and HPV16, respectively, and weakly with the HPV18 L2 protein. In contrast, ME2 detected neither the HPV18 L2 protein in Western blots nor the HPV16 and HPV18 L2 proteins in ELISAs (Table 1). The different cross-reactivities of the sera recognizing the same epitope on the HPV33 L2 protein may reflect differences in the fine tuning of the various L2-specific antibodies.

The rabbit antisera (K17 and K18) showed cross-reactivity with the L2 protein of the genital HPV types when tested in Western blots. The K17 serum was weakly reactive with HPV11 and HPV16 L2 and showed stronger reactivity with the HPV18 L2 protein. In ELISAs, however, the serum had a comparable degree of reactivity with all three L2 proteins. The rabbit antiserum K18 recognized the L2 fusion protein of HPV16 better than those of HPV11 and HPV18 in both assays (Fig. 4C; Table 1).

Preabsorption of the polyclonal antisera with HPV33 L2 fusion proteins each carrying only one of the characterized epitopes allowed us to distinguish between the different antibody specificities with respect to cross-reactivity with L2 proteins of other HPV types. When antiserum K18 was preabsorbed with fusion protein G107/117 or G95/112, cross-reactivity with the L2 proteins of HPV11, -16, and -18 was reduced (not shown). Preabsorption with a mixture of fusion proteins G33F1a (amino acids 82 to 94) and G102/117 or with G33F1b (amino acids 95 to 259) alone completely abolished any cross-reactivity of antiserum K18 in Western blots and ELISAs. The same result was obtained when G102/117 was used (Fig. 4D; Table 1). The preabsorption rendered the polyclonal antiserum monospecific for the mapped epitopes 82 to 94 or 117 to 130 and thereby type specific for HPV33.

Conversely, preabsorption of antiserum K18 with fusion proteins G33F1a and G117/130 carrying only type-specific epitopes completely eliminated the reactivity toward these proteins but left the cross-reactivity unchanged (data not shown).

In conclusion, the antigenic determinants carried by amino acids 82 to 94 and 117 to 130 of L2 are HPV33-specific epitopes for the rabbit antiserum K18, whereas antibodies binding to the overlapping epitopes present in amino acids 102 to 112 or 107 to 117 are responsible for the type-restricted cross-reactivity of the antiserum. The type-specific character of epitope 82 to 94 can be confirmed by preabsorption of antiserum K17 and of guinea pig antisera ME4 and ME5 with fusion protein G33F1b (amino acids 95 to 259) (not shown).

Immunization with a synthetic peptide yields type-specific antisera. In an attempt to obtain a type-specific polyclonal antiserum to the HPV33 L2 protein, a peptide comprising the L2 amino acid sequence 117 to 130 was synthesized, coupled to ovalbumin, and used for immunization of two rabbits. The antisera obtained (K28 and K29) were assayed for cross-reactivity with L2 proteins of several HPV types in Western blots. As shown in Fig. 5 for antiserum K28, fusion proteins including the peptide sequence used for immunization or the entire L2 sequence analyzed (G33L2) were strongly positive, whereas no reactivity could be observed with L2 from HPV1, -8, -11, -16, and -18. The same results were obtained with antiserum K29 (not shown). This result shows that the L2 amino acid sequence from 117 to 130 induces a type-specific immune response in animals both in the context of a larger L2 fragment synthesized in *E. coli* and as a synthetic peptide.

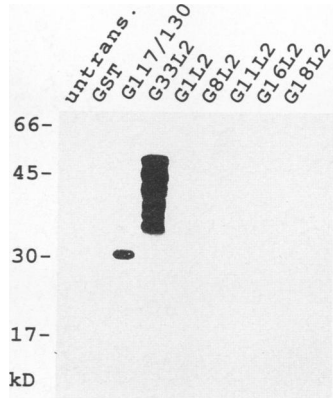

FIG. 5. Type-specific reactivity of rabbit antiserum K28 raised against a peptide comprising HPV33 L2 amino acids 117 to 130 (corresponding to deletion mutant G117/130). Shown is a Western blot of GST/L2 fusion proteins of HPV33 (G33L2), HPV1 (G1L2), HPV8 (G8L2), HPV11 (G11L2), HPV16 (G16L2), and HPV18 (G18L2) reacted with rabbit antiserum K28 (diluted 1:1,000). untrans., untransformed lysate of *E. coli* MC1061 cells.

DISCUSSION

In this work, we have identified and mapped type-specific and cross-reactive antigenic determinants on the minor capsid protein encoded by L2 of HPV33 by using bacterially expressed fusion proteins. The humoral immune response found in guinea pigs was directed toward a single epitope located between amino acids 98 and 107 when amino acids 82 to 259 of the L2 protein were expressed at the amino terminus of β-Gal. In tests with L2 proteins from other HPV types, cross-reactivity was observed with HPV11 (varying between 17 and 52%), HPV18 (2 to 12%), and HPV16 (3 to 30%). No cross-reactivity was seen with the L2 proteins from HPV1 and HPV8. A comparison of the amino acid sequences in the region from 98 to 107 reveals very little similarity of HPV33 to HPV1 and HPV8. HPV1 and HPV8 carry an 11-amino-acid insertion in this region (Fig. 6). The most striking difference between HPV11, -16, and -18 compared with HPV33 is the substitution of a proline for a serine residue in HPV33 at position 102. It is surprising that HPV11, showing the highest degree of cross-reactivity, is less similar to HPV33 than to HPV16. A comparison of carboxy-terminally adjacent amino acids shows a conserved isoleucine residue in HPV11 which is substituted for valine in HPV16. This finding indicates that the isoleucine at position 108 might influence the binding of the antibody to the epitope, whereas residue 98 is probably not recognized by the antibodies.

A more complex picture was seen when a smaller L2 fragment expressed at the carboxy terminus of protein A was used for immunization of either rabbits or guinea pigs. In this context, almost the entire L2 sequence was antigenic. With use of one of the rabbit sera for mapping, two overlapping epitopes in the conserved region between amino acids 102 and 117 were revealed. Antibodies directed against these epitopes cross-react with HPV11, -16, and -18 but not with HPV1 and -8. Cross-reactivity of the K18 antiserum was highest with the HPV16 L2 fusion protein (26%) compared with HPV11 L2 (15%) and HPV18 L2 (13%). This result is in

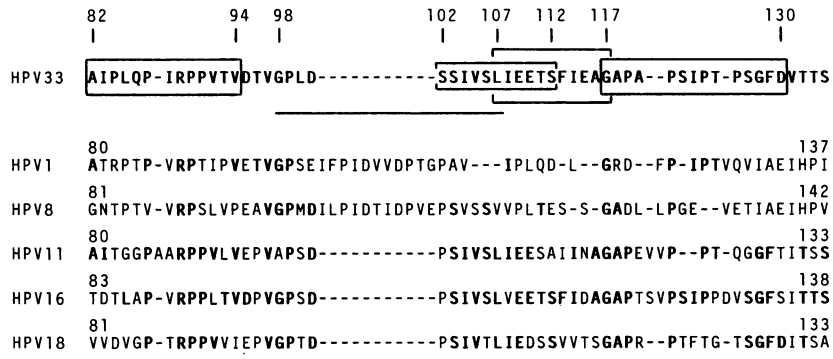

FIG. 6. Alignment of the HPV33 L2 amino acid sequence containing the antigenic sites mapped with the homologous regions of L2 proteins of different HPV types. Gaps in the alignment are indicated by hyphens. The epitope (amino acids 98 to 107) reactive to guinea pig antisera ME1 to ME3 is underlined; type-specific epitopes detected by rabbit antiserum K18 are boxed; cross-reactive epitopes detected by antiserum K18 are indicated by brackets. The figures in the HPV33 sequence refer to the amino acid boundaries of the epitopes. Amino acids identical to the HPV33 sequence are in boldface.

agreement with the various degrees of sequence homology in this region (Fig. 6).

Two regions, amino acids 82 to 94 and 117 to 130, induce a type-specific immune response, with the main response directed to amino acids 117 to 130. Type specificity refers to the lack of cross-reactivity with the L2 proteins of HPV1, -8, -11, -16, and -18. A comparison of the amino acid sequences in these regions is in agreement with this result. Epitope 82 to 94 is conserved at the carboxy-terminal six amino acids, but the amino-terminal seven amino acids show no homology among the HPV types studied. In addition, the HPV types tested either show hardly any sequence homology in epitope 117 to 130 (HPV11 and HPV18) or have amino acid insertions in this region (HPV16).

The use of a conjugated synthetic peptide corresponding to L2 amino acids 117 to 130 as an immunogen yielded high-titered, HPV33-specific antisera. This result confirmed our findings obtained with larger, bacterially expressed fusion proteins; epitope 117 to 130 is a major antigenic determinant and induces a type-specific immune response in rabbits. These antisera should prove useful for probing tissue for productive HPV33 infection.

The L2 amino acid sequence 1 to 80 has not been analyzed in this work since this region is highly conserved among HPVs and is therefore unlikely to carry type-specific antigenic determinants. Fusion proteins carrying the L2 sequences downstream of amino acid 259 tend to aggregate. Work on the epitopes in this L2 segment is now in progress.

The methods used in our experiments allow the identification of linear epitopes only. Recent work of several groups has pointed out that humans often develop anti-HPV antibodies directed against conformational epitopes (48). However, these antibodies are readily destroyed when tissue is prepared for immunohistochemistry or ELISA. Therefore, antibodies recognizing linear epitopes are more useful for diagnostic purposes.

Linear epitopes on the minor capsid protein recognized by human antibodies have been mapped for several HPV types. In analogy to our findings, cross-reactive epitopes were identified between L2 amino acids 106 to 128 and 103 to 127 of HPV6 and HPV11, respectively, whereas a type-specific epitope was located between amino acids 110 to 139 on the HPV18 L2 protein (20, 60). The corresponding region of the HPV16 L2 protein does not seem to induce an immune response in humans. We do not yet know whether the HPV33 L2 epitopes mapped for animal sera induce an immune response in humans. We are currently testing sera from cervical carcinoma patients for reactivity with these epitopes.

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