Cancer of the cervix is the second most common cancer among women worldwide (Clifford et al., 2003). Infection with oncogenic human papillomavirus (HPV), most frequently HPV16, is the most significant risk factor in the aetiology of cervical cancer, as shown by the extremely high frequency (99.7%) of HPV DNA detected in cervical malignancies (Walboomers et al., 1999). Both in vitro and in vivo studies show that the functions of the E6 and E7 proteins of the ‘high-risk’ HPVs are essential for cellular transformation (Bedell et al., 1987; Herber et al., 1996; Matlashewski et al., 1987; Song et al., 1999; Vousden et al., 1988; Watanabe et al., 1989). The viral oncoproteins E6 and E7 from ‘high-risk’ HPVs have been shown to alter pathways involved in cell-cycle control, interacting with and neutralizing two important tumour suppressors, p53 and Rb (Vousden et al., 1988). The loss of activity of these two tumour suppressors results in an uncontrolled continuous cellular proliferation leading to cell transformation. HPV16 E6 (16E6) is a small protein composed of 151 or 158 aa, depending on the initiation codon used. 16E6 contains two zinc-binding domains and a C-terminal PDZ-binding domain (Mantovani & Banks, 2001). The help of the cellular ubiquitin-ligase E6AP, 16E6 mediates the degradation of p53 (Scheffner et al., 1990). So far, the regions of E6 involved in binding to either p53 or E6AP have mostly been investigated by performing point mutations or deletions of E6 (Cooper et al., 2003; Foster et al., 1994; Gewin & Galloway, 2001; Li & Coffino, 1996; Liu et al., 1999; Pim & Banks, 1999). As the three-dimensional structure of 16E6 is unknown, such mutations can unpredictably affect the folding of 16E6. In this light, we chose a different approach to address these questions by testing the effects of 16E6-specific monoclonal antibodies (mAbs) on the binding of 16E6 to p53 or E6AP. Our group previously reported three mAbs, 1F1, 6F4 and 4C6, that recognize epitopes in the N-terminal region of E6, located between the residues Q3 and R8, that are able to block the degradation of p53 in vitro (Giovane et al., 1999; Masson et al., 2003; Schwalbach et al., 2000). In the current work, we generated antibodies against the second zinc-binding domain of 16E6 (16ZD2) and used them in conjunction with the previously described 6F4 antibody to test their inhibitory potential on the interaction of 16E6 with p53 and E6AP, as well as on the degradation of p53.

The 16ZD2 domain (see Fig. 1b for sequence) was expressed fused to maltose-binding protein (MBP) in E. coli. mAbs were obtained by injecting the MBP–16ZD2 fusion protein, purified as previously described (Ristriani et al., 2001), into BALB/c mice. Three different 16ZD2-specific mAbs (1F5, 3B8 and 3F8) were isolated after ELISA screening with purified 16ZD2 protein. The HPV type specificities of these antibodies were investigated by Western blot analysis. For this purpose, 1 μg purified fusion protein consisting of the ZD2 domain from HPV types 5, 6, 8, 11, 16, 18 and 33 fused...
to MBP was analysed by SDS-PAGE followed by Western blotting. mAb 3F8 bound to both 16ZD2 and 33ZD2, whereas 1F5 and 3B8 were strictly specific to 16ZD2 (Fig. 1a). To characterize these new E6 antibodies further, synthetic overlapping peptides covering the 16ZD2 sequence were used in ELISA tests as competitors for

**Fig. 1.** Characterization of anti-16ZD2 antibodies. (a) HPV type specificities. One microgram of ZD2 domain from HPV types 5, 6, 8, 11, 16, 18 and 33 fused to MBP was analysed by SDS-PAGE followed by Western blotting. The resulting blots are shown with the indicated antibodies, revealed using phosphatase-labelled anti-mouse Igs. (b) Epitope mapping of anti-ZD2 mAbs. Overlapping peptides were tested for competition for binding of the anti-ZD2 antibodies to MBP–ZD2 in an ELISA. The peptide sequence of the protein used to immunize the mice is represented on top, with arrows pointing to the cysteine residues involved in zinc binding. Numbering is according to the 151 aa HPV16 E6 sequence. The various synthetic peptides tested are represented as horizontal bars. The results are summarized on the right; _, no competition; +, 50% inhibition of binding to MBP–ZD2; ++, 100% inhibition of binding to MBP–ZD2. (c) Further definition of the epitopes of mAbs 3B8, 1F5 and 3F8 using phage peptide display. For each antibody and following the third round of selection, 20 clones were sequenced and the amino acid sequence of the presented peptides deduced. Only distinct sequences obtained are represented. Sequences of the different peptides bound by the antibodies were aligned with the matching sequence of 16E6 (and sequence of E6 from HPV33 in the case of 3F8) and derived consensus sequences are indicated. The 16ZD2 sequence is shown at the bottom with its secondary structure: S, β-Strand; H, α-helix; L, loop.
16ZD2 binding. For this analysis, MBP–16ZD2 protein, coated at 200 nM, was detected with the three anti-16ZD2 mAbs at 30 nM in the presence or absence of the competing peptides at 10 μM. The results of this experiment (Fig. 1b) revealed that the epitopes of 3B8, 1F5 and 3F8 were distinct and located within a stretch of 33 aa. Thereafter, to define more precisely the epitopes of the anti-16ZD2 mAbs, we used phage peptide display technology and a dodecapeptide library (PhD12; New England Biolabs) as previously described (Choulier et al., 2002). After three rounds of panning, sequences of the selected clones were determined and the deduced amino acid sequences aligned with that of 16E6 (Fig. 1c). Sequences recognized by 3F8, which binds both 16E6 and 33E6 (Fig. 1a), were also aligned with the sequence of 33E6. The epitopes of the three 16ZD2-specific mAbs were located within a 16-residue stretch (Q116 to R131) overlapping the second α-helix and the second β-strand of the 16ZD2 secondary structure recently assigned by nuclear magnetic resonance (Y. Nominé and others, unpublished results).

To investigate whether these new mAbs could recognize 16E6 in a cellular context, the coding sequences of 16E6 and 16ZD2 were cloned into pEGFP-C3 (Clontech). HeLa cells were transfected with pEGFP-E6 or pEGFP-ZD2. Two days later, cells were fixed and analysed for EGFP fluorescence and immunofluorescence as previously described (Sibler et al., 2003) with the anti-16E6 antibodies at a concentration of 60 nM. The immunofluorescence signal associated with mAb 6F4 was strong, specific and displayed a similar pattern to that produced by EGFP–16E6 fluorescence (Fig. 2a). The three anti-16ZD2 mAbs also allowed the detection of 16E6 or 16ZD2 in transfected cells by immunofluorescence. The reactivity and specificity of the three 16ZD2-specific antibodies were also validated by immunoprecipitation. The 158 aa 16E6 coding sequence was inserted into pTL1, a derivative of the vector pSG5 (Green et al., 1988). Immunoprecipitation of 16E6 was carried out 48 h after transfection of pTL1–E6 into COS-1 and HeLa cells. This experiment was performed as previously described (Deryckere & Burgert, 1996) with the anti-16E6 antibodies (100 pmol) coupled to protein A–Sepharose following [35S]methionine and [35S]cysteine labelling (Promix; Amersham Biosciences) and NP-40 lysis. The four anti-16E6 mAbs immunoprecipitated two protein species, most likely corresponding to the 151 and 158 aa forms of E6 from transfected COS-1 and transfected HeLa cells (Fig. 2b and c, filled arrows). Immunoprecipitation from HPV16-transformed CaSkI cells using mAbs 6F4, 3B8 and 3F8 also allowed the detection of the CaSkI endogenous 16E6 protein (Fig. 2c). As none of the ZD2-specific antibodies could bind to HPV18 E6 (Fig. 1a), which is expressed in HeLa cells, we expected to immunoprecipitate E6 only from transfected cells or CaSkI cells. However, mAb 1F5 immunoprecipitated a non-specific protein from non-transfected HeLa, transfected HeLa and CaSkI cells in the same molecular mass range as E6 (Fig. 2c, asterisk). With the exception of 1F5, the E6-specific mAbs immunoprecipitated two protein species from CaSkI cells (Fig. 2c, open arrows) migrating slightly more slowly than the E6 proteins immunoprecipitated from transfected HeLa cells (Fig. 2c, filled arrows). The difference in mobility between the endogenous CaSkI E6 and the transfected HeLa E6 is likely to be due to a difference in post-translational modifications, which remain to be investigated.

After validating the E6-binding activity of these new antibodies in cells, we investigated their inhibitory potential on the binding of E6 to p53 and E6AP, and on the degradation of p53. Two different E6-binding sites on p53 have been reported (Li & Coffino, 1996). One, located in the p53 core domain, depends on E6AP and is required for p53 degradation. This interaction can be demonstrated by pull-down of p53 by 16E6 fused to the glutathione S-transferase protein (GST–E6). We first examined the effects of the anti-16E6 mAbs on the binding of 16E6 to the E6AP-dependent site of p53. This experiment was performed with the anti-p53 antibody pAb 1801 (Banks et al., 1986) as previously described (Crook et al., 1991) with the following modifications: 10 μL 35S-labelled 16E6 was pre-incubated for 1 h at 4 °C with 200 pmol anti-E6 mAb in 200 μL LSAB (100 mM NaCl, 100 mM Tris/HCl pH 8·0, 1% NP-40) containing a mix of protease inhibitors (Complete; Roche) before the addition of 10 μL 35S-labelled p53 and 30 μL rabbit reticulocyte lysate (containing endogenous E6AP) and a further incubation of 1 h at 4 °C. Proteins complexed with p53 were then immunoprecipitated using 10 μl agarose-coupled pAb 1801 (Santa Cruz Biotechnology). The co-immunoprecipitation of p53–E6 was efficiently demonstrated (Fig. 3a, lane 5). The three anti-16ZD2 mAbs did not affect 16E6–p53 binding (Fig. 3a, lanes 7–9), whereas pre-incubation of 16E6 with the 6F4 antibody completely blocked the p53–E6 interaction (Fig. 3a, lane 6, disappearance of the E6 band). Next, we investigated the effect of the 16E6-specific antibodies on the E6AP-independent binding of p53 to GST–E6 (Fig. 3b). This experiment was performed as previously described (Li & Coffino, 1996) except for the pre-incubation or not of glutathione–Sepharose 4B beads (Amersham Biosciences) bearing the GST–E6 fusion protein with the anti-16E6 mAbs (200 μmol) for 1 h at 4 °C with shaking before the addition of 35S-labelled p53. In contrast to the preceding experiment, the three anti-16ZD2 mAbs inhibited binding between 16E6 and p53, whereas the 6F4 antibody did not affect the interaction. The three anti-16ZD2 antibodies also inhibited the pull down of in vitro-translated 16E6 by the p53 C terminus (aa 360–383) fused to GST (Fig. 3c), thus confirming that anti-16ZD2 mAbs selectively inhibit the E6AP-independent binding of 16E6 to p53. We thus showed that the two sets of antibodies targeting either the N terminus or the 16ZD2 domain of 16E6 have strictly opposite effects on the two distinct 16E6–p53 interactions.
Fig. 2. Immunodetection of 16E6 in transfected HeLa and COS-1 cells. (a) Binding properties of the three different anti-16ZD2 mAbs tested in immunofluorescence experiments on HeLa cells transfected with either pEGFP-E6 or pEGFP-ZD2. The green fluorescence of EGFP is visualized in the upper panel and the same fields were analysed for antibody binding as shown by the red fluorescence in the middle panel. The green and red fluorescence are merged in the lower panel. (b) Immunoprecipitation of E6 with 6F4, 3B8, 1F5 or 3F8 from COS-1 cells transfected (COS-1 T) or not (COS-1 NT) with pTL1-E6. The E6 protein precipitated from transfected COS-1 cells migrates as two bands indicated by arrows. (c) Immunoprecipitation of E6 with 6F4, 3B8, 1F5 or 3F8 carried out from HeLa cells transfected (HeLa T) or not (HeLa NT) with pTL1-E6 and from CaSki cells. The E6 protein precipitated from transfected HeLa cells migrates as two bands indicated by filled arrows and the E6 protein precipitated from CaSki cells migrates as two bands indicated by open arrows. mAb 1F5 precipitates a non-specific band (*) from CaSki cells and HeLa cells, transfected or not.
Fig. 3. Neutralizing effects of anti-16E6 mAbs on the interaction between 16E6 and p53 or E6AP. (a) Co-immunoprecipitation of 16E6 with p53. 16E6 and p53 proteins, produced by in vitro transcription and translation, were incubated together with protease inhibitors and the indicated mAbs. Thereafter, the p53–E6 complex was immunoprecipitated with the anti-p53 antibody pAb 1801 covalently bound to Sepharose and analysed by SDS-PAGE and autoradiography. Control lanes with p53 and 16E6 input were run in parallel. The control lane 3 indicates the absence of cross reactivity between pAb 1801-agarose and 16E6. (b) Effect of 16E6 mAbs on p53 binding to GST–E6. The different antibodies were used to compete with binding of p53 to GST–E6 as indicated. The analysis was performed by GST pull down, SDS-PAGE and autoradiography. A control lane with 10% input of p53 was run in parallel (lane 1). The percentage of p53 binding to GST–E6 relative to the control without antibody (lane 3) was quantified on a Bio-Imager FX (Bio-Rad). (c) Effect of anti-16E6 mAbs on 16E6 binding to the C-terminal part of p53. The in vitro-translated 16E6 protein was incubated with GST protein fused to the C terminus of p53 (GGSRAGSSHKLKKGGQSTSRHKK) (GST–p53C-ter). The different antibodies were used to compete for binding of 16E6 to GST–p53C-ter as indicated. The analysis was performed by GST pull down, SDS-PAGE and autoradiography. A control lane with 10% input of 16E6 was run in parallel (lane 1). The percentage of E6 binding to GST–p53C-ter relative to the control without antibody (lane 2) was quantified on a Bio-Imager FX (Bio-Rad). (d) Effect of 16E6-specific mAbs on 16E6 binding to E6AP. The in vitro-translated 16E6 protein was incubated with GST protein fused to E6AP. The different antibodies were used to compete for binding of 16E6 to E6AP as indicated. The analysis was performed by GST pull down, SDS-PAGE and autoradiography. A control lane with 10% input of 16E6 was run in parallel (lane 1). The percentage of E6 binding to GST–E6AP relative to the control without antibody (lane 2) was quantified on a Bio-Imager FX (Bio-Rad). (e) In vitro degradation of p53 by 16E6. 16E6 and p53 proteins, produced by in vitro transcription and translation, were incubated together for 3 h with or without pre-incubation of 16E6 with the indicated mAbs. Control reactions without 16E6 (lane 1) or without antibody (lane 2) were run in parallel and the analysis was performed by SDS-PAGE followed by autoradiography.
We investigated the blocking potential of the anti-16E6 mAbs on this interaction by pull down of radiolabelled 16E6 with the GST–E6AP fusion protein (Huibregtse et al., 1993). Ten microlitres of 35S-labelled 16E6 were pre-incubated or not with 100 pmol anti-16E6 mAbs for 1 h at 4 °C before the addition of glutathione–Sepharose 4B beads bearing the GST–E6AP protein. Pre-incubation of 16E6 with the mAb 3F8 strongly decreased the level of precipitated 16E6 (Fig. 3d). mAb 1F5 also reduced the binding of 16E6 to GST–E6AP but to a lesser extent, whereas 6F4 and 3B8 provoked no inhibition of binding. We subsequently investigated the potential of the 16ZD2-specific mAbs to interfere with p53 degradation (Fig. 3e). This was performed as previously described (Giovane et al., 1999). The three 16ZD2-specific mAbs did not affect the degradation of p53; however, pre-incubation of 16E6 with the 6F4 antibody completely neutralized the degradation of p53, as shown previously (Giovane et al., 1999).

We have reported here the generation and characterization of three mAbs directed to the second zinc-binding domain of the HPV16 E6 protein. These new reagents can detect E6 in transfected cells by either immunoprecipitation or immunofluorescence. These antibodies, together with the previously described 6F4 antibody (Giovane et al., 1999), were used as potential inhibitors of E6AP binding, p53 binding and p53 degradation. This work is the first functional analysis of 16E6 binding to p53 and E6AP using anti-16E6 antibodies. We clearly demonstrated the importance of a small 16 aa stretch within the 16ZD2 region for E6AP-independent binding to the C terminus of p53. Our results also highlight the importance of the N terminus of 16E6 in both p53 degradation and E6AP-dependent binding to the core domain of p53, as previously indicated by mutational analysis (Cooper et al., 2003; Crook et al., 1991; Foster et al., 1994; Liu et al., 1999). The interference of two 16ZD2-specific mAbs with E6AP binding was somewhat unexpected, as a short stretch within the N-terminal region of 18E6 situated far from the ZD2 epitopes is sufficient for binding to E6AP (Pim & Banks, 1999) and N-terminal deletions of 16E6 abrogate this binding (Cooper et al., 2003). Also it was surprising that the inhibition of 16E6 binding to E6AP by 16ZD2-specific antibodies hardly affected the degradation of p53. However, in agreement with our results, the mutation I128T of 16E6, directed at a central residue of the 3F8 epitope, also abolishes binding of 16E6 to E6AP without fully inhibiting p53 degradation (Liu et al., 1999). From these apparently contradictory data (Cooper et al., 2003; Liu et al., 1999; Pim & Banks, 1999), we hypothesize that alteration of the region around I128, by either mutation or antibody binding, can provoke a conformational change in the E6AP-binding site in the N-terminal region of 16E6 that modifies the E6–E6AP interaction. The reason why perturbations of E6AP binding mediated by that region with mutation or the use of mAbs is not sufficient to block the proteasome-mediated degradation of p53 might be due to either the involvement of other ubiquitin ligases in the degradation of p53, as is the case for other E6 targets (Grm & Banks, 2004; Pim et al., 2000), or a reduced stability of the dimeric complex E6–E6AP compared with that of the trimeric complex E6–E6AP–p53.

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References


