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Antibody response to a viral transcriptional regulator

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Abstract The E2 transcriptional activator of the human papillomavirus regulates the expression of most viral transcripts. Its binding to specific target DNA sequences involves large conformational changes in the interacting macromolecules. The high stability of the E2:DNA complex prompted us to analyze the role of macromolecular interactions and adjuvant emulsions in the appearance of conformation-specific antibodies. We demonstrate that immunization with free or DNA-complexed E2 emulsified in an oil-in-water adjuvant elicits a humoral response shifted to the recognition of discontinuous epitopes. Epitope mapping and functional analysis of the generated anti-E2 mAbs reveals that two separate antibodies populations can be obtained: those able to form a stable ternary complex with protein and DNA, and those which recognize the DNA-binding surface of the transcription factor, interfering with E2 binding to DNA.

Key words: Antibody; Conformational epitope; Antigen binding; Transcription factor; Adjuvant

1. Introduction

The E2 transcriptional activator of the human papillomavirus (HPV) regulates the expression of most viral transcripts, and plays an auxiliary role in viral DNA replication [1]. Infection with the high-risk strains (HPV-16, 18, 31 and 33) is strongly associated with various cancers of the ano-genital tract, particularly cervical carcinomas. The E2 C-terminal DNA-binding and dimerization domain (E2C) binds to particular recognition sequences on the viral genome. It was demonstrated that binding of recombinant HPV-16 E2C to its cognate double-stranded 18 bp oligonucleotide (site 35) induces a conformational change in the protein, including a large stabilizing effect [2]. Besides, spectrofluorometric studies show that this interaction is extremely stable, with a dissociation constant of about 0.2 nM [3].

Macromolecular interactions are crucial for stabilizing or changing the conformation of the interacting partners. These changes are detected by the immune system, which develops specific antibodies against the newly exposed conformational epitopes. For example, the interaction of the HIV envelope protein gp120 with the extracellular domain of CD4 and the CCR5 chemokine receptor provokes a marked conformational change on the HIV gp120 and gp41 proteins, resulting in fusion of the virus and T cell membranes [4]. As a consequence, B cells secreting antibodies specific to these new conformational epitopes start to proliferate.

Given the high stability of the E2C:site 35 complex [2], and the described conformational changes that occur upon complex formation, this complex constitutes an excellent model to study the immunogenic effect of a protein:DNA complex on the generation of conformation-specific antibodies against the interacting macromolecules. In a previous work [5] we have shown that immunization with this protein:DNA complex allowed us to obtain sequence-specific, high-affinity anti-oligonucleotide antibodies. In this work we describe the effect of adjuvant composition and complex formation on the induced anti-E2C response. Besides, we show that the generated anti-E2C monoclonal antibodies (mAbs) recognize two main different surfaces on the transcription factor.

2. Materials and methods

2.1. E2C and DNA

The C-terminal 80-amino acid DNA-binding domain of the E2 protein from HPV-16 was expressed and purified as a soluble folded dimeric protein as previously described [3]. The sequence of the A chain of the synthetic oligonucleotide (IDT, USA) is 5′-GTAACCGAAATCGGTTGA-3′, corresponding to the E2 site 35 in the HPV-16 genome. Fluoresceinated site 35 (FITC–site 35) contains a fluorescein molecule attached to the 5′ end via a 6-carbon linker of the B chain. Biotinylated site 35 (Bio-site 35) contains a biotin molecule attached to the 5′ end of the A chain. Single stranded oligonucleotide concentration determination and annealing were carried out as described [3].

2.2. Immunization protocols

For E2C immunizations, each mouse received intraperitoneally (IP) 16 μg of the protein emulsified in the oil-in-water MPL®+TDM Adjuvant System (MPL) [6] (E2C/MPL) or in the water-in-oil Complete Freund Adjuvant (E2C/FA) [7]. Second and third boosters were administered with similar doses at intervals of 20-days. Incomplete Freund Adjuvant was used in second and third boosters of the FA protocol. Mice immunized with the E2C:site 35 complex received IP 25 μg of the 1:1 mixture emulsified in MPL (E2C:site 35/MPL).

2.3. Production and purification of antibodies and Fabs

Splenocytes obtained from the immunized mice with the highest titers were fused with a NSO mouse plasmacytoma cell line following established techniques [8]. Hybridoma cultures producing anti-DNA- and anti-E2C-specific immunoglobulins were detected by enzyme-linked immunosorbent assay (ELISA) and cloned to ensure monoclonality. mAb IgGs were prepared following standard procedures [9].

2.4. Heat denaturation of E2C

Purified E2C protein in 50 mM sodium acetate, pH 5.6, was incu-

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Abbreviations: E2C, E2 C-terminal DNA-binding and dimerization domain; mAb, monoclonal antibody; Site 35, double-stranded 18 bp oligonucleotide; IP, intraperitoneal administration; TBS, Tris-buffered saline; BSA, bovine serum albumin

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bated for 1 h at 85°C, and then centrifuged for 15 min at 13 000 rpm. Protein concentration of the sample before and after the treatment remained unchanged. Fluorescence spectra of untreated and heat-denatured E2C were recorded in an Aminco Bowman series 2 luminescence spectrometer. Excitation wavelength was fixed at 280 nm, and the emission spectrum was recorded from 310 to 410 nm. The temperature was set at 25 ± 0.1°C.

2.5. ELISA assays

ELISA plates were sensitized with the different antigens for 1 h at room temperature in Tris-buffered saline (TBS), unless specified. The different coating antigens used were 0.5 μg/well E2C, 0.5 μg/well of thermally denatured E2C in 50 mM sodium acetate, pH 5.6, 0.5 μg/well of a 1:1 E2C:site 35 complex and 1 μg/well of E2C peptides. In the last case, the plate was incubated overnight at 37°C. Sera from mice were diluted in bovine serum albumin (BSA)/TBS, and their reactivity was developed by incubating with peroxidase-conjugated polyclonal antibodies to murine IgG Fc fragment (Accurate, USA).

2.6. Electrophoretic mobility shift assay

Samples containing 25 mM Bis-Tris–HCl, pH 7.0, 200 mM NaCl, 1 mM dithiothreitol (DTT), 8% glycerol, 2.5 μM E2C and the different anti-E2C IgG mAbs (4 μM maximal concentration of binding sites) were incubated for 2 h at room temperature. Next, 2.5 μM 5′ fluorescein–site 35 oligonucleotide was added and let to equilibrate for 30 min more in a final volume of 15 μl. Mixtures were loaded in a 6% polyacrylamide native gel containing 2.5% glycerol, 25 mM Tris–HCl, pH 8.3, 190 mM glycine, 1 mM DTT. The gel was resolved at 5 V/cm for approximately 2 h. Fluorescein bands were detected by UV transillumination and documentation (UVP transilluminator system, GDS8000).

2.7. Biosensor analysis of mAbs effect on protein–DNA interaction

The effect of the anti-protein mAbs on E2C binding to the site 35 oligonucleotide was analyzed in an Iasys plus Affinity Sensor Biosensor from ThermoLabsystems. Immobilization of streptavidin was carried out using the EDC/NHS Coupling Kit. Fifty μl of buffer (25 mM Bis-Tris–HCl, pH 7.0, 200 mM NaCl, 1 mM DTT, 1% BSA, 0.005% Tween 20) were placed in the cuvette and 2.2 μl of Bio-site 35 oligonucleotide (5 μg/ml final concentration) was let to equilibrate for approximately 20 min. The change in resonance position noted was 10 arcsec response units. Binding of 0.1 μM E2C (final concentration) with or without previous incubation for 2 h with increasing concentrations of different IgG anti-E2C mAbs was followed for 5 min. In any case, E2C binding to Bio-site 35 was considered as the difference in arcsec response between t_0 and $t_{5\,min}$. At the beginning of each experiment, E2C binding to the immobilized oligonucleotide was considered as the maximal response.

3. Results and discussion

Despite the importance of adjuvants for raising an immune response, limited information concerning the effects they cause on antigen conformation is available in the literature. We have previously shown that immunization with the E2C:site 35 protein:DNA complex induces a strong anti-DNA polyclonal response, prompting us to production of mAbs [5]. Since we had used the oil-in-water MPL+TDM adjuvant to emulsify the protein:DNA complex, we further analyzed whether the presence of the cognate DNA or alternatively the composition of the adjuvant had affected both the immunogenicity of the protein and the pattern of reactivity of the induced anti-E2C antibodies. To this purpose, a comparative study of the antibody response elicited by three different immunization protocols was carried out. The animals were immunized with the E2C:site 35 complex (E2C:site 35/MPL) or E2C alone (E2C/MPL) in MPL+TDM adjuvant, or with free E2C in the water-in-oil Freund Adjuvant (E2C/FA).

As can be seen in Fig. 1B, E2C is highly immunogenic. E2C emulsified in Freund Adjuvant renders high IgG anti-E2C titers (1/400 000), whereas E2C emulsified in MPL elicits titers similar to the E2C:DNA complex in the same adjuvant (1/150 000 average titers). E2 has been stated as the most immuno-reactive of all the HPV-16 proteins [10]. In accordance with that report, our results show that high anti-E2C titers were developed in the sera of all immunized mice, despite the immunization protocol applied.

The effect caused by two different adjuvant compositions on the pattern of reactivity of the elicited antibodies was tested by comparing sera titers against the native and denatured forms of E2C. Fig. 1A shows the tryptophan fluorescence spectra of the native and heat-denatured E2C. The decrease in intrinsic tryptophan fluorescence and the shift to the red of the center of mass indicate that the protein is irreversibly denatured by high temperature at acidic pH (see Section 2, [11]). As shown in Fig. 1B, MPL shifts the response towards the recognition of discontinuous epitopes on E2C, irrespective of the presence of the cognate oligonucleotide (compare E2C/MPL and E2C:site 35/MPL titration curves). In contrast, Freund Adjuvant causes at least a partial denaturation of the protein, as noticed by the high polyclonal sera reactivity against heat-denatured E2C (ELISA titers 1/10 000 and 1/400 for E2C/FA and E2C/MPL, respectively).

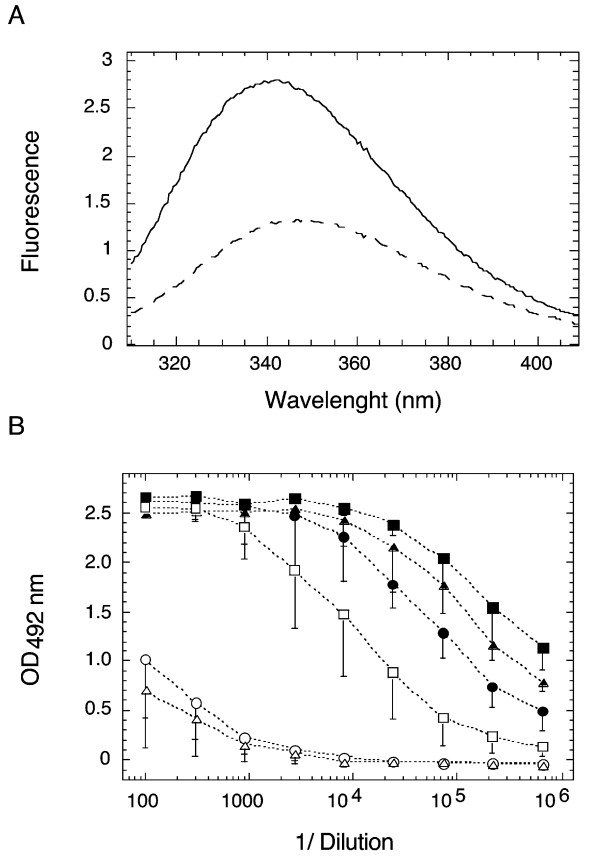


Fig. 1. A: Fluorescence tryptophan spectra of native (solid line) and heat-denatured (dashed line) E2C. B: Polyclonal IgG response towards native (closed symbols) or heat-denatured (open symbols) E2C. Sera titration curves of mice immunized with free E2C and E2C complexed with DNA in MPL+TDM adjuvant (E2C/MPL, circles; E2C:site 35/MPL, triangles, respectively), or with free E2C in Freund Adjuvant (E2C/FA, squares).

From a E2C:site 35/MPL-immunized mouse, we made a fusion experiment to obtain monoclonal anti-E2C and anti-DNA antibodies. All 92 hybridoma culture supernatants showed IgG anti-E2C:DNA and anti-E2C reactivity, whereas 15/92 cultures exhibited IgG anti-site 35 oligonucleotide reactivity as determined by indirect ELISA. From those cultures, we have cloned and studied six anti-E2C mAbs (named ED5, ED12, ED13, ED15, ED19 and ED23), all of them from the IgG_1, k isotype.

In coincidence with the analysis of the polyclonal response, most mAbs obtained from E2C:site 35/MPL-immunized mice also recognize discontinuous epitopes. As shown in Fig. 2A, five of the six anti-E2C mAbs (ED5, 12, 13, 15 and 19) exhibit a marked decrease in reactivity following thermal denaturation of the protein. Accordingly, they did not react with E2C-derived peptides covering the entire E2C sequence (see Fig. 2B). By contrast, ED23 was the only mAb that recognized both native and denatured E2C. Moreover, it strongly reacted against a 10 amino acids long peptide mapping to the C-terminus of E2C, comprising residues 349–358 of the polypeptide chain.

It was previously demonstrated that sera of animals immunized with lysozyme emulsified in Freund Adjuvant strongly reacted with both the native and denatured forms of the protein [13]. The author hypothesized that some denaturation of the protein may have occurred during emulsification, since antisera of animals immunized with the protein alone did not react with the denatured antigen. On the other hand, immunization with the CD4–gp120 complex in Syntex adjuvant (oil-in-water emulsion similar in composition to MPL) allowed the production of neutralizing anti-gp120 mAbs [14], most of which were completely unable to bind denatured gp120. The authors argued that this pattern of reactivity was the result of the CD4–gp120 complex interaction, which preserved the conformational structure of gp120. However, they did not discuss a potential influence of the nature of the adjuvant as another potential reason. Thus, based on our experimental results we postulate that the aqueous nature of the MPL+TDM adjuvant system is the key factor for preserving the native conformation of the viral transcriptional regulator during the immunization process.

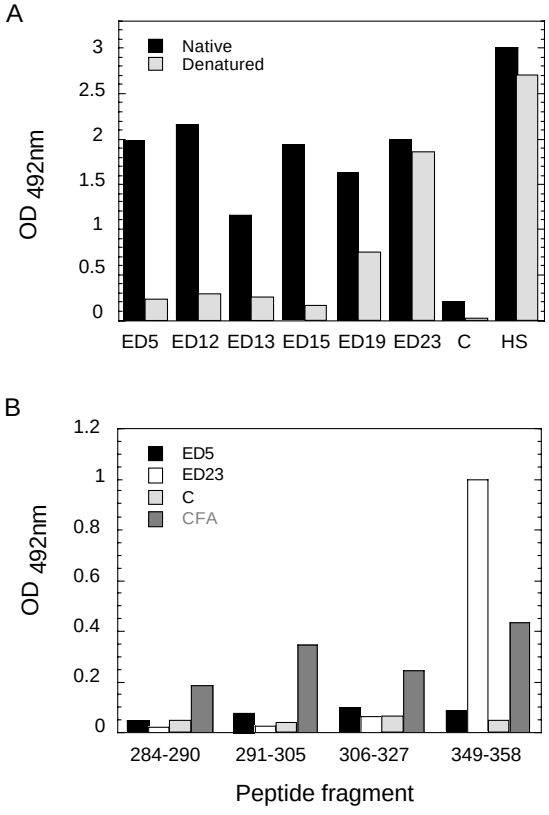
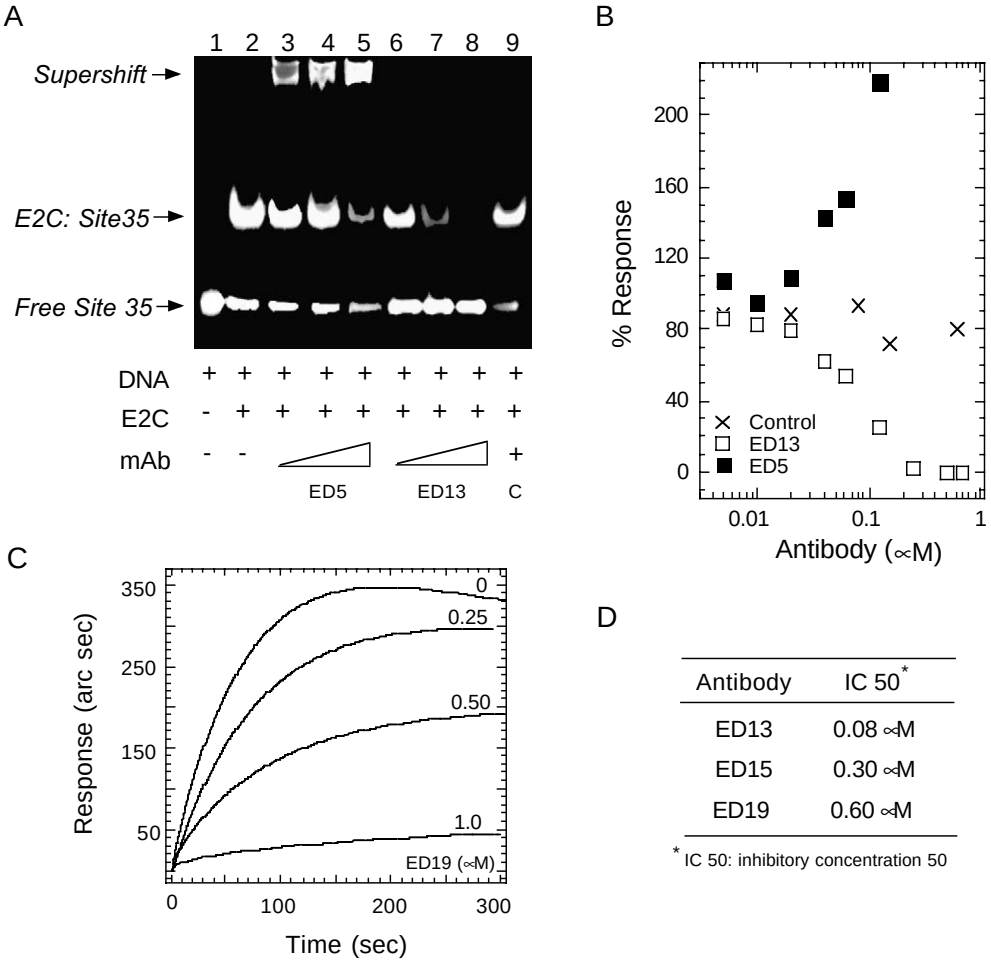


Fig. 2. A: ELISA reactivity of anti-E2C mAbs against the native and heat-denatured forms of E2C. Sera from unimmunized (C) and hyperimmunized (HS) mouse were used as controls. B: mAb reactivity against E2C peptides. Peptide amino acid assignment was performed according to the PDB database [12]. mAbs ED12, 13, 15 and 19 showed the same pattern of reactivity as ED5 (data not shown). FA, serum from an E2C/FA-immunized mouse. Ascitic fluids of the different mAbs and control sera were used at 1/100 dilution.

Since blocking of the interaction of DNA-binding proteins to their specific recognition sequences is a known antibody activity [15–16], we tested the ability of our conformation-sensitive anti-E2C mAbs to interfere with the transcription factor binding to its cognate oligonucleotide in an electrophoretic gel-shift binding assay. As shown in Fig. 3A, the fluorescein-labeled site 35 18 bp oligonucleotide band is shifted upon E2C binding (lane 2). Of the six anti-E2C mAbs assayed, mAbs ED5, ED12 and ED23 were able to produce a supershift of the E2C:FITC–site 35 complex band (lanes 3–5 for ED5 as an example), indicating that these mAbs bind an epitope non-related to the E2C DNA-recognition surface. In contrast, the other three anti-E2C mAbs interfere with the band shift produced by E2C (lanes 6–8 for ED13 as an example), producing an increase of the free oligonucleotide band.

To further confirm this result, we analyzed the effect of the mAbs on E2C binding to immobilized biotinylated site 35 in a biosensor cuvette. Addition of E2C to the immobilized oligonucleotide produces an increase in the signal indicating specific binding. Pre-incubation of E2C with mAb ED5 produces a concentration-dependent increase in the signal obtained for the protein:DNA complex, being two-fold higher at equimolar protein:antibody concentration (Fig. 3B). This increase clearly indicates the generation of a ternary complex, since in a biosensor the signal is proportional to the molecular weight of the interacting macromolecule. The same results were obtained with mAb ED23 (data not shown). On the contrary, pre-incubation of E2C with mAbs ED13, ED15 and ED19 leads to a concentration-dependent inhibition of binding observed as a decrease in the signal (Fig. 3B,C). Moreover, different inhibitory concentrations (IC_{50}) were found for each mAb, probably due to distinct affinity constants (see Fig. 3D). Thus, these antibodies are able to efficiently inhibit E2C binding to DNA, suggesting they recognize an epitope on or overlapped to the DNA-binding surface of the transcription factor.

As demonstrated above, mAb ED23 binds a continuous epitope comprising the residues 349–358. This epitope is located at the opposite side of the DNA-combining site of E2C and includes a turn and a β-strand (see red areas in Fig. 4A). Besides, this mAb produces a strong inhibition on HRP-labeled ED5 IgG binding to E2C in an ELISA assay (results not shown), suggesting that mAb ED5 recognizes an epitope overlapped to that of mAb ED23. In agreement with this, gel filtration experiments demonstrated that these mAbs recog-

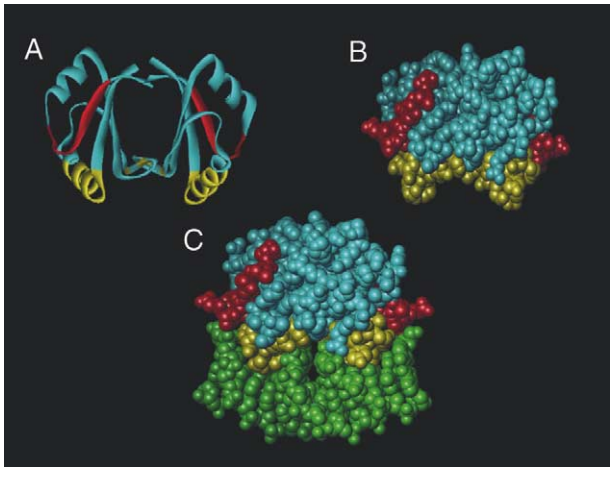


D

Antibody	IC 50*
ED13	0.08 ∝M
ED15	0.30 ∝M
ED19	0.60 ∝M

* IC 50: inhibitory concentration 50

Fig. 3. A: Interaction of anti-E2C mAbs with the E2C:DNA complex in an electrophoretic mobility shift assay. E2C protein (2.5 μM) was incubated with 1.0, 2.0 and 4.0 μM antibody combining sites for 2 h prior 5′ fluoresceinated-site 35 probe (2.5 μM) addition (C), a non-related IgG at 4 μM. B: Effect of antibody:E2C interaction on protein:DNA complex formation as measured by a biosensor. Percentual response of E2C binding to the immobilized oligonucleotide after incubation with increasing amounts of anti-protein mAbs. mAbs ED5 and ED13 are shown as examples (see Section 2). C: IAsys raw data for different concentrations of mAb ED19 are depicted as an example to illustrate the inhibitory effect of anti-E2C mAbs on DNA binding. D: Table showing the observed IC_{50} for the different anti-E2C mAbs. IC_{50} was calculated as the IgG concentration that renders a 50% of E2C binding response as compared with the control without antibody. Pre-incubation with unlabeled site 35 oligonucleotide leads to total inhibition, even at low nanomolar concentrations (data not shown).



nize a repeated epitope on the homodimeric transcription factor (data not shown), pointing out to the highlighted red surfaces (Fig. 4). In contrast, DNA-binding interfering mAbs should recognize an epitope that coincides or overlaps with the DNA-binding surface of the protein. As illustrated in the figure, this surface forms a continuous area with two symmetric protruding α-helices (yellow-colored residues). Taken

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Fig. 4. Antigenic topography of human HPV-16 E2C. Graphical models of free (panels A and B) and DNA-bound (panel C) HPV-18 E2 homologous protein, indicating two clearly defined surface areas based on antibody recognition. Diagrams were generated using Turbo Frodo and the ViewerProTrial 42 software from deposited crystallographic coordinates [17]. The repetitive ED5-like recognition area is colored in red (residues 351–360 in the HPV-18 E2 protein). Residues of E2C that contact DNA are highlighted in yellow. A: Ribbon representation of the HPV-18 E2 DNA-binding domain. B,C: Space-fill representation colored as in A.

together, these results suggest that we have obtained two different anti-E2C mAbs populations, depending on which of the two major surfaces of the transcriptional regulator they recognize.

In the last years, a preliminary characterization of polyclonal antibodies against overlapping synthetic peptides covering this protein has been reported [18]. mAbs against the homologous BPV-1 E2 protein have also been produced and tested for their ability to interfere with E2 functions in vivo and in vitro [15]. Among these antibodies, only one mAb directed against the C-terminal domain was able to inhibit E2 binding to DNA. Furthermore, concentration-dependent inhibition of DNA replication in vitro by this antibody was also observed. Interruption of transcription factor DNA binding by antibodies may have at least two explanations [16,19]. First, steric hindrance of the DNA-binding domain by the antibody may block its interaction with DNA. Second, antibody binding might prevent essential conformational changes in the transcription factor that would be required for its biological functions. Further analyses of these protein–protein interactions are needed to understand the effect these mAbs produce over E2. Moreover, structural studies of these complexes would be the basis for rational design strategies aimed at developing therapeutic compounds [20] that modify binding of E2 to its specific recognition sequences on the papillomavirus genome.

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References

[1] McBride, A., Romanczuk, H. and Howley, P.M. (1991) J. Biol. Chem. 266, 18411–18414.
[2] Lima, L.M. and Prat Gay, G. de (1997) J. Biol. Chem. 272, 19295–19303.
[3] Ferreiro, D.U., Lima, L.M.T.R., Alonso, L.G., Goldbaum, F.A. and Prat Gay, G. de (2000) Biochemistry 39, 14692–14701.
[4] Moore, J.P., Trkola, A. and Dragic, T. (1997) Curr. Opin. Immunol. 9, 551–562.
[5] Cerutti, M.L., Centeno, J.M., Goldbaum, F.A. and Prat Gay, G. de (2001) J. Biol. Chem. 276, 12769–12773.
[6] Ulrich, J.T. and Myers, K.R. (1995) in: Vaccine Design: The Subunit and Adjuvant Approach (Powell and Newman, Eds.), Vol. 6, pp. 495–524, Plenum Press, New York.
[7] Vogel, F.R. and Powell, M.F. (1995) in: Vaccine Design: The Subunit and Adjuvant Approach (Powell and Newman, Eds.), Vol. 6, pp. 141–228, Plenum Press, New York.
[8] Galfré, G.A. and Milstein, C. (1981) Methods Enzymol. 73, 3–46.
[9] Goldbaum, F.A., Cauerhff, A., Velikovsky, C.A., Llera, A.S., Riottot, M. and Poljak, R.J. (1999) J. Immunol. 162, 6040–6045.
[10] Hassen, E., Chaieb, A., Bouaouina, N., Khairi, H., Remadi, S. and Chouchane, L. (2001) Mod. Asp. Immunobiol. 2, 8–12.
[11] Foguel, D., Silva, J.L. and Prat Gay, G. de (1998) J. Biol. Chem. 273, 9050–9057.
[12] Hegde, R.S. and Androphy, E.J. (1998) J. Mol. Biol. 284, 1479–1489.
[13] Scibienski, R.J. (1973) J. Immunol. 111, 114–120.
[14] Kang, C.L., Hariharan, K., Nara, P.L., Sodroski, J. and Moore, J.P. (1994) J. Virol. 68, 5854–5862.
[15] Kurg, R., Parik, J., Juronen, E., Sedman, T., Abroi, A., Liiv, I., Langel, U. and Ustav, M. (1999) J. Virol. 73, 4670–4677.
[16] Orten, D.J., Strawhecker, J.M., Sanderson, S.D., Huang, D., Prystowsky, M.B. and Hinrichs, S.H. (1994) J. Biol. Chem. 269, 32254–32263.
[17] Kim, S.S., Tam, J.K., Wang, A.F. and Hegde, R.S. (2000) J. Biol. Chem. 275, 31245–31254.
[18] Gauthier, J.-M., Dillner, J. and Yaniv, M. (1991) Nucleic Acids Res. 19, 7073–7079.
[19] Thompson, N.E., Strasheim, L.A., Nolan, K.M. and Burgess, R.R. (1995) J. Biol. Chem. 270, 4735–4740.
[20] Marasco, W.A. (2001) Curr. Top. Microbiol. Immunol. 286, 587–594.