BIOCHEMISTRY

 Volume 46, Number 37 September 18, 2007

New Concepts

The N-Terminal Module of HPV16 E7 Is an Intrinsically Disordered Domain That Confers Conformational and Recognition Plasticity to the Oncoprotein

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Received April 26, 2007; Revised Manuscript Received August 8, 2007

ABSTRACT: The HPV16 E7 oncoprotein is an extended dimer, with a stable and cooperative fold, but that displays properties of "natively unfolded" proteins. Two regions of conserved sequence are found in E7 proteins, where the N-terminus (1−40) includes the retinoblastoma tumor suppressor binding and casein kinase II phosphorylation sites. A fragment containing the highly acidic N-terminal half shows an apparently disordered conformation by far-UV−circular dichroism (CD) at neutral pH, and its hydrodynamic radius is much larger than a neutral peptide of the same length. Trifluoroethanol and micellar concentrations of sodium dodecyl sulfate stabilize a much more helical structure at pH 4.0 than at pH 7.5, while submicellar concentrations of the detergent yield a β-strand. The shape, pH, and temperature dependence of the CD spectrum at pH 7.5 are indicative of a poly proline type II structure. This structure is stabilized by phosphorylation, which would translate into increased transforming activity in the cell. Thus, the intrinsically disordered properties of the N-terminal module of E7 are responsible for the structural plasticity of the oncoprotein. Although the domain is not a compact and cooperatively folded unit, it is a bona fide functional domain, evolved to maintain a dynamic but extended structure in the cell. These properties allow adaptation to a variety of protein targets and expose the PEST degradation sequence that regulates its turnover in the cell, a modification of which leads to the accumulation of E7 species with consequences in the transformation process.

Papillomavirus-related infections are a worldwide threat, in particular because of their direct link to cervical cancer, a major cause of death among women. The E7 oncoprotein from the "high risk" strain 16 raises substantial interest since it is the most frequently found strain associated with cervical cancers, followed in frequency by HPV-18 (*1*). Although E7 has been identified as the major transforming protein and its function was described over 15 years ago (*2*), the number of cell interacting partners described increases every year (*3*) and structures of the C-terminal domain of strains HPV 45 and 1A were solved only recently (*4*, *5*).

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HPV16 E7 bears properties that resemble natively unfolded polypeptides (*6*), but the isolated globular C-terminal structure (*4*), far-UV circular dichroism (CD) spectrum, and cooperative unfolding (*7*) support a folded conformation. We have reported that HPV16 E7 is a stable and extended Zn binding dimer in solution ($E7_2$). The unusual non-globular characteristics we described for HPV16 E7 and the pH-induced conformational transitions accompanied by exposure of hydrophobic patches were proposed as the basis for the possible binding to multiple cellular targets (*8*).

We have also described that when the zinc atom of $E7_2$ is removed by treatment with a chelating agent, the protein

10.1021/bi7007917 CCC: $37.00
Published on Web 08/23/2007

	CR1		CR2					
				LXLXE		CKII		
HPV 16 E7	MHGDTPTLHEYMLDL		..QP.E.TTD	LYCYE	QLND	SS	EEE.DE	IDG
HPV 18	---PKA--QDIV-H-		EP-N.-IPV-	-L-H-	--S-	.-	---N--	---
HPV 6	---RMV--KDIV---		..--PD.PVG	-H---	--V-	--	-D-V--	V--
HPV 11	---RLV--KDIV---		..--PD.PVG	-H---	--E-	--	-D-VDK	V-K
aa	1	15	16					40
Ad 5-E1A	/-FEP-----.LY--		..V-.-.VI-	-T-H-	AGFP	P-	DD-.--	
aa	37	49	116				137	
SV40 Tag	/REESLQ-MD.L-G-		NAFN.-.E.N	-F-S-	EM.P	--	DD-ATA	
aa	7	19	99				116	

FIGURE 1: The N-terminal region (CR1 and CR2) of HPV E7 proteins (strains 16, 18, 6 and 11); regions in the adenovirus E1A protein and the large TAg of SV40. Acidic residues are indicated in bold, and the binding motif to Rb and serines that are phosphorylated by CKII are marked in boxes. (−) shows residues identical to those in HPV16 and (.) are gaps inserted for alignment. The potential PEST sequence according to the local enrichment of critical amino acids D, E, P, S, or T (*39*), as well as the motif's hydrophobicity, is shown underlined.

readily assembles into homogeneous high molecular weight spherical particles (E7SOs, *8*). E7SOs are highly stable and cooperatively folded and display chaperone activity on citrate synthase and luciferase, both standard chaperone substrates, at substoichiometric concentrations. For the assembly to take place, the protein undergoes secondary structure rearrangements with concomitant consolidation of tertiary structure. These oligomers have the N-terminus exposed to the solvent and are capable of binding both Congo Red and thioflavin T, reporters of repetitive β-sheet structures found in amyloid conformation, a fact that is shared with other chaperones (*9*).

The N-terminal "domain" of approximately 40 residues of HPV E7s was defined by sequence alignment and displays homology to regions of adenovirus E1A and SV40 T antigen (*10*) (see Figure 1) and contains a high proportion of acidic residues, translated into a theoretical pI of 3.5. One of the early identified targets of the E7 oncoprotein, the retinoblastoma tumor suppressor "pocket" protein (Rb) (*11*), binds to a region located in the N-terminal half, and a structure of a peptide containing the Rb binding site bound to Rb was described (*12*). In addition, HPV16 E7 presents a consensus site for CKII phosphorylation on its N-terminal half, adjacent to a poly glutamic (poly-E) sequence (see Figure 1). It has been reported that phosphorylation in serines 31 and 32 is important for transformation and critical to promote S-phase entry (*13*), with no reported effect on the Rb binding activity (*13*). Mutation of these serines and the consensus site for the kinase resulted in a defective phosphorylation protein with decreased transforming ability (*13*, *14*).

In this work, we carried out a detailed investigation of the conformational properties of the HPV16 E7 terminal domain, E7(1−40),[1] in solution. We found that it is responsible for the nonglobular properties of E7, it contains residual structure at neutral pH, and studies demonstrate that phosphorylation introduces a structural modification in the peptide. We describe E7(1−40) in terms of a functional "intrinsically disordered" or "natively unfolded" domain (*15*−*17*) whose flexibility and fine conformational transitions confer target adaptability to the oncoprotein.

EXPERIMENTAL PROCEDURES

The HPV16 E7 gene was amplified by PCR and cloned into plasmid pTZ18u under the T7 promoter. The resulting plasmid was transformed into BL21(DE3) *Escherichia coli* strain for expression. Inclusion bodies containing HPV16 E7 were resuspended in 50 mM Tris-HCl, pH 7.5, 10 mM DTT, 8.0 M urea, loaded onto a Q-HyperD column (BioSepra, France), purified as described (*7*), and dialyzed against 10 mM sodium phosphate pH 7.0 and 1 mM DTT for storage.

A 40-residue peptide corresponding to the N-terminal half of HPV16 E7, E7(1−40), and the double phosphorylated peptide in serines 31 and 32, E7(1−40)pp, were obtained from the Keck facility (Yale University), purified by reverse phase HPLC, and submitted to mass spectrometry. The peptides were dissolved in water pH 8.0 to a concentration of approximately 900 μM and stored at −70 °C. Quantification was carried out by absorbance at 276 and 220 nm in HCl.

SEC experiments were carried out in a Superdex 75 (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM Tris HCl buffer pH 7.5 or sodium formiate pH 4.2 as indicated. No differences in elution volumes for the peptides were observed when working with these buffers at 0.5 M NaCl. The samples were incubated for 1 h before injection, and the elution of the peptides were monitored at 220 nm. The column was calibrated with the following standard globular proteins: BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), ribonuclease A (13.4 kDa) from a gel calibration kit (Pharmacia Biotech, Uppsala, Sweden), protein chymotrypsin inhibitor CI2 (7.3 kDa), and its N-terminal portion, a peptide of 40 residues, CI2(1−40), used as a model for an unfolded polypeptide (*18*). The calibration with molecular weight markers, void volume (V_0) and total volume ($V_0 + V_i$) by loading Blue Dextran and acetone, respectively, were determined at each pH and a similar slope was obtained for the log MW vs volume of elution. All experiments were carried out at 25 °C.

CD measurements were carried out on a Jasco J-810 spectropolarimeter (Jasco, Japan) employing a scan speed of 20 nm/min, a band-pass of 1 nm, and an average response time of 4 s. All spectra were an average of at least 10 scans. The temperature was kept at 20 °C using a Peltier temperature-controlled sample compartment unless indicated. Spectra were measured at 10 μM E7 and 20 μM E7(1−40) on a 0.1 cm path length cell. For pH dependence experiments, E7 in 10 mM of sodium formiate (pH = 2.5, 3.0, 3.5, 4.0); sodium acetate (pH = 4.5, 5.0, 5.5, 6.0) or Tris-HCl (pH = 7.5) and 20 mM E7(1−40) in 10 mM citrate-phosphate (pH 3.2, to 7.25) buffers were used. Temperature denaturation curves (ranging from 2 to 60 °C) of E7(1−40) and E7(1−40)pp were carried out in Tris HCl buffer pH 7.5 recording spectra every 2 °C (2−10 °C) and every 5 °C (10−60 °C) and monitored following the amplitude of negative ellipticity band at 218 nm.

The critical micelle concentration (CMC) for sodium dodecyl sulfate (SDS) was calculated by following fluorescence of 1-anilino-8-naphthalene-sulfonate (ANS) to be 4.8 mM in 20 mM Tris HCl pH 7.5 and 5.0 mM for sodium formiate pH 4.0, in accordance with tabulated values for buffers with a similar ionic strength (*19*). Experiments were carried out at 20 μM E7(1−40) in 10 mM Tris HCl pH 7.5 and sodium formiate pH 4.0, as indicated, at 25 °C, and 1 mM SDS was used to obtain a submicellar concentration of the detergent and 10 mM for a micellar concentration.

[1] Abbreviations: E7(1−40): HPV16 E7 terminal domain, residues 1−40.

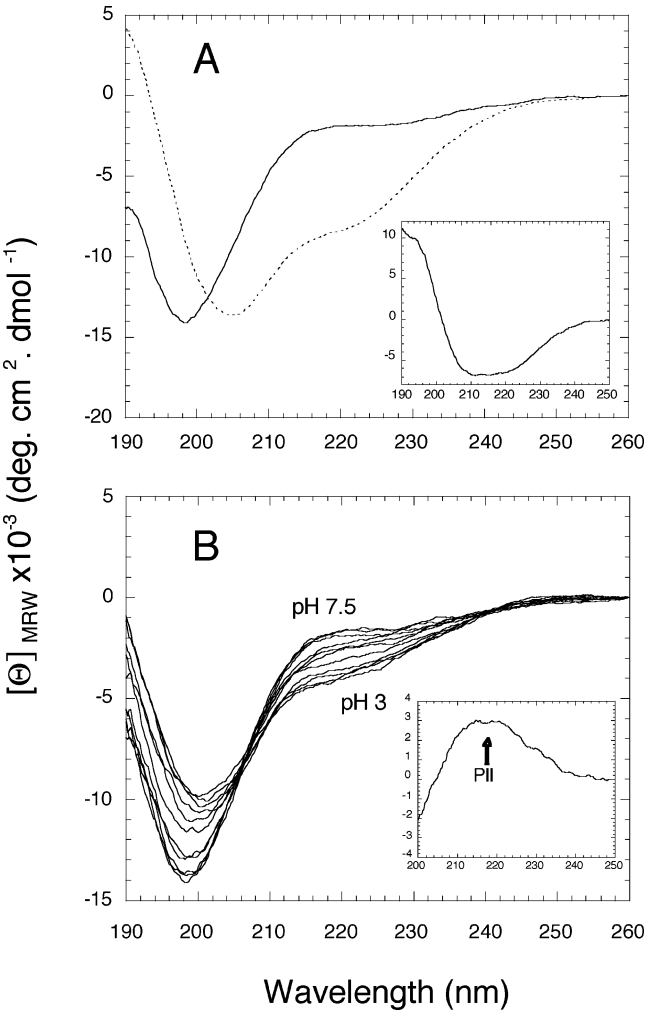

FIGURE 2: Conformational transitions in E7 and E7(1−40) triggered by pH modifications. (A) Circular dichroism of E7 (dotted line) and E7(1−40) (solid line) in 10 mM phosphate buffer pH 7.0 and 10 mM buffer Tris HCl pH 7.5, respectively. Inset: differential spectrum between HPV16 $E7_2$ and E7(1−40). (B) Far-UV−CD spectra of E7(1−40) at different pH (see Experimental Procedures). Inset: Differential spectrum between E7(1−40) at pH 7.5 and pH 3.0.

For 2,2,2-trifluoroethanol (TFE) (ICN, Biomedicals Inc.) experiments, the peptide was dissolved in 0−65% TFE (volume of TFE added/total volume added) and 20 mM Tris HCl pH 7.5 or sodium formiate pH 4.2 as indicated in a 0.5 cm path length cell. Helical populations were estimated from measured mean residue ellipticities at 222 nm, $[\Theta]_{222}$, taking zero for 0% helix and −39 500 (1−2.57/*N*) for 100% (*20*). ΔG^{TFE} values were estimated from TFE titrations using the model for TFE−water−peptide according to the equation proposed in ref *21*. The fitting was performed using the Kaleidagraph package (Adelbeck software).

RESULTS

The far-UV CD spectrum of the E7 dimer ($E7_2$) at pH 7.5 is dominated by a minimum at 205 nm, with a negative band at around 220 nm (Figure 2a, dotted line). The band at 220 nm is indicative of α-helix content, and the minimum at 205 can be the result of a combination of an α-helix (208 nm) and disordered structure (200 nm). When observing the spectrum of the N-terminus of E7, it is reminiscent of that of a disordered polypeptide with a minimum around 200 nm (Figure 2a, full line). To analyze the structural contribution of the E7(1−40) domain to the $E7_2$ far-UV−CD, the subtraction of spectra was carried out and presents only the α-helix spectral characteristics, suggesting that the apparently disordered components in the E7 dimer, manifested by the minimum at 205 nm, are introduced by the N-terminal module (Figure 2a, inset), in agreement with the solved structure of HPV 1A E7 where the N-terminal region is absent in the crystal (*5*) and HPV 45 E7 N-terminus not analyzable by NMR (*4*). The C-terminal domain would correspond to the different spectra and has substantial α-helix, which dominates the spectrum.

The E7(1−40) peptide contains an unusual amount of acidic side chains, and we considered that it may display the characteristics of poly-E peptides in solution (*22*). It presents a minimum at 198 nm, a negative band at 228 nm, and a band at around 218 nm at pH 7.5, all of which disappear as the result of neutralization of the acidic side-chains upon pH decrease (Figure 2b), in agreement with a poly proline type II (PII) structure (*23*, *24*). The N-terminus could thus be responsible for E7's conformational behavior toward pH; it undergoes a conformational transition within a physiological pH range that could correspond to an induction in α-helix and/or loss of PII structure. The difference spectrum between E7(1−40) at pH 7.5 and pH 3.0 displays the positive distinctive band at 218 nm present in PII models (Figure 2b inset). E7(1−40) has a theoretical pI of 3.5 with 30% of acidic residues, and despite this, it does not aggregate around pH 3.5. The fragment shares the conformational transition observed for E7 as a function of pH (not shown).

Far-UV−CD spectra at 10 and 100 μM E7(1−40) are identical, stressing the absence of changes in molecularity (not shown).

E7 is a polypeptide of 11 kDa and migrates in sodium dodecyl sulfate−polyacylamide gel electrophoresis (SDS−PAGE) as a protein of apparent molecular weight of 19 kDa (*7*, *25*), while E7(1−40) displays the expected electrophoretic mobility for a peptide of 4.6 kDa (not shown). To establish whether the E7 N-terminal half has the hydrodynamic volume for a peptide in an extended conformation, we carried out gel filtration experiments of E7(1−40) and compared it with a model peptide comprising the first 40 residues of the chymotrypsin inhibitor 2, CI2(1−40), which does not have a folded conformation, is not extended, nor does it contain an excess of negatively charged residues (*18*). At pH 7.5, E7(1−40) elutes at 15 mL, while CI2(1−40) does so at 17 mL, indicating that at neutral pH, E7(1−40) is extended (Figure 3). E7(1−40) elutes with the hydrodynamic volume of a globular protein of 25 kDa, equivalent to a Stokes radius of 20.9 Å, a value even larger than the predicted for a "natively unfolded" polypeptide of this size. In addition, we carried out pulse field gradient NMR experiments at 1 mM E7(1−40), and from the diffusion coefficient the domain was shown to be monomeric, with a calculated stokes radius of 21.26 Å, expected for an extended fragment of that number of residues (not shown). When the same experiment is carried out at pH 4.0, the hydrodynamic volume of CI2(1−40) stays unmodified, but E7(1−40) elutes at a larger volume from the column, strongly suggesting that the conformational change induced at low pH yields a more compact structure (Figure 3). This result is in excellent agreement with the observed conformational transition by CD, where the molar

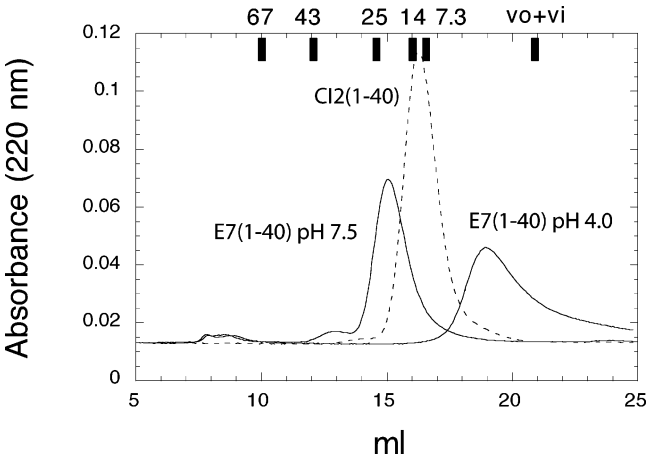

FIGURE 3: E7(1−40) in size exclusion chromatography (SEC). SEC of E7(1−40) (solid line) and CI2(1−40) (dotted line) in TrisHCl pH 7.5 (black) and sodium formiate pH 4.0 (gray). Void volume (V_o), $V_o + V_i$ and elution volumes for globular molecular weight markers in kDa are indicated with bars.

ellipticity at 220 nm decreased when the pH is lowered (not shown).

It is well documented that addition of detergents such as SDS induce conformational transitions in model peptides that allow the stabilization of either α-helix or β-sheet elements depending on the concentration of the detergent and the nature of the peptides (*26*, *27*). When E7(1−40) is placed in a solution containing 10 mM SDS, substantial elements of secondary structure can be stabilized at low pH, while little or no change is observed at pH 7.5 (not shown). Above the CMC, the detergent induces α-helix, as the typical double minimum at 208 and 220 nm in the spectra indicates (Figure 4a), and submicellar concentrations of SDS stabilize β-sheet-like structures, as judged by the minimum at 217 nm (Figure 4b). Therefore, the E7 N-terminus is capable of adopting different types of secondary structures depending on the pH and the amount of detergent added.

TFE is able to stabilize pre-existing α-helical populations in peptides (*28*, *29*). To investigate how neutralization of the acidic residues affects the formation of α-helical structure in E7(1−40), we carried out TFE titrations at different pHs. The development of the characteristic negative bands around 208 and 222 nm indicates that regions of α-helix structure are more represented at low pH (not shown). The isodichroic point in the transition supports a two-state equilibrium approximation (*21*). For the helix−coil transition, ΔG represents the fraction of α-helix in water and in TFE, while the *m* value describes the effectiveness of the solvent in the interconversion between the two states in terms of the differential exposure to the solvent (*30*, *31*), both calculated from titrations in Figure 5 (see Experimental Procedures). At pH 7.5, the peptide reaches only 14% α-helix in TFE, while at pH 4.0, 29% of helix is stabilized. The stability of α-helix structures present in the peptides at both pH values are coincident (∼1.5 kcal mol^{-1}), which together with similar *m* values (0.5 ± 0.05 kcal mol^{-1} M^{-1} for pH 7.5 and 0.6 ± 0.05 kcal mol^{-1} M^{-1} for pH 4.0) provide a strong indication that the regions involved in the nucleation of the α-helix are equivalent. At neutral pH, poly-E residues adopt a PII conformation, favored by the charge repulsion. Although the propagation of helix is more efficient at low pH, the energetic barrier for the residual structure in water is independent of

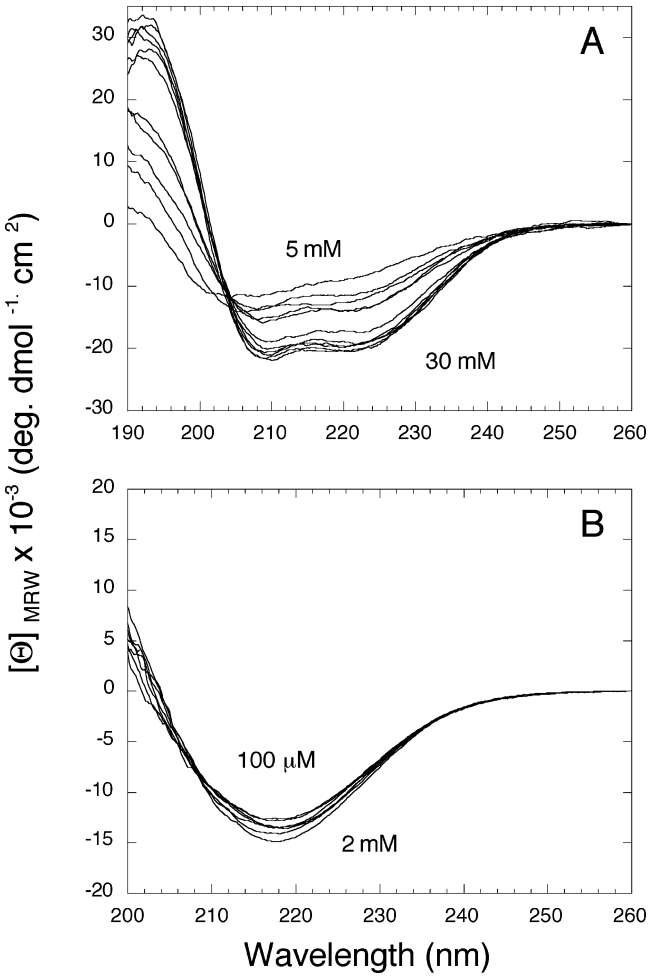

FIGURE 4: Effect of detergent addition on E7(1−40). (A) Far-UV−CD of E7(1−40) in sodium formiate pH 4.0 adding micellar concentrations of SDS, ranging from 5 to 30 mM. (B) Peptide in sodium formiate pH 4.2 adding submicellar concentrations of SDS, ranging from 100 μM to 2 mM.

pH, suggesting that the residues involved in the PII structure appear not to be involved in the nucleation of the α-helix in water.

To investigate the effect of phosphorylation of serines 31 and 32 on the conformational equilibriums of E7(1−40), we synthesized the double-phosphorylated N-terminal fragment (E7(1−40)pp). Both E7(1−40) and E7(1−40)pp appear disordered, as the far-UV suggests (Figure 6A). However, their spectra show considerable differences: E7(1−40)pp shows a positive band at 218 nm (Figure 6A, inset) and displays a more negative molar ellipticity value at 198 nm, strongly suggesting that its PII content is stabilized compared to the unphosphorylated species (*24*).

The PII helix is known to be stabilized at lower temperatures (*32*). As the temperature increases, the PII structure present in E7(1−40)pp is disrupted, the positive band at 218 nm lost, and the minimum around 198 nm shifted upward (Figure 6b). E7(1−40)pp displays a higher content of PII at 5 °C than the unphosphorylated peptide as reported by its molar ellipticity at 218 nm; around 35 °C the PII content is equivalent for both peptides (not shown).

Regions of PII in peptides can be stabilized by addition of the denaturant guanidine chloride (GdmCl), which shifts the equilibrium to the PII population (*24*, *33*). E7(1−40) shows such behavior when titrated with increasing denaturant

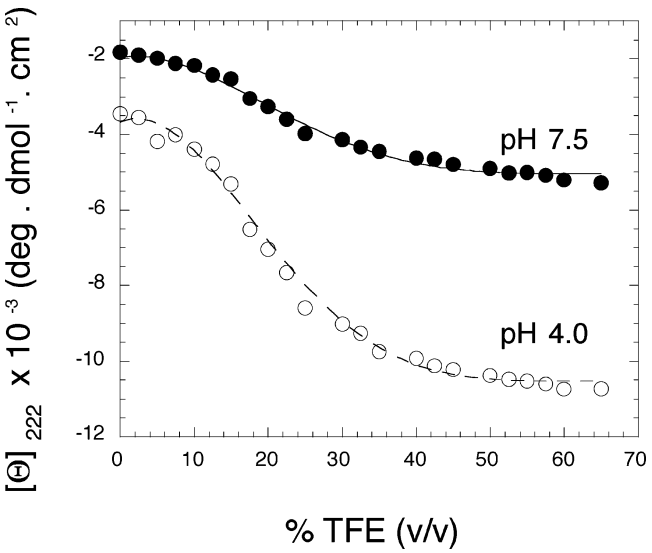

FIGURE 5: Stabilization of α-helical populations in E7(1−40) by TFE. Molar ellipticity of E7(1−40) at different TFE concentrations, ranging from 0 to 65% v/v, measured at 220 nm for pH 7.5 (solid circles) and pH 4.2 (open circles). Titration curves were fitted to a two-state equilibrium model (*21*) for obtaining the Δ*G* of α-helix formation in water (∼1.5 kcal mol^{-1}), showing *m* values of 0.5 ± 0.05 kcal mol^{-1} M^{-1} at pH 7.5 and 0.6 ± 0.05 kcal mol $^{-1}$ M $^{-1}$ at pH 4.0.

concentrations since the positive band at around 218 nm increases with GdmCl concentration (Figure 6c). In contrast, only a small change is observed for E7(1−40)pp (Figure 6d), where the starting point of the titration has a more positive value. This result strongly suggests that the residual structure of the phosphorylated species in the absence of the stabilizing GdmCl already contains a higher proportion of PII. Thus, the presence of the phosphate modifications in S31 and S32 stabilizes regions of the PII structure. The unavoidable low signal-to-noise ratio due to the combination of a small change in ellipticity and the presence of the chaotrope introduces a large error for estimating a Δ*G* parameter (*24*). Nevertheless, the differences in the final values of molar ellipticity around 218 nm attained by both peptides in 6 M GdmCl clearly indicate a larger content of PII in E7(1−40)pp.

DISCUSSION

The N-terminal 40 residues of HPV E7 proteins were defined as a domain based on sequence homology, suggesting that the oncoprotein is modular, something that was confirmed by recent structural studies. We found that the isolated domain, the E7(1−40) fragment, cannot form a compact and cooperative fold containing canonical secondary and tertiary structure. However, we showed that the N-terminal module governs many of the remarkable properties of E7 and that it can be considered a bona fide domain, even in the absence of a compact-cooperative structure.

The far-UV CD spectrum of E7(1−40) resembles that of a disordered polypeptide. However, phosphorylation, temperature, and pH alter the spectrum substantially, indicating the presence of a PII type of structure, that is, a negative band at 198 nm and a positive band at 218 nm. In addition to previous studies with model peptides (*23*), we have recently correlated CD changes with NMR, validating the assignment of PII structure by the former technique (*24*). Intracellular pH is modified along the epithelium during a carcinogenic process together with the levels of E7 detected and its activity (*3*). When exposed to an acidic environment, E7(1−40) undergoes a structural transition triggered by the neutralization of its acidic residues, similar to the full-length HPV16 E7 dimer (*7*). A decrease in the CD signal at 220 nm at low pH is indicative of the acquisition of α-helix. Moreover, as pH decreases, the CD indicates that the PII content tends to disappear.

HPV16 $E7_2$ was shown to present a hydrodynamic value larger than the one predicted for a globular protein of 22 kDa. It displays an extended and thermostable structure persistent in SDS−PAGE, which provides an explanation for its anomalous electrophoretic behavior (*7*). The E7 C-terminal domain structure was recently solved by NMR, and its N-terminus was described as unfolded in solution (*4*), conferring the protein an enlarged radius in agreement with the properties we previously described (*7*). Here, we show that E7(1−40) is not just disordered but exists in an extended conformation when compared to a disordered neutral peptide. On the basis of previous reports on polypeptides adopting PII conformations (*34*), we propose that the PII structure, stable at neutral pH and stabilized by electrostatic repulsion of negative side chains, is responsible for the extended structure. It is known that when poly-E model peptides are neutralized, a conformational modification takes place such that the peptide adopts a more compact behavior and displays a hydrodynamic radius smaller than a disordered peptide of the same size.

Our results demonstrate that E7(1−40) contains the noncanonical PII structure alternating with more disordered regions, with the capacity to adopt different conformations depending of the environment and finely tuned by pH. TFE induces a α-helix in E7(1−40) longer at pH 4.0 than at pH 7.5, and the Δ*G* of helix formation is independent of pH, indicating that the nucleation sites of the helix are the same in both cases and do not correspond to the residues of poly-E in a PII conformation. At pH 4.0 and below, the negatively charged residues are neutralized, the charge repulsion decreases, and more α-helix is formed.

In addition, the α-helix was also stabilized at micellar concentrations of SDS at low pH. On the other hand, when the detergent concentration is below the CMC, *β*-like far-UV−CD spectra are obtained at lower but not at higher pH. This result is striking because it shows that when the PII is disrupted, E7(1−40) can undergo different structural transitions compatible with promiscuous binding. SDS or TFE confer environmental conditions to the peptide that could mimic the effect generated by the proximity to other proteins, membranes, or long-range interactions with the C-terminal domain. The capacity of E7(1−40) to undergo α-helix to *β*-sheet transition shows the functional plasticity that this domain confers to the protein.

We had shown that HPV16 E7 undergoes oligomerization exposing its N-terminus to the solvent and displays chaperone activity (*9*). An accessible disordered region is a feature shared by classical protein chaperones and chaperonines, where "structural disorder" has been shown to provide a significant solubilizing effect, preventing aggregation of the underfolded partner (*35*).

Alpha-synuclein was shown to be characterized by a remarkable conformational plasticity, adopting a series of different conformations depending on the environment. For

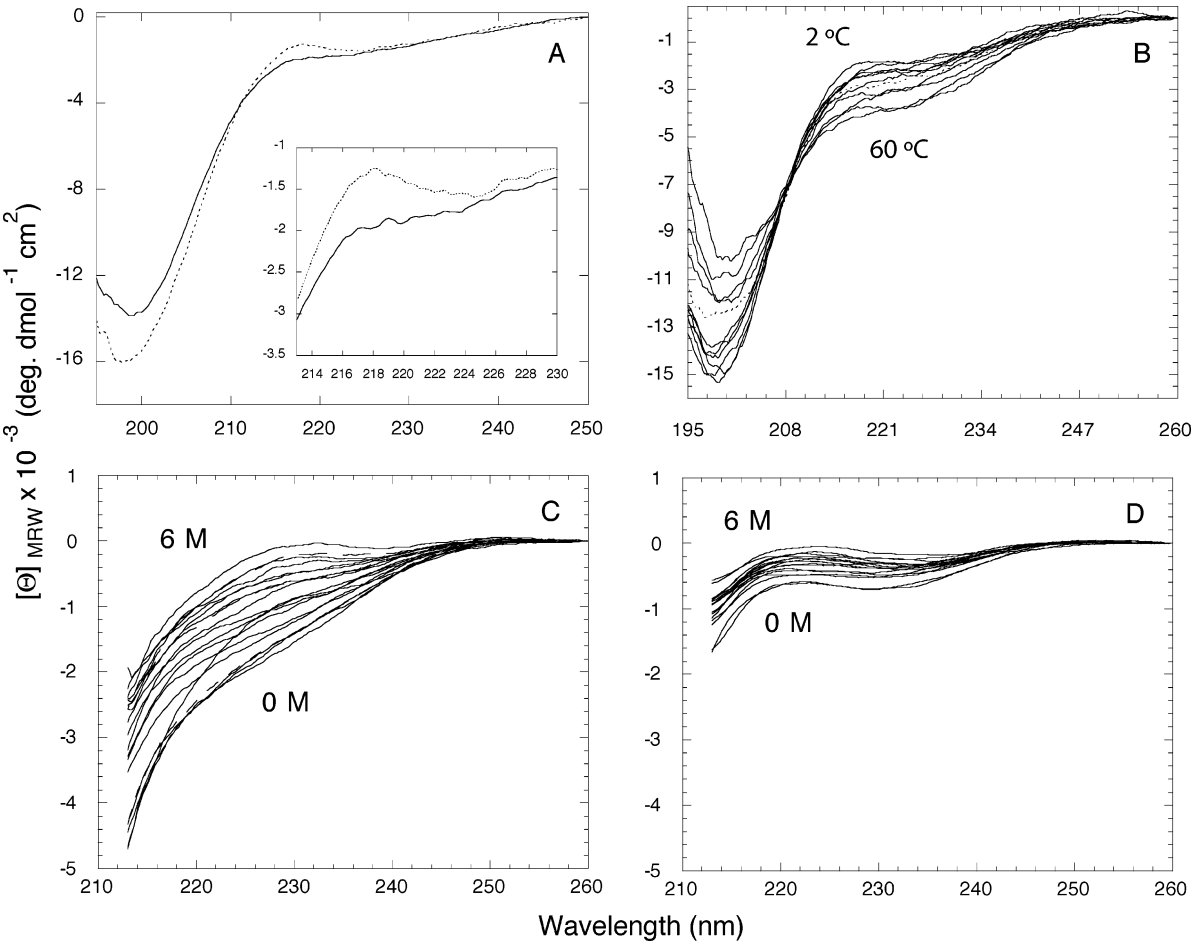

FIGURE 6: Far-UV circular dichroism of the E7(1−40) peptide and the double phosphorylated form. (A) Far-UV−CD spectra of E7(1−40) (solid line) and E7(1−40)pp (dotted line) at pH 7.5. Inset: Amplification of spectra showing a differential band around 218 nm. (B) E7(1−40) spectra measured at different temperatures ranging from 2 to 60 °C. (C, D) Far-UV−CD spectra of E7(1−40). (C) and E7(1−40)pp (D) in the presence of different concentrations of GdmHCl at pH 7.5.

example, this protein may either stay substantially unfolded or adopt an amyloidogenic partially folded conformation or fold into α-helical or β-structural species, both monomeric and oligomeric. Furthermore, it might form several morphologically different types of aggregates, including oligomers (spheres or doughnuts), amorphous aggregates, and/or amyloid-like fibrils. On the basis of the peculiarities of this astonishing conformational behavior, the concept of a protein-chameleon was introduced (*36*).

HPV16 E7 is phosphorylated by CKII at serines 31 and 32, adjacent to the region-rich in acidic residues that we assign as PII structure (Figure 1). PII conformations are described as major structural features in unfolded states or in exposed regions of proteins (*34*), and they play a key role in defining extended and accessible conformations for chemical modification and proteolysis (*37*). We show that E7(1−40)pp displays a higher content of PII compared to the unphosphorylated peptide. We have recently shown that disruption of PII in the PEST degradation sequence of BPV1 C-terminal domain of the E2 transcriptional regulator leads to unstable polypeptides susceptible of degradation by the proteasome machinery. We determined that this was caused by local thermodynamic destabilization of structure by phosphorylation, uncovering a role for phosphorylation beyond a mere tag to be recognized by the degradation machinery (*24*). In the case of E7, phosphorylation of serines 31 and 32 stabilizes the PII and therefore could have a stabilizing effect toward protein degradation. Such local modification could render the polypeptide resistant to cleavage by proteases in general and become populated. The correlation found between structural stability and this post-translational modification may be essential for protein function.

HPV16 E7 was described as a short-lived protein, and its N-terminus is essential for degradation by the proteasome (*38*). The E7 N-terminus scores high as a PEST sequence, known to target proteins for phosphorylated mediated degradation (Figure 1) (*39*). Native proteins need to be unfolded for degradation, and their global or local stabilization prevents their proteolysis (*40, 41*). The structural features of E7(1−40) are compatible with those found in degradation initiation sites and provide the necessary marginal stability for a fast and efficient proteolysis. Regulation of proteolytic degradation by a disruption or stabilization of local protein structure is a fast and effective way to control the activities of polypeptides (*24*). It has been shown that phosphorylation is essential for E7 transforming action, and therefore this post-translational modification could be a suitable regulator of E7 activity.

An important experimental conclusion from this work is that the N-terminal module of HPV16 E7 is not a structural domain in the classical sense, that is, it is not an independent cooperatively folded unit, but an "intrinsically disordered" domain. However, it is a functional domain, evolved to

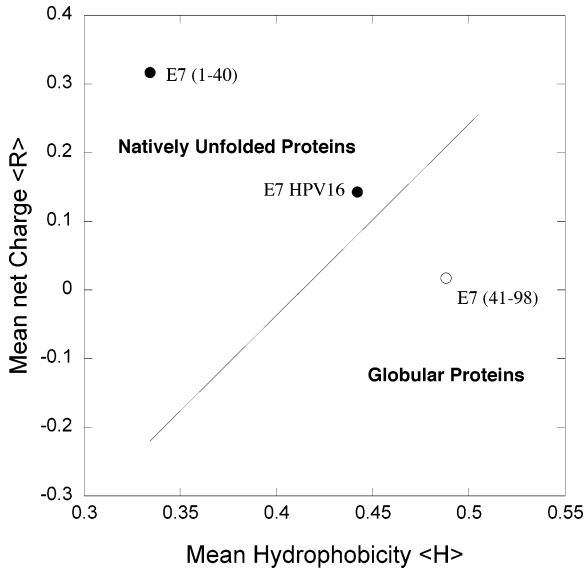

FIGURE 7: Charge-hydrophobicity phase space. The solid line in this figure represents the border between intrinsically unstructured and native proteins proposed by Uversky (*16*). The $<H>$ value for the E7 species were calculated with the equation: $<H> = (<R> + 1.151)/2.785$ (*6*), where $<H>$ is the mean hydrophobicity and $<R>$ is the mean net charge of the polypeptide. Solid circles show polypeptides that are localized within the natively unfolded region, while open circles are globular proteins.

maintain a dynamic structure in the cell, allowing a marginal stability that regulates its turnover and structural plasticity required to accommodate to a number of targets, with or without a role in HPV-mediated transformation. Sequence analysis classifies HPV16 E7 as a "natively unfolded protein" presenting high net charge coupled with low mean hydrophobicity (see Figure 7) (*42*). It is known to interact with several targets (*43*). The existence of natively unfolded modules plays a key role in cancer-associated proteins (*44*), where 79% of the latter and 66% of cell-signaling related proteins contain predicted disordered regions of 30 residues or longer (*16*).

The HPV16 E7 N-terminus can thus be described as an "intrinsically disordered" or "natively unfolded" domain. It is an extended, charged, but plastic structural domain, which is likely to optimize the speed of interaction and allows less specific, still selective, binding to multiple cellular host proteins. Moreover, the fact that E7(1−40) displays PII-like behavior is another issue shared with the described "natively unfolded" domains where PII has been proposed as its predominant structure (*34*). What is clear is that the domain does not adopt a random coil conformation that other protein fragments lacking these properties would adopt. This dictates first how the half-life and thus the transforming activity of the E7 oncoprotein are regulated. It also explains its target promiscuity and the chaperone holdase properties we described for E7SOs (*9*). It remains to be established whether counterpart oncoproteins from other DNA tumor viruses share these properties.

ACKNOWLEDGMENT

M.G.A. holds a studentship from University of Buenos Aires. G.P.G. is a career investigator from Conicet. The work was supported by a grant from ICGEB CRP ARG0102, and the ANPCyT (PICT 01-10944), Argentina.

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BI7007917