The E6 Protein

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Introduction

The E6 protein is regarded to be a multifunctional protein for the following reasons: First, it is a transcriptional transactivator, or coactivator [1–2]; for example, it is known to heterologously transactivate the adenovirus E2 promoter [3]. In some instances, namely with the MMLV LTR and the CMV intermediate early promoter, E6 behaves as a weak transcriptional repressor [4]. These activities, which are observed for both high-risk (16 and 18) and low-risk (6 and 11) PVs, do not require p53 binding or degradation. Two groups have noted an interaction with the homologous upstream regulatory region [5–6]. The transcriptional transactivator role of E6 may be coupled to its ability to transform and immortalize cells; on the other hand, low-risk HPVs can transactivate but have virtually no transforming capability [7].

Second, E6 binds to cellular proteins, in particular E6AP and E6BP, thereby modulating normal cellular processes. The E6-E6AP complex can bind *in vitro to* p53 [8] or to E6BP [9]. Deregulation of the p53 growth suppressor function appears in some experiments to be separate from the E6 involvement in the degradation of p53 [10–11]. Binding to several other cellular proteins, some of which have kinase activity, has been recently reported [12].

Third, E6 targets the degradation of p53[13–18], a process that requires a ternary complex between E6, E6AP and p53 [17]. Low-risk HPVs, such as HPV-6 and HPV-11, do not have the capacity to degrade p53.

Fourth, E6 of high-risk HPVs induces immortalization of primary cell cultures, and E6 of low-risk HPVs augments the ability of E7 to immortalize cells. With certain HPVs, for example HPV-6, the E6 and E7 proteins alone are weak at promoting immortalization, but coupled are potent; greater potency is seen when 16E6 (*i.e.*, HPV-16 E6) is coupled with 6E7 or 6E6 with 16E7 [19]). Independent of p53 or E7, E6 can transform NIH 3T3 cells in culture; it remains to be seen whether E6BP is essential for this property. Some E6 proteins have oncogenic properties but do not interact with p53 (see references in [9]).

There are many uncertainties underlying these observations, a result of the fact that the E6 protein is not well-characterized. E6 is present at extremely low levels in transformed cells, and no cellular homolog of E6 has been found. A small number of PVs—BPV-3, BPV-4, BPV-6—do not possess an E6 [20].

The E6 protein is a basic nuclear and cytoplasmic protein of about 18 kDa [21]. It contains four cysteine arrays that constitute two relatively large zinc fingers, both of which are required for full function. Most PVs have tandem ATGs of which the second (151 aa form) usually, but not always, is the start [21–23]. A protein of 158 residues can be generated from HPV16 *in vitro* and probably *in vivo*. E6 is a highly variable protein that nevertheless contains invariant and conserved motifs. Foster and coworkers argue that ". . . no linear sequence convincingly distinguishes the high-risk from the low-risk HPV E6 proteins" [16].

E6 (and E7) transcription is negatively affected by E2 overexpression. Further regulation is achieved through splice donor sites in some types that give rise to truncated forms of E6, denoted E6$*$I, E6$*$II, and E6$*$III [1,24–30]. The significance of the protein fragments generated from these various transcripts is in question; Shirasawa and colleagues propose that E6$*$I could serve as a transactivator [29]. Because the binding activities of E6 have not been observed for the truncated forms, others argue that these protein fragments are not themselves important, but that the splicing shifts the balance in

favor of E7 translation [1]. It is not clear to what extent splicing patterns affect tumor progression. Increased levels of E6 and E7 mRNA are reported for integrated viral forms [31]. The significance of a possible E6-E7 fusion protein is also in question [18,32].

Many of the effects summarized below, especially involving p53 binding and degradation, are *in vitro* observations. *In vivo* effects are reported for E6 and p53 by Lechner et al. [11] and by Foster et al. [16]. In the latter study, effects were seen when levels of E6 were undetectable, which is to say that the levels didn't always correlate with the magnitude of the effects. *In vivo* effects are also reported for E6 and E6BP [9].

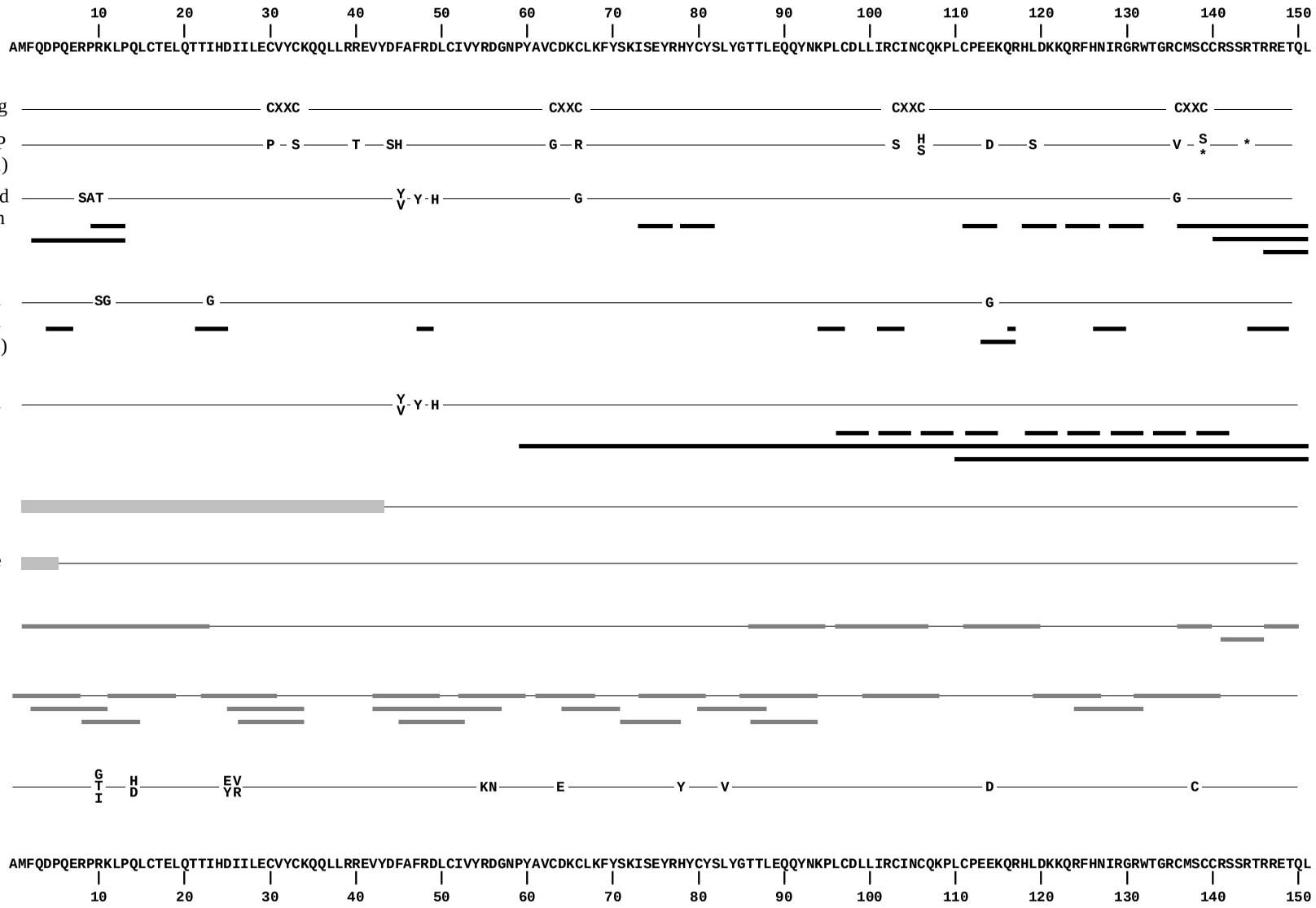
Some recent review articles that discuss the E6 protein are those of Mansur and Androphy [33], Scheffner et al. [17], and Vousden [34]. For a review of p53, see Zambetti and Levine [35].

In the following summary of E6 structure-function relationships, HPV-16 E6 is the basis for the observations unless stated otherwise. Mutations from wild-type (WT) to variant will be symbolized by C66G, for example, where C (cysteine) is the normal residue at position 66 that has been replaced by G (glycine). Deletion mutants are indicated in the figure by bold lines.

A. Zinc Binding Four cysteine arrays—two potential zinc fingers with relatively large loops characteristic of a certain class of transcriptional transactivators—are characteristic of human and animal papillomavirus E6 proteins and do indeed bind zinc [36-37]. Glycine substitutions introduced for C66 (C66G), C136 (C136G), and both simultaneously, led Kanda et al. to conclude that C66, but not C136, was essential for *in vitro* zinc binding by 16E6 [38]. DNA binding was unaffected by any of these substitutions. The C66 substitution, but not C136, also reduced the amount of nuclear E6. Both mutations led to a loss of enhancement of E7-induced cellular transformation.

BPV-1E6 cysteine mutants have been constructed by Vousden and coworkers: C17P, C20S, C50G, C53R, C90S, C93S, C124V, and C128S (BPV-1 coordinates). All but one of these, C50G, eliminated transforming capability and E6BP binding (see below) [39]. Vousden's C20S, C??S and C124V were shown by Lamberti and coworkers to be transformation defective, leading the latter group of investigators to conclude that E6 transcriptional properties may be concordant with transforming capability [2].

B. E6BP and E6AP Binding. E6 binding to cellular proteins can be considered separately from p53 binding and degradation (**C.** for several reasons: 1) E6 can bind to E6BP (ERC-55/E6BP) in the absence of p53; 2) BPV-1 E6 binds both E6BP and E6AP but not p53; 3) E6 binding to E6AP is a prerequisite for p53 binding; and 4) E6, E6BP and E6AP can form a ternary complex [9]. The only study to date of specifically E6BP and E6AP binding has been conducted with the BPV-1E6 mutants constructed by Vousden et al. [39]; the following summary has been condensed from Table 1 of that study by Chen et al. [9].


10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
AMFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLLRREVYDFAFRDLCIVYRDGNPYAVCDKCLKFYSKISEYRHYCYSLYGTTLEQQYNKPLCDLLIRCINCQKPLCPEEKQRHLDKKQRFHNIRGRWTGRCMSCCRSSRTRRETQL
Zinc binding
CXXC
CXXC
CXXC
CXXC
A
E6BP, E6AP binding (BPV1)
P – S
T
SH
G – R
S
H
S
D
S
V – S
*
*
B
p53 binding and degradation
SAT
Y
V
Y – Y – H
G
G
C
p53 transcription transactivation (HPV18)
SG
G
G
D
E6 transcription transactivation
Y
V
Y – Y – H
E
E6 truncations
F
E6 half-life
G
B-cell epitopes
H
T-cell epitopes
I
Natural Variants
G
T
I
H
D
EV
YR
KN
E
Y
V
D
C
J
AMFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLLRREVYDFAFRDLCIVYRDGNPYAVCDKCLKFYSKISEYRHYCYSLYGTTLEQQYNKPLCDLLIRCINCQKPLCPEEKQRHLDKKQRFHNIRGRWTGRCMSCCRSSRTRRETQL
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150

E6 Protein

TABLE 1 E6BP and E6AP Binding

BPV-1 Mutation	Growth [39]	E6BP Binding	E6AP Binding
WT	+ + +	+++	+++
I41T	+ + +	+++	+++
C128S	+ + +	+++	++
R46S	+	+	+
Y47H	+	+	+
C50G	+	+	+
C17P	−	-	-
C20S	−	-	-
C53R	−	-	+
C90S	−	-	-
C93H	−	-	-
C93S	−	-	+
H105D	−	-	-
R116S	−	-	+
C124V	−	-	-
C127∗	−	-	-
S134∗	−	-	+

∗ signifies an internal stop.
BPV-1 cysteines are at 17,20,50,53,90,93, and 124,127. The above mutations have been placed relative to the corresponding 16E6 cysteines in the figure. Values between 90 and 100, +++; between 25 and 90, ++; between 10 and 25, +; less than 10, -

C. P53 Binding and Degradation An array of single amino acid replacements and deletion mutants in 16E6, as well as chimeric E6s, have been studied by Crook and colleagues, with respect to *in vitro* p53 binding (using the immunoprecipitation assay of Werness et al., Science 248:76–79, 1990), p53 degradation, and transcriptional transactivation [40]. Various substitutions in the cysteine motifs, C63H, C103G, C136H, and C136G, had no effect upon E6-p53 complex formation. 16E6 deletion mutants that created truncations at residues 59 and 110 indicated the involvement of amino acid residues C-terminal to residue 59, and further deletion analysis pinpointed the site to residues 106 to 115, with weaker effects resulting from changes between 123 and 132. Seven of ten residues in this region, according to [40], are conserved among all genital HPV types, and nine of ten among oncogenic types.

On the other hand, the triple mutant R8S-P9A-R10T, that effectively replaces high-risk E6 residues with low-risk E6 residues, eliminated inhibition of p53 transactivation*in vivo* in the study by Mietz et al. [41]. Because 6E6 and 11E6, which have these residues, bound p53 at 30–40% wild-type 16E6 levels, Crook et al. inferred the principal binding site to be around 106–110. In the work of Foster et al., however, p53 binding was not observed for several mutants in the N-terminal half of E6, including the R8S-P9A-R10T triple mutant [16]. A brief summary of the latter set of findings is given below (adapted from Table 1 of [16]). The authors investigated both the 158 aa and 151 aa forms of 16E6; all coordinates utilize the second methionine, the N-terminus of the 151 form, as +1. Some results of Crook et al. (Tables 1–5 of [40]), using the same defects and same plasmids (158 aa form), are shown in parentheses.

TABLE 2 p53 binding and degradation.

E6 Protein form	p53 binding (relative to 16E6 wt 158 aa)	p53 degradation (relative to 16E6 wt 158aa)
158 aa form		
16E6	100% (100)	100%(96)
Δ9-13	0[a]	0[a]
F45Y-F47Y-D49H	0(196)	0(8)
Δ73-77	0(89)	0(98)
Δ78-82	0(79)	0
Δ111-115/N127K	0(6; N127)	0(11)
Δ118-122	0(38)	2(98)
Δ123-127	0(55)	0(68)
Δ128-132	0(42)	0(59)
C66G	0	0
C136G	0(88)	0
C66G/C136G	0	0
151 aa form		
16E6	120	50
Δ2-12	0	NS
R8S-P9A-R10T	0	0
F45V	29	25
Δ136-151	NS	NS
Δ140-151	35	25
Δ146-151	33	67
other		
6E6	0(39)	0(0)
6/16E6[b]	0(113)	0(0)
16/6E6[c]	0(32)	0(81)
16E∗I	NS	NS

[a] no activity over background
[b] 60 aa from 6E6, 92 aa from 16E6
[c] 59 aa (plus 7aa) from 16E6, 90 aa from 6E6
Values in parentheses are from [40], all others are from [16]

The p53 binding results of Foster et al. are clearly in disagreement with those of Crook et al., although both groups studied many of the same mutants and both employed an immunoprecipitation assay for complex formation. The two studies are in general agreement about the contribution of E6 residues in the C-terminal region around 106-115 to p53 binding. The differences concern the role of the cysteines, the role of N-terminal and central region residues, and the binding ability of 6E6. The discrepancy involving the F45Y-F47Y-D49H triple mutant is especially glaring. The data of Foster et al. argues for parallelism between p53 binding and degradation, whereas the data of Crook et al. supports the hypothesis that the two can be decoupled.

The two sets of results also differ with regard to p53 degradation, although both agree that sequences within the N-terminal half are important. The work of Foster et al. not only examines p53 binding and degradation *in vitro*, but also the levels of endogenous p53 in cells expressing the

E6 constructs and the ability of E6 to abrogate actinomycin D-induced growth arrest. Their *in vivo* results agree with their *in vitro* results [16]. Mutational analyses bearing on p53 degradation targeted by 18E6 are reported by Pim et al. [42]: the NPAEK stretch of 18E6 (defined by the ΔF mutant), which corresponds to residues 111 to 115 of 16E6, is found to be important. Other 18E6 regions of importance are RFHNI (defined by the ΔG mutant), which corresponds to the identical stretch in 16E6, 124–128;the FAF stretch (defined by the ΔA mutant), which corresponds to the identical residues in 16E6 at 45-47; and finally the region at the N-terminal end corresponding to the 16E6 site of Mietz et al. [41]. The discrepancies between [40] and [16] remain unresolved. Most likely, regions of both the N- and C-terminal halves of E6 affect the structural interactions with p53, E6AP and E6BP.

D. E6 Abolition of p53 Transcriptional Transactivation p53 can either stimulate or repress gene expression. Lechner and colleagues noted that the 16E6 mutant F45Y-F47Y-D49H, which binds p53 but does not target its degradation (see section C.) was as capable as wild-type 16E6 of abrogating p53-mediated transcriptional repression [11]. Another 16E6 mutant, Δ106–110, which doesn't bind p53 but which has E6 transactivating capabilities (section E. below), does not display activity in this same assay. Pim and colleagues [42] analyze several 18E6 mutants for their ability to abolish p53-mediated transcriptional activation, with the following results: the wild-type 18E6 and Δ47–49 gave 21-fold reductions, whereas mutants Δ94–97, Δ116–117, Δ21–252, Δ101–104, Δ113–117, Δ126–130, Δ144–149, Δ4–7, R10S-P11G, T23G, and P114G gave reductions of 6-fold or less.(Cysteines in 18E6 are at 32,35, 65,68, 105,108, and 138,141. To translate the 18E6 positions into 16E6 coordinates, as has been done in the figure, 18E6 C32 is roughly equivalent to 16E6 C30.)

The fact that the Δ47–49 mutant behaves like wild-type 18E6, but does not target p53 degradation, leads the authors of [42] to conclude that the two activities of E6 are independent. The results of [42] appear to be in agreement with those of [11].

E. Transcriptional Transactivation Crook and coworkers utilized the mutants in their p53 binding and degradation study (C. above) to also evaluate activation of the adenovirus E2 promoter[40]. Again, premature termination of 16E6 at residues 59 and 110 gave an early indication that sequences C-terminal to 110 would be involved in transactivation. The active site was inferred to be between residues 123 and 132, since deletion mutants Δ123-127 and Δ128-132 displayed a five-fold reduction in CAT activity, whereas mutants Δ96-100, Δ101-105, Δ106-110, Δ111-115, Δ118-122, Δ133-137, Δ138-142, and F45Y-F47Y-D49H had 5018E6, 6E6, 16/6E6 and 6/16E6 were all close to the 16E6 wild-type activity; 16E6 early termination mutants 59 and 100 were severely reduced.

In light of the above results, the authors of [40] conclude that transcriptional transactivation, facilitated by residues 123–137, does not overlap with the principal site for p53 binding, residues 105–110. This conclusion could not be reached from the data of Foster et al. [16](see section C above). Because F45Y-F47Y-D49H has normal transactivating ability but does not contribute to the degradation of p53 (see section C above), the authors of [40] also conclude that transcriptional regulatory function is not coupled to p53 degradation.

In the work of Pim and colleagues, a large stretch of the C-terminal half of 18E6 is implicated in EJ-ras related immortalization of BMK cells [42]. HPV-18 E6 mutants that did not interact with p53 were transformation competent, implying that 18E6 possesses p53-independent transforming activity.

F. E6 Truncated Proteins With HPV-16 a donor splice site internal to the E6 orf will produce truncated E6 forms, E6∗I, E6∗II and E6∗III. The donor splice site at nt 226 will lead to an E6∗I fragment of 43 aa counting from the second ATG in the E6 ORF (50aa counting from the first)[1]. The terminal amino acids would be VY, gained from the new frame after splicing at nt 409. The E6∗II fragment would have the same 41 (or 48) N-terminal residues plus IIKNT, translated from the frame after the acceptor splice site at nt 526. E6∗III would also have the same 41 (or 48) N-terminal residues, but would end in QQRSILS taken from the frame after the splice at nt 3358.

An 18E6∗ consists of the N-terminal 43 amino acids plus 14 residues acquired through translation after the acceptor site at nt 411 [43].

HPV-33 E6∗ fragments can be inferred from the work of Snijders et al.[27] The E6∗I fragment would consist of the 60 N-terminal residues plus 18 residues gained from the frame following the acceptor site at nt 509. The E6∗II fragment would also consist of the N-terminal 60 residues, but would

acquire 3 residues from the frame following the acceptor site at nt 785. The E6∗III fragment would have a differnt 6 C-terminal residues resulting from the frame following the acceptor splice at nt 3351.

G. E6 Lifetime Wild-type 18E6 was determined to have half-lives of 4 and 2 hours for the nuclear and membrane fractions, respectively, in insect cells. Alteration of the first five amino acids greatly increased the half-lives, to 30 and 8 hours, respectively, without promoting a shift in the cellular localization [44]. The half-life of E6 in a cervical carcinoma cell line (SiHa) was found to be bimodal [21].

H. E6 B-Cell Epitopes Gao et al. expressed 16E6 peptides in a recombinant vaccinia vector and evaluated the responses to these in mice: six peptide fragments, 86–95, 96–100, 111–120, 136–140, 141–146, and 146–150 were found to be antigenic [45]. Tosi and coworkers show that the 11 C-terminal residues of 16E6 elicit type-specific antibodies [46]. A significant IgA response, but not an IgG response, to the 16E6 peptide KPLCDLLIRCINCQKPLCPEE was strongly associated with cervical cancer [47]. The C-terminal 23 amino acids of the 18E6∗ protein (see section F above) are reported to be antigenic [43]; 9 of these residues would derive from the first exon of the truncated E6.

Antibodies to N-terminal peptides, aa 1–23 and aa 8–37, were also found to be markers for HPV-16 associated invasive cancer [48]. HPV-16 infected individuals with CIN and uninfected individuals with invasive cervical cancer do not show antibodies to these peptides. The first 18 amino acids of the N-terminus of 18E6 synthesized in E.coli were also found to be antigenic [49].

Stacey and colleagues confirm the antigenicity of the N-terminal residues [50]. While peptides from the zinc-finger domains were not antigenic (probably because the peptides cannot mimic the rigid structure), the linker region between the two fingers is antigenic.

I. E6 T-Cell Epitopes The 16E6 peptide encompassing residues 42 to 57 and peptides nested within that stretch were studied by Strang and others [51]. PBMC from healthy asymptomatic individuals responed to one or another portion of this region. Gao et al. report the proliferative response to peptide 131-140, and show that the response is highly haplotype-specific [45]. The N-terminal peptide `KLPQLCTEL` (residues 11 through 19, or 18 through 26 numbering from the first `ATG` start) is a naturally-processed HLA-A0201-restricted peptide [52]. Binding of 16E6 nonameric peptides to five different HLA-A allelic molecules was investigated by Kast and coworkers [53], with the the following results.

HLA-A	16E6 Bound Peptide(s)/High	/Low
A1 (A∗0101)	73–81 (`ISEYRHYCY`)	
A2.1 (A∗0201)	45–53 (`FAFRDLCIV`)	–1–8 (`AMFQDPQER`)
	11–19 (`KLPQLCTEL`)	19–27 (`LQTTIHDII`)
A3 (A∗0301)	52–60 (`IVYRDGNPY`)	68–76 (`KFYSKISEY`)
	119–127 (`HLDKKQRFH`)	26–34 (`IILECVYCK`)
	–1–8 (`AMFQDPQER`)	82–90 (`SLYGTTLEQ`)
	86–94 (`TTLEQQYNK`)	35–43 (`QQLLRREVY`)
A11 (A81101)	86–94 (`TTLEQQYNK`)	35–43 (`QQLLRREVY`)
	–1–8 (`AMFQDPQER`)	
	52–60 (`IVYRDGNPY`)	
	26–34 (`IILECVYCK`)	
	73–81 (`ISEYRHYCY`)	
A24 (A∗2401)	80–88 (`CYSLYGTTL`)	37–45 (`LLRREVYDF`)
	124–132 (`RFHNIRGRW`)	42–50 (`VYDFAFRDL`)

Peptides of 8 and 10 amino acids gave the following high-binding results:

HLA-A	E16E6 Peptide(s)
A1	70–78 (YSKISEYRHY)
A2.1	22–31 (TIHDIILECV)
A3	99–108 (LLIRCINCQK)
A11	61–68 (AVCDKCLK)
	2–11 (FQDPQERPRK)
	25–34 (DIILECVYCK)
	85–94 (GTTLEQQYNK)
	99–108 (LLIRCINCQK)
A24	64–71 (FYSRIREL)

The five peptides that bound A2 (A∗0201) either weakly or strongly were further evaluated for their immunogenicity [54]. Of these, only one, TIHDIILECV, evoked a strong *in vivo* response in mice and all gave a weak *in vitro* response with PBMC from healthy A∗0201 donors.

A suboptimal E6 epitope for H2-Kb, YDFAFRDL, was improved by substituting an isoleucine for the aspartate in the second position of this epitope [55]. The peptide RPRKLPQL has been studied as an HLA B-7 restricted peptide [56].

J. 16E6 Natural Variants. Natural variation of HPV-16 E6 has been observed to date at eleven sites: R10G [57], R10T [58], R10I [57,58], Q14H [58], Q14D [57,58], D25E [58], D25Y [57], I27V [57], I27R [58], R55K [58], D56N [58], D64E [57], H78Y [57,58], L83V [57,58], E114D [57], and S138C [57] (Part I, 1995 compendium). Five distinguishable lineages of HPV-16 have been proposed, European, Asian, Asian-American, African-1, and African-2 (see Wheeler and Icenogle, Part III, 1995 compendium).

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